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Investigations on the Role of the Gut Microbiota in Hippocampal Neuroplasticity and Behaviour Throughout the Lifespan

Thesis presented by

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under the supervision of

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Prof. John F. Cryan

For the degree of Doctor of Philosophy

February, 2022

Table of contents

Table of contents **I**

Declaration **IV**

Author Contributions **IV**

Acknowledgments **VI**

Publications and presentations **VIII**

Abstract **XI**

Chapter 1: General Introduction **13**

1.1 The gut microbiota 14
1.2 The dynamics of the gut microbiota across the lifespan 16
1.3 The microbiota-gut-brain axis 26
1.4 The hippocampus 42
1.5 The microbiota-gut-hippocampus axis 48
1.6 Hippocampal neurogenesis 50
1.7 Hippocampal neurogenesis throughout the lifespan 58
1.8 Gut and microbially derived factors influencing hippocampal neurogenesis 65
1.9 Hypothesis and overall objectives 69

Chapter 2: Microbiota from young mice counteracts selective age-associated behavioural deficits **72**

2.1 Abstract 73
2.2 Introduction 74
2.3 Methods 75
2.4 Results 88

2.5 Discussion 100
2.6 Supplemental information 101

Chapter 3: Microbiota consortium derived from cognitively-impaired elderly patients does not impair cognition when transplanted into young rats 122

3.1 Abstract 123
3.2 Introduction 124
3.3 Methods 128
3.4 Results 139
3.5 Discussion 146

Chapter 4: Understanding the relationship between early life stress-induced gut microbiota changes and hippocampal neuroplasticity 149

4.1 Abstract 150
4.2 Introduction 151
4.3 Methods 154
4.4 Results 159
4.5 Discussion 171
4.6 Supplemental material 176

Chapter 5: Sex differences in the effects of caesarean section birth on neurogenesis in the juvenile hippocampus 177

5.1 Abstract 178
5.2 Introduction 179
5.3 Methods 184
5.4 Results 189
5.5 Discussion 195
5.6 Supplementary Material 202

Chapter 6: General Discussion 203

6.1 General Summary 204

6.2 Neurogenesis, the gut microbiota, and the aging brain – Where are we now? 207
6.3 Early life gut microbiota: Priming for future brain health 211
6.4 The diet-microbiota-gut-brain axis: Towards novel interventions 219
6.5 The future of microbiota-based therapeutics for improving disrupted brain plasticity 220
6.6 Perspectives and conclusions 228

References **230**

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

Dr. Marcus Boehme, Ms. Loreto Olavarria-Ramirez, Dr. Marta C. Neto, and Dr. Enrique Morillas aided me in collecting and preparing the faecal inoculum.

Dr. Marcus Bohme conducted and scored the Y-maze, conducted the Morris water maze which I scored, aided in conducting the intestinal motility test, and me aided in microbiota and blood sample collection along with Mr. Patrick Fitzgerald. Dr. Enrique Morillas conducted and scored the novel object test. Dr. Caitlin Cowen aided in conducting the 3-chamber social interaction test which I scored.

Dr. Fiona Crispie performed the microbial DNA extraction and ran 16S sequencing.

Dr. Thomaz F.S. Bastiaanssen performed the microbiota analysis, hippocampal metabolomics analysis and hippocampal transcriptomics analysis, along with Ms. Rachel Fitzgerald and Mr. Simon Spichak.

Dr. Marcus Boehme, Dr. Marcel van de Wouw, Dr. Gerry Moloney, Dr. Marzia Sicchetti, Dr. Francisco Donoso, Mr Andreu Gual-Grau, and Mr. Nathaniel Ritz aided me in designing, performing, analysing, or interpreting peripheral immune data.

Dr. Marcus Boehme aided me in staining and imaging hippocampal microglial cells, which I analysed.

Chapter 3

Dr. Tarini Ghosh performed the bioinformatics analysis and conception of the microbial consortia. Dr. Paola Pellanda grew the microbial consortia.

Chapter 4

Dr. Francisco Donoso and Dr. Sian Egerton performed the animal experimentation.

Dr. Sian Egerton and Dr. Catherine Stanton performed the microbial DNA extraction and 16S microbiome sequencing.

Dr. Thomaz Bastiaanssen assisted in performing the gut microbiome analysis and correlation analysis.

Chapter 5

Dr. Silvia Aburto, Dr. Shane Morely, and Mr. Nathanial Ritz aided me in the caesarean section surgeries and administration of antibiotics.

Ms. Joan Osayande aided me by performing some of the hippocampal RNA extractions and some of the hippocampal DCX staining, which I imaged and analysed.

Mr. Samuele Laudani aided me in performing some of the hippocampal PCRs, which I analysed.

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Publications and presentations

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   * = authors contributed equally

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3. S. Spichak*, Katherine E. Guzzetta*, O.F. O'Leary, G. Clarke, T.G. Dinan, J.F. Cryan (2018). Without A Bug's Life: Germ-Free Rodents to Interrogate Microbiota-Gut-Neuroimmune Interactions. Drug Discovery Today 28, 79-93. https://doi.org/10.1016/j.ddmod.2019.08.002
   * = authors contributed equally

Manuscripts Submitted/in preparation

4. Katherine E. Guzzetta, OF O'Leary, JF Cryan. Gut-Derived Signals Regulate Adult Hippocampal Neurogenesis and Neurodegenerative Conditions. [*In Preparation*]

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6. Katherine E. Guzzetta, Thomaz F.S. Bastiaanssen, Francisco Donoso, Sian Egerton, Catherine Stanton, Olivia F. O'Leary, and John F. Cryan. Understanding

the relationship between early life stress-induced gut microbiota changes and hippocampal neuroplasticity. [*In Preparation*]

7. Katherine E. Guzzetta, Samuele Laudani, Joan Ossayande, Maria Aburto, Marta Brocka, Paula Sanchez, Francisca Villalobos Manríquez, Silvia Cabre, Shane Morely, Nathaniel Ritz, John F. Cryan, and Olivia F. O'Leary. Sex differences in the effects of caesarean section birth on neurogenesis in the juvenile hippocampus. [*In Preparation*]

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Conference posters and oral presentations

1. Katherine E. Guzzetta. Microbiota from young mice counteracts selective age-associated behavioral deficits. *Smart AgeWorkshop, Karolinska Institute, Sweden*, Oct 2021 [*Invited speaker*]

2. Katherine E. Guzzetta. Faecal Microbiota Transplantation Improves Cognitive Deficits in the Aging Murine Brain. *Targeting Therapeutics for Brain Disorders, Cork, Ireland*, June 2021 [*Oral Presentation*]

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neuroimmunity, physiology, and behavior in germ-free mice. *The Society for Neuroscience Annual Meeting, Chicago, USA*, Oct 2019

Abstract

Trillions of microorganisms inhabit the gastrointestinal tract, and while their importance in host digestion is well established, these microbes have recently been shown to harness the ability to influence neuroplasticity within the hippocampus of the brain, as well as hippocampus-dependent behavioural processes. The hippocampus is one of the few brain regions in which new neurons are born throughout life, an ability that is critical for specific cognitive and emotional processes, making it imperative to understand the biological controls of hippocampal neurogenesis.

Hippocampal neuroplasticity is shaped by factors such as aging, diet, and psychological stress. However, the underlying mechanisms by which these factors influence neuroplasticity in the hippocampus are not fully known. In parallel, the gut microbiome is highly sensitive to diet and stressful life events, however it is unknown whether the gut microbiota is an active mediator in the impact of these factors on hippocampal neurogenesis. Furthermore, the gut microbiota is initially seeded at birth and undergoes compositional and functional changes as its host ages, though whether it is a contributor to the processes of brain aging, including the declining cognition and hippocampal plasticity observed in aging, has previously not been demonstrated.

Herein, this thesis explores the relationships between the gut microbiome and host hippocampal neuroplasticity and related behaviours throughout the lifespan, including in early life following caesarean section birth, early life stress, and/or microbiota-targeted dietary interventions, and in aging using preclinical rodent models. This thesis demonstrates for the first time that transferring the gut microbiota from young to aged mice is sufficient to restore aging-related deficits in hippocampus-dependent spatial memory and learning, as well as some components of the hippocampal metabolome and transcriptome. Interestingly, there was no effect on age-associated decline in hippocampal neurogenesis. However, this thesis also realised that transferring a consortium of bacteria identified as highly abundant in elderly patients with mild cognitive decline could not induce cognitive deficits in young rat recipients, suggesting a limit to the potential of the aging-related gut microbiota to impact host cognition. Furthermore, this thesis demonstrated that early life stress induced changes

in the gut microbiome composition and predicted functional ability within the gut-brain axis, as well as the expression of genes related to hippocampal neuroplasticity. Some of these early life stress-induced alterations were ameliorated with the microbiota-targeted dietary supplementation of fish oil or various polyphenols, highlighting the importance of diet within the microbiota-gut-brain axis. Finally, it was demonstrated that the mode of birth, which has a strong influence on the early life gut microbiome, can lead to critical changes in dorsal hippocampal neurogenesis, effects which were sex-dependent and exacerbated by early life antibiotic exposure.

Taken together, the findings within this thesis highlight the importance of the gut microbiota in shaping hippocampal neuroplasticity and related behaviours of its host. Moreover, these effects are complex, but reveal potential opportunities for novel microbiota-targeted therapeutics throughout the lifespan.

Chapter 1

General Introduction

1.1 The gut microbiota

Trillions of microorganisms reside in the human gastrointestinal tract (Figure 1.1), where they impact the digestion of nutrients and maintenance of health of their host (Cryan *et al.* 2019b). Comprised of bacteria, archaea, fungi, protists, nematodes, and viruses included phages, the collective *gut microbiota* is a highly dynamic community. In healthy adults, the bacterial phyla *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Verrucomicrobia*, and *Actinobacteria* dominate the gastrointestinal tract (Human Microbiome Project 2012). Roughly 90% of the bacterial species found in the human gut classify under the phyla *Bacteroidetes* (including the genera *Prevotella* and *Bacteroides*) or *Firmicutes* (such as *Lactobacillus, Enterococcus*, *Ruminococcus* and *Clostridium*) (Human Microbiome Project 2012; Duenas *et al.* 2015). These microscopic companions reside within the gut throughout the life of the host, although the diversity of the gut microbiome and the relative abundance of each microbe is constantly changing (Johnson *et al.* 2019).

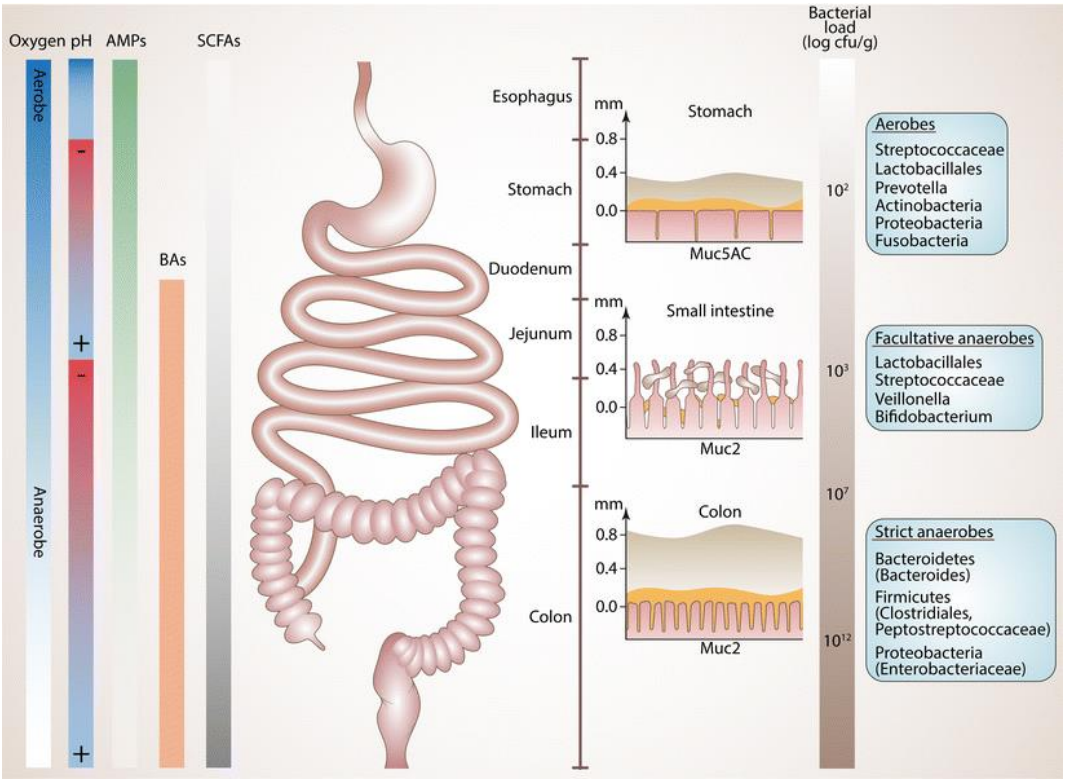


Figure 1.1. The biogeography of the gastrointestinal tract. The left panel depicts endogenous factors determining the microbiome composition within segments of the gastrointestinal tract including oxygen,

pH, bile acids (BA), antimicrobial peptides (AMPs), and short-chain fatty acid concentration. The middle schematic depicts the gastrointestinal tract and the regional mucus layer architecture, including the inner solid mucus layer (amber) and the outer loose mucus layer (grey) The right panel highlights dominant taxa and bacterial lode within different gut segments. Figure obtained from (Gorkiewicz & Moschen 2018).

1.1.1 Measuring the gut microbiota

The improvements in genetic sequencing technologies over the last few decades have allowed for the ability to determine the diversity and relative abundance of genera or species present within a microbial population. Today, two types of microbial sequencing are commonly performed in the field of gut microbiome research: 16S sequencing, a more cost-effective method which allows for observations to the genus level; and shotgun metagenomic sequencing, which allows for resolution down to the strain level, although it is more expensive (Cryan *et al.* 2019b). Once sequenced, methods involving bioinformatics are used to generate descriptions of the microbial communities, including the diversity of the microbiome within a sample (α-diversity) or between samples (β-diversity), and the relative abundance of each bacterium within a sample (Figure 1.2). Through these methods, it is possible to investigate differences in the gut or fecal microbial communities between different individuals or within the same individual over time by collecting multiple samples.

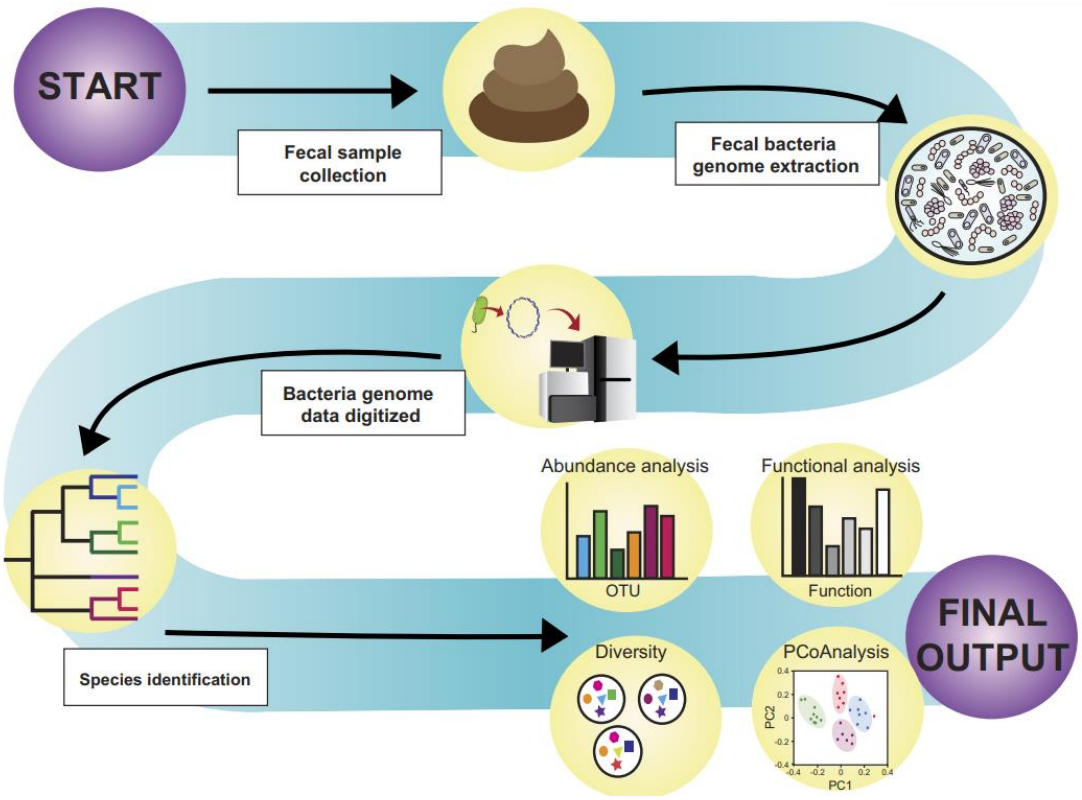


Figure 1.2. The sequential steps of microbiome analysis. Analysis of the microbiota is conducted by extracting the bacterial genome then seuqncing it to create digital records. The species or genera are then identified and used to assess the diversity, abundance, and functional capacity of the microbiome. Abbreviations: principal component (PCo); operational taxanomic unit (OTU). Figure from (Cryan et al. 2019b).

1.2 The dynamics of the gut microbiota across the lifespan

Multiple factors can influence the composition, function, and diversity of the gut microbiota, including genetics, diet, stress, mode of birth, and the age of the host (Figures 1.3, 1.4 and 1.5).

1.2.1 Early life

It is largely believed that birth is the time when microbial colonization of the gastrointestinal tract first occurs, although there are ongoing disputes about whether the uterine environment harbours microbiota or whether it is sterile. Regardless of whether a uterine microbiota exists, the mode of birth has a substantial impact on the early postnatal microbiome (Figure 1.3). The mode of delivery, be it vaginal birth or caesarean section, plays a large role in the initial seeding of the gut microbiota

(Dominguez-Bello *et al.* 2010; Bokulich *et al.* 2016; Shao *et al.* 2019; Morais *et al.* 2020). The initial microbiome of babies born by caesarean section is more comparable to that of the mother's skin, while the gut microbiome of vaginally born babies more closely resembles the mother's vaginal microbiome (Dominguez-Bello *et al.* 2010). These differences in the gut microbiota do not normalize quickly; the impact of caesarean section birth on the gut microbiome is evident into early adolescence (Bokulich *et al.* 2016; Shao *et al.* 2019). Regardless of birth mode, microbes that colonize the gut early in life are known to shape the long-term gut microbiota composition of the host (Martinez *et al.* 2018).

Although the definition of a healthy early life gut microbiome is difficult to define, distinct shifts occur in the gut microbiome during early life postnatal development. Shortly after birth, the gut microbiome commonly includes high relative abundances of bacteria belonging to the families *Bifidobacteriaceae*, *Enterobacteriaceae*, and *Clostridiaceae*, and relatively low abundances of *Ruminococcaceae* and *Lachnospiraceae* families (Fallani *et al.* 2011; Bokulich *et al.* 2016). Over time, bacterial diversity increases, and strict anaerobic species dominate the gut. By the age of 1-3 years old, around the timeframe of weaning, or the transition to solid food consumption, the overall diversity of the gut resembles that of an adult, though it is still rather unique in the relative abundances of each bacterium (Palmer *et al.* 2007; Koenig *et al.* 2011; Yatsunenko *et al.* 2012). This expansion in the gut microbiota around the timeframe of weaning has been demonstrated in rodents to trigger beneficial immune priming and protection against immunopathological developments later in life (Al Nabhani *et al.* 2019).

The composition of the gut microbiota from middle childhood through adolescence remains uniquely distinct from the adult gut microbiome. In healthy 7–12-year-old children, the gut microbiota was enriched in genes associated with supporting developmental processes, such as vitamin and folate synthesis, and anti-inflammatory properties (Hollister *et al.* 2015). While there is limited human microbiome research around the period of adolescence, one study involving healthy 11-18 year olds observed that older adolescents displayed gut microbiota more similar to healthy

adults, although they were still distinct, suggesting the development to the adult microbiome state occurs later in life (Agans *et al.* 2011). Generally, the gut microbiota of adolescents showed increased relative abundances of the genus *Bifidobacterium* and *Clostridium*, and lower abundances of *Prevotella* and *Sutterella*, showing similarities to the childhood microbiota and suggesting a gradual transition occurs into adulthood (Hopkins *et al.* 2001). The human adult gut microbiota is considered relatively stable compared to the early life gut microbiota, although external conditions, such as diet, drug consumption, stress, and exercise, can impact upon it (Figure 1.5).

In addition to birth mode (Dominguez-Bello *et al.* 2010) and the transition to solid foods (Fallani *et al.* 2011), numerous other factors influence the early life gut microbiome. Diet and nutrition (Ho *et al.* 2018), antibiotic use (Bokulich *et al.* 2016), stress (Gur *et al.* 2015), maternal gestational diet and weight (Seppo *et al.* 2019), premature birth (Fouhy *et al.* 2019), illness (Kan *et al.* 2019), birth location (Combellick *et al.* 2018), and pet ownership (Azad *et al.* 2013) all exert effects on the developing gut microbiome. However, through the course of development, the reflection of some of these factors in the gut microbiome disappears (Cowan *et al.* 2020). While these early life marks upon the gut microbiota may normalize over time, there still may be lingering consequences of disturbing the early life gut microbiota which persist into adulthood.

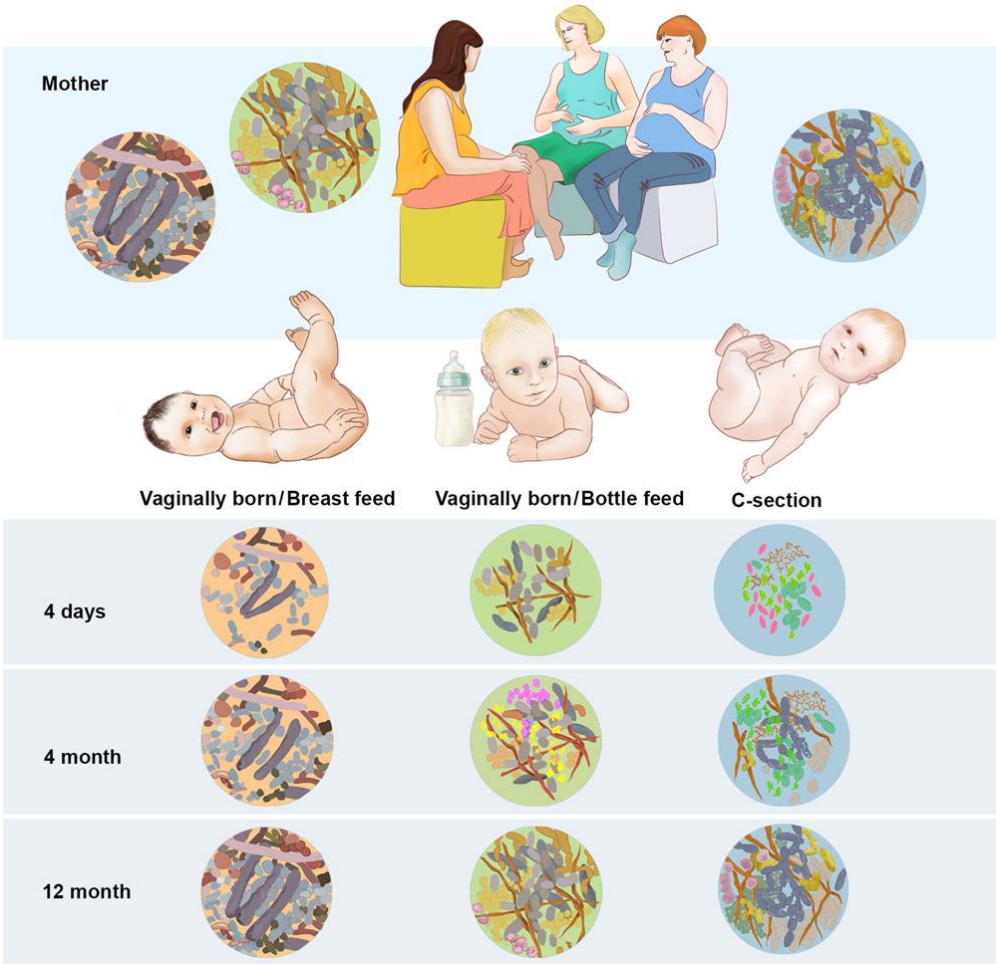


Figure 1.3. The development of the early life gut microbiota is strongly influenced by birth mode and diet. The impacts of caesarean section and early life diet on the gut microbiome persist beyond the first year of life (Backhed et al. 2015).

1.2.2 Aging

Aging is the gradual process of deterioration of multiple homeostatic functions, characterized by alterations to cellular and genetic factors including cellular senescence, stem cell exhaustion, mitochondrial dysfunction, oxidative stress, telomere attrition, inflammation, altered neurotransmitter signalling and receptor sensing, and a reduction in the birth of new neurons in the brain (Klempin & Kempermann 2007; Lopez-Otin *et al.* 2013). The aging gut displays altered physiology, gastric motility, and degenerative changes (Konturek *et al.* 2015), which may underlie the distinct alterations in the gut microbiota which occur throughout aging (Figure 1.4).

While the gut microbiota's composition remains relatively stable in adulthood, as an individual ages, distinct shifts occur in its relative abundance and diversity, and once again the gut microbiota enters a period of increased plasticity (Claesson *et al.* 2011). Although there is increasing research regarding the dynamics of the gut microbiome during aging, studies in different populations do not always agree as to what is the standard trajectory of the gut microbiome during the aging process, perhaps due to cultural, environmental, or geographical differences, or differences in methodology (Biagi *et al.* 2010; Claesson *et al.* 2012a; Odamaki *et al.* 2016; Biagi *et al.* 2017; Salazar *et al.* 2017). Furthermore, the window of time at which an adult gut microbiome transitions to that of an elderly individual is not as clearly defined compared to that which occurs during the transition from an infant gut microbiome to an adult. Fascinatingly, the gut microbiota of centenarians displays unique features compared to younger aged individuals, although studies examining centenarians in different geographical locations found dissimilar results in terms of which species of microbiota are more or less abundant in centenarians (Biagi *et al.* 2010; Odamaki *et al.* 2016; Biagi *et al.* 2017), highlighting the potential importance of differences in genetics, culture, diet, or other lifestyle factors. For instance, Biagi and colleagues found a depletion of core microbial taxa, including *Roseburia, Faecalibacterium,* and *Bacteroides* in Italian centenarians (Biagi *et al.* 2016). Intriguingly, the trajectory of the gut microbiota throughout aging correlated with survival in a large-scale clinical analysis (Wilmanski *et al.* 2021). Longer surviving elderly individuals displayed a reduction in core bacterial genera during aging, while the retention of a high abundance of *Bacteroides* or having low microbial uniqueness was able to predict a poorer survival likelihood four years later (Wilmanski *et al.* 2021).

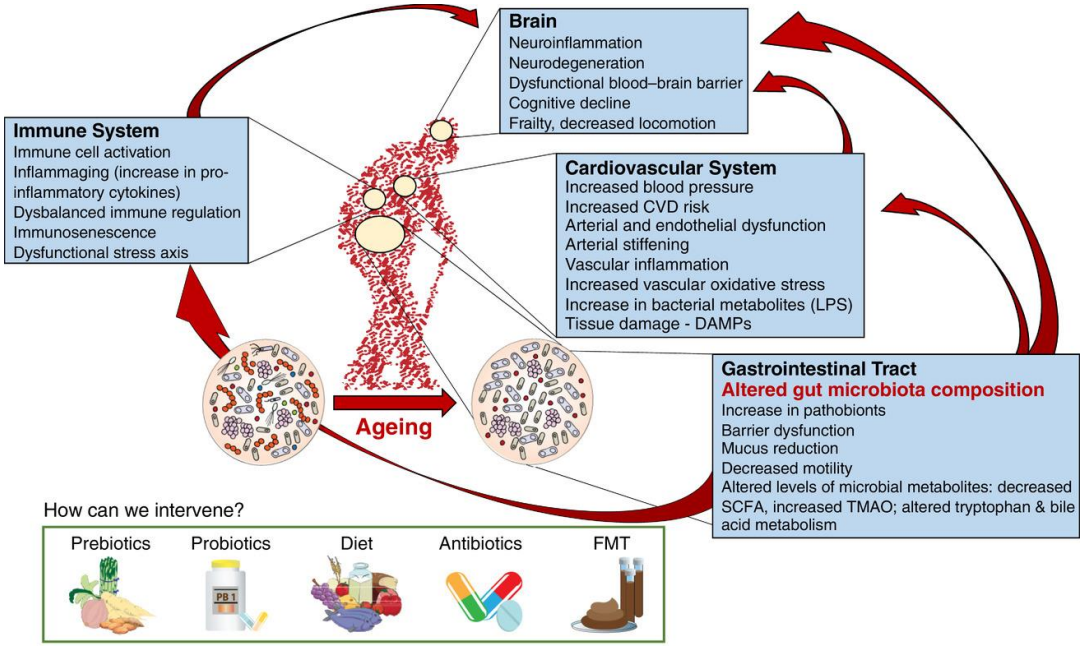


Figure 1.4. The aging process yields immense consequences on the body, including changes in the gastrointestinal microbiota composition, immune, cardiovascular, gastrointestinal, and brain health. CVD, cardiovascular disease; LPS, lipopolysaccharide; DAMPs, damage-associated molecular patterns; SCFA, short-chain fatty acid; TMAO, trimethylamine N-oxide; FMT, faecal microbiota transplant. Figure from (Cryan et al. 2019a)

1.2.3 Factors influencing the gut microbiota

In addition to aging, the gut microbiome is extraordinarily sensitive to inputs from the environment, and rapidly changes based on factors such as diet, antibiotic exposure, and stress (Figure 1.5). Furthermore, biological sex can influence the composition of the gut microbiome.

1.2.3.1 Diet

Diet rapidly restructures the human gut microbiome (David *et al.* 2014). The daily variation in human diet exerts a strong influence on the day-to-day variation in the gut microbiome, and strongly depends on the type of food consumed by the host (Johnson *et al.* 2019). Diets high in fat have been found to increase gut bacteria that can induce systemic inflammation, including *Bilophila wadsworthia* (Devkota *et al.* 2012; Feng *et al.* 2017), while reducing *Bacteroidetes* along with overall microbiome diversity (Hildebrandt *et al.* 2009).

Live bacteria supplementation, through oral intake or dietary consumption of fermented foods can also directly influence the gut microbiome of the host, and these bacteria are termed *probiotics* when they exert a beneficial effect (Azad *et al.* 2018). Furthermore, specific micronutrients, including some polyphenols, fish oil, and some fibres, are also called *prebiotics* as they beneficially influence the gut microbiome by supporting healthy gut microbial processes (Peredo-Lovillo *et al.* 2020). Dietary fibre is comprised of a collection of various carbohydrate polymers found primarily in fruits, vegetables, whole grains, and lentils, which may exert different effects within the gastrointestinal tract (Berding *et al.* 2021b). Fibres influence the gut microbiome in a diverse manner, with various studies showing that fibres increase microbial diversity and promote the growth of beneficial bacteria, including *Akkermansia, Bifidobacterium, Faecalibacterium, Lactobacillus, Prevotella, and Roseburia*, while reducing the abundance of potentially pathogenic bacteria, such as *Enterobacteriaceae* (Walker *et al.* 2011; Salonen *et al.* 2014; Holscher *et al.* 2015; Kovatcheva-Datchary *et al.* 2015; Tap *et al.* 2015; Vanegas *et al.* 2017; Berding *et al.* 2021b). Polyphenols are phytochemicals that include flavonoids and nonflavonoids, and are found in abundance in fruits and vegetables, whole grains, nuts, cocoa and coffee, olive oil, red wine, and green tea (Neveu *et al.* 2010). Polyphenols have been shown to encourage the growth of beneficial bacteria, including *bifidobacteria* and *lactobacilli* and reduce the abundance of bacteria with pathogenic potential including *C. histolyticum* and *C. perfringens* in a manner that is dose dependent (Ma & Chen 2020). Furthermore, specific polyphenols have been shown to exert unique effects on the gut microbiome (Donoso *et al.* 2020). For instance, consumption of cocoa, which is high in flavanols, increased the abundance of *Prevotella, Blautia,* and *Faecalibacterium prauznitzii*, while decreasing *Bacteroides*, *Clostridium*, and *Staphylococcus* (Sorrenti *et al.* 2020), while red wine, which is very rich in resveratrol, increased the abundance of *Prevotellaceae_NK3B31*, *Barnesiella*, and *Phascolarctobacterium* (Le Roy *et al.* 2020). Beyond fiber and polyphenols, polyunsaturated fatty acids, such as ω-3, which is highly abundant in fish oil, can increase microbial diversity and contribute to the growth of beneficial butyrate-producing bacteria, including *Bifidobacterium, Lachnospira, Lactobacillus,* and *Roseburia* (Watson *et al.* 2018).

1.2.3.2 Stress disrupts the gut microbiome

Stress, such as acute or psychological stress, occurs when an organism is alarmed due to a perceived threat. Stress alters an organism's homeostasis, triggering the hypothalamic–pituitary–adrenal (HPA) axis and initiating a release of cortisol, or corticosterone in rodents. Furthermore, stress has been shown to impact the gut microbiome (Tannock & Savage 1974). Stress during early life, including maternal separation stress, is notorious for reducing *Lactobacillus* abundance in the gut of young, otherwise healthy primates (Bailey & Coe 1999). Moreover, the guts of infants exposed to prenatal stress harboured reduced levels of *Lactobacillus* and *Bifidobacterium* than non-stress peers (Zijlmans *et al.* 2015). *Lactobacillus* appears rather sensitive to various types of stress, as this reduction in *Lactobacillus* has also been observed following restraint stress and acute social stress in rodents (Galley *et al.* 2014). On the other hand, the early-life microbiota also contributes to an individual's response to stress. Not only does the maternal microbiome correlate strongly with the hyperactivity of the HPA axis in offspring, but also transferring maternal vaginal microbiota from stressed mouse mothers to naïve male pups has been shown to perturb the pup's stress response in adulthood (Jasarevic *et al.* 2018). Furthermore, mouse pups born to mothers with high perceived stress during pregnancy leads to distinct gut microbiota in the offspring (Jasarevic *et al.* 2017).

In addition to early life stress, stress later in life also alters the gut microbiome. Stress induces volatility in the adult rodent and human gut microbiome, with higher microbiome volatility correlating to increased perceived stress during exam periods, and decreased social interaction in rodents exposed to social defeat stress (Bastiaanssen *et al.* 2021). Moreover, long-term psychosocial stress increases the amount of *Helicobacter pylori* in the mucosa of the mouse stomach (Guo *et al.* 2009), a bacteria known for its infectious ability to destroy stomach lining. Meanwhile, people suffering from post-traumatic stress disorder appear to have a lower abundance of *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia* compared with trauma-exposed individuals (Hemmings *et al.* 2017).

1.2.3.3 Antibiotics and other drug consumption

Antibiotics are drugs designed to kill bacteria and can be either broad-spectrum or more targeted. Although commonly prescribed antibiotics are typically only administered over a period of three to seven days, short-term antibiotic injection leaves lasting marks on the host microbiome which are, in some cases, evident even several months after antibiotic exposure (Korpela *et al.* 2016). While the impact of antibiotics on the gut bacteria are unsurprising, recent research has revealed that hundreds of interactions exist between non-antibiotic drugs and the gut microbiome (Javdan *et al.* 2020; Vich Vila *et al.* 2020). For instance, several antipsychotics and antidepressants were found to alter the relative abundance of specific microbes in rats (Cussotto *et al.* 2019). Some drugs that are not classified as antibiotics express antimicrobial properties. Oral administration of the common antidepressant drug fluoxetine eliminated the genera *Prevotella 7*, *Prevotella 9* and *Succinivibrio* (Cussotto *et al.* 2019) and reduced levels of *Lactobacillus johnsonii* (Lyte *et al.* 2019) in the rodent gut microbiome. Interestingly, the gut microbiome is altered in patients suffering from the stress-related disorder depression, and preclinical models of depression (Tillmann *et al.* 2019).

1.2.3.4 Exercise

Exercise over several weeks restructures the gut microbiome, promoting butyrate-producing bacteria and increases systemic butyrate in rodents (Matsumoto *et al.* 2008; Evans *et al.* 2014; Kang *et al.* 2014). Furthermore, the gut microbiomes of professional male rugby players show increased alpha diversity along with reduced levels of *Bacteroides* and *Lactobacillus* compared to sedentary, lean controls (Clarke *et al.* 2014). Furthermore, the gut microbiomes of women who exercised for at least three hours per week were enriched for *Faecalibacterium prausnitzii*, *Akkermansia muciniphila,* and *Roseburia hominis* (Bressa *et al.* 2017). Initiating endurance exercise training induced a similar growth in *Akkermansia muciniphila* after 6 weeks in previously sedentary, overweight women (Munukka *et al.* 2018).

1.2.3.5 Biological Sex

Studies examining the early life gut microbiota indicate males have reduced microbial alpha diversity in their first 30 days of life, accompanied by increased *Clostridiales* and reduced *Enterobacteriales* compared to female babies (Cong *et al.* 2016). Furthermore, boys display increased *Bifidobacterium* abundance at the first day of life (Nagpal *et al.* 2017). However, large-scale population studies have not displayed sex-specific differences in gut microbiota composition or diversity of adult healthy humans (Mueller *et al.* 2006; Human Microbiome Project 2012; Odamaki *et al.* 2016), indicating that sex-differences in the gut microbiome are time-sensitive. Nonetheless, enterotype classification of individual's gut microbiomes revealed that males were more three times more likely to be classified into community type D, which is characterized by having higher relative abundance of *Prevotella* and reduced *Bacteroides* (Ding & Schloss 2014). Meanwhile, the gut microbiome of females were more likely to be classified as type C, having higher levels of *Alistipes, Faecalibacterium,* and *Ruminococcaceae* and reduced relative abundance of *Bacteroides* and *Prevotella* (Ding & Schloss 2014). Furthermore, levels of serum sex hormones testosterone and oestradiol correlated with the diversity of the gut microbiome in men and women, and were related to the relative abundance of specific microbial genera, including *Slackia* and *Butyricimonas* in women and *Aceinetobacter, Dorea, Ruminococcus,* and *Megamonas* in men (Shin *et al.* 2019). Still, the role of sex in influencing the gut microbiota in disease states throughout the lifespan should be given further attention (Jaggar *et al.* 2020).

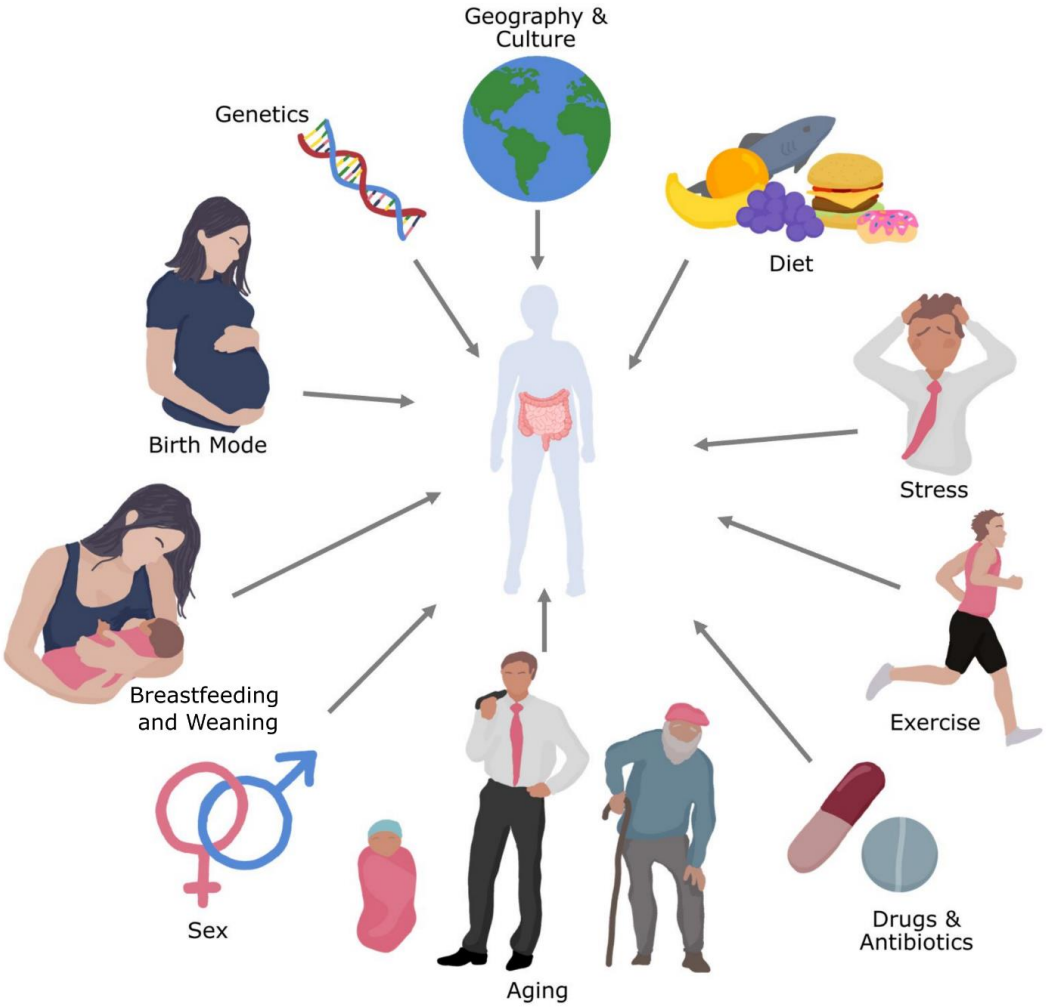


Figure 1.5. Factors influencing the gut microbiome. Multiple factors can influence the composition, function, and diversity of the gut microbiome. These factors are inheritable, such as genetics, biological sex, geography, and culture; lifestyle-independent, including birth mode and age; and lifestyle-dependent, such as diet, breastfeeding, weaning, stress, exercise, and drug consumption.

1.3 The microbiota-gut-brain axis

While microbes in the gastrointestinal tract are constantly surveying, adapting, and responding to their environment, they are also active participants in maintaining homeostasis within their host. Thousands of years of coexistence and coevolution have allowed gut-dwelling microbes and their mammalian host to interact with one another, sending chemical messages via metabolites, hormones, and neurotransmitters over neural, endocrine, and immune pathways. Investigations into these pathways, along with the use of germ-free rodents and microbiota perturbations, such as antibiotics,

have allowed for deeper understandings of how the gut microbiota shape biological functions in the host throughout life, including in the brain.

Animals and their microbiota share a complex evolutionary history; humans have never naturally existed without microbes. Over the millennial of evolution, the gut microbiota has adapted to take advantage of the various signalling mechanisms existing between the gastrointestinal tract and the brain. Studies involving antibiotics, faecal microbiota transplantation, microbiota-targeted dietary interventions, such as probiotics and prebiotics, and/or germ-free (GF) rodents, which are aseptically isolated from birth and reared without any microbiota, have revealed vast alterations in brain development and complexity depending on the gut microbiome (Spichak & Guzzetta *et al.* 2018). For instance, studies utilizing GF mice have revealed that the gut microbiota is able to influence the blood-brain barrier (Braniste *et al.* 2014) and growth of new adult hippocampal neurons (Ogbonnaya *et al.* 2015; Scott *et al.* 2020), though it is still not fully understood how.

1.3.1 Pathways of microbiota-gut-brain communication

The gut microbiota can communicate with the brain through a variety of pathways, including neurological, immunological, and metabolic pathways. Some of these mechanisms are highlighted in Figure 1.6.

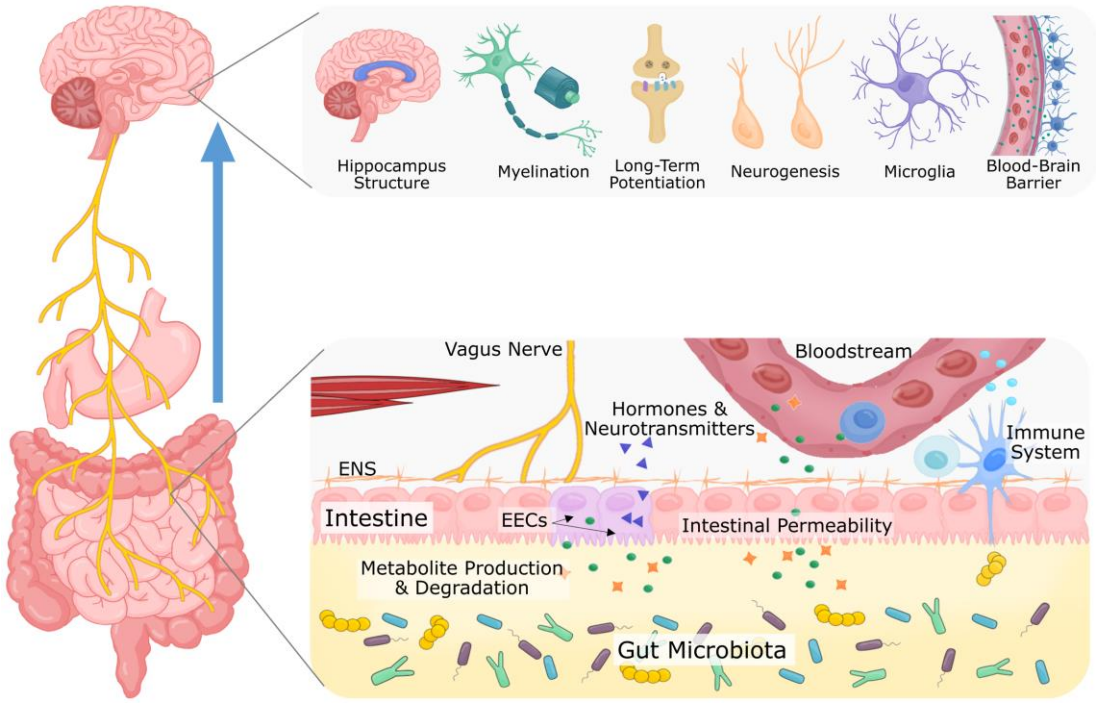


Figure 1.6. Mechanisms of microbiota-gut-brain signalling. Microbe-derived signals can be transmitted to the brain through neuronal, endocrine, and immune pathways. The vagus nerve allows for direct neuronal transmission of gut signals. Local immune cells, including monocytes and T cells, can traffic to the brain. These immune cells are highly sensitive to microbial inputs, which can alter their function. The gut microbiota can also produce and modulate metabolites, including neurotransmitters, hormones, and short-chain fatty acids which can contribute to local processes or be relayed to the brain via the bloodstream. The gut microbiota has been shown to contribute to differences in myelination, neurogenesis and neuroplasticity, long term potentiation, microglia function, the permeability of the blood brain barrier, and differences in hippocampus structure, which offers some mechanistic insight into how the gut microbiota may regulate host brain function and behaviour. ENS is the enteric neervous system. EECs are enteroendocrine cells.

1.3.1.1 Vagus nerve signalling

The vagus nerve is the quickest mode of gut-brain communication. Comprised of around 80% afferent and 20% efferent fibres, the vagus nerve sends vital information from the gastrointestinal tract to the brain, and *vice versa*. Although vagal afferent fibres penetrate to all layers of gastrointestinal wall, the vagus nerve does not extend past the epithelial layer (Wang & Powley 2007), and thus is not believed to directly contact the gut microbiota (Bonaz *et al.* 2018). Still, germ-free mice showed decreased excitability of gut sensory neurons, directly implicating the microbiota in neuronal activity (McVey Neufeld *et al.* 2013).

The use of subdiaphragmatic vagotomy in murine studies has made it increasingly clear that certain strains of gut bacteria can take advantage of the vagus nerve gut-brain signalling pathway to influence host behaviour and mood. For instance, the ability for *Lactobacillus rhamnosus JB1* to relieve stress-induced behavioural changes and decrease the density of microglia in the rodent hippocampus was blocked following vagotomy (Bercik *et al.* 2011b; Bravo *et al.* 2011b; Liu *et al.* 2021). The ability of *Lactobacillus rhamnosus JB1* to alleviate the impact of stress on behaviour may relate to its ability to reduce the expression of the $GABA_{B(1b)}$ gene in the hippocampus (Bravo *et al.* 2011b), a receptor subunit which mediates stress resilience and the production of new hippocampal neurons (O'Leary *et al.* 2014). Furthermore, vagotomy also blocked changes in behaviour and prefrontal cortex gene expression that were observed in rodents following treatment with either *Lactobacillus intestinalis* or *Lactobacillus reuteri (Wang et al. 2020)*. Vagotomy also prevented the pro-cognitive and electrophysiological effects of 2'-Fucosyllactose, a prebiotic known to promote healthy intestinal bacteria (Vazquez *et al.* 2016). By preclinically examining Fos immunoreactivity, it has been shown that the pathogenic bacteria *Campylobacter jejuni* can stimulate cell bodies in vagal afferents, which project to the nucleus tractus solitarius (NTS) in the brain (Goehler *et al.* 2005). These gut-derived vagal signals can also influence memory, learning, and anxiety, which may allow the GI tract to contribute to a variety of mental illnesses (discussed in further detail later). For instance, projections from the nucleus tractus solitarius to the nucleus accumbens and basolateral amygdala can modulate memory consolidation following physiological arousal (Roozendaal *et al.* 1999; Kerfoot *et al.* 2008). Anxiety, fear and avoidance behaviours can also be impacted by activation of NTS-derived projections to the bed nucleus of the stria terminalis and central amygdala (Bienkowski & Rinaman 2013).

1.3.1.2 Immune signalling

The gut mucosal immune system is constantly surveying the gut microbiota for pathogenic threats. Therefore, the gut microbiota can impact the relative number, function, migration, and cytokine secretion of specific subsets of gut-associated

immune cells including monocytes (Mohle *et al.* 2016), regulatory and helper T cells (Mazmanian *et al.* 2008; Medina-Rodriguez *et al.* 2020), B cells, and dendritic cells (Fung 2020). Some of these immune cells, such as granulocytes, macrophages, and T cells, can circulate to the brain where they may relay gut-derived messages or respond to problems in the nearby environment (Fung 2020). Additionally, lipopolysaccharide is generated by the gut microbiota and can induce inflammatory responses, along with depressive-like behaviours in rodent models and clinical studies (Dantzer *et al.* 2008; Lawson *et al.* 2013).

In germ-free mice, the innate and adaptive immune system are largely underdeveloped, highlighting the importance of a microbiota for healthy gut-immune signalling. Perturbances in the features of circulating immune cells have also been observed in rodents following antibiotic treatment and were sufficient to induce changes in immunity, behaviour, and survival of newly born hippocampal neurons in the brain. For instance, antibiotic treatment in mice diminished the number of infiltrating $Ly6C^{hi}$ monocytes in the brain, leading to a decline in newborn neuron survival and disrupted hippocampal-dependent short-term spatial and object recognition memory, which were rescued by adoptive transfer of $Ly6C^{hi}$ monocytes (Mohle *et al.* 2016).

In addition to $Ly6C^{hi}$ monocytes, T cells have emerged as fundamental players in the microbiota-gut-immune-brain axis. One subset, γδ17 T cells, are particularly important for patrolling barrier sites, including the gut, where they initiate inflammatory reactions in response to pathogenic threats via cytokine production. γδ17 T cells also traffic to the meninges of the brain, where they can contribute to cognitive processes and anxiety-like behaviour (Alves de Lima *et al.* 2020) and short-term memory (Ribeiro *et al.* 2019), through proinflammatory cytokine interleukin (IL)-17 signalling to glial cells (Ribeiro *et al.* 2019) and cortical neurons (Alves de Lima *et al.* 2020). γδ17 T cells are particularly sensitive to microbial depletion via antibiotic treatment, which disrupted the transcription of IL-17 in meningeal γδ17 T cells (Alves de Lima *et al.* 2020). Furthermore, T cells have been implicated in the gut microbiota's ability to influence the neurological recovery of the host following

ischemic stroke. Bacterial priming of intestinal dendritic cells leads to an expansion of intestinal regulatory T cells and a suppression of IL-17^{+} γδ17 T cells, allowing for the microbial regulation of the post-stroke neuroinflammatory response (Benakis *et al.* 2016). Recently, segmented filamentous bacteria were revealed to influence depressive-like behaviour though the induction and promotion of T helper 17 (Th17) cells. Mice lacking these bacteria do not have Th17 cells patrolling their small intestine (Ivanov *et al.* 2009), and are resistant to Th17-cell mediated induction of depressive-like behaviour (Medina-Rodriguez *et al.* 2020). Microbiota-T cell communication is also bidirectional. For instance, depleting Foxp3+ regulatory T cells lead to an increase in the abundance of Firmicutes in mice (Kehrmann *et al.* 2020), highlighting the delicate homeostasis between gut microbiota and host immune cells.

While widely understudied in the context of the microbiota-gut-brain axis, B cells have been increasingly shown to be sensitive to gut microbiota signals and may play an active role in relaying these signals to the brain. Recently it was demonstrated that the exposure of germ-free mice to specific microbial taxa shapes the immunoglobulin repertoire of B cells in a temporal manner, thereby impacting their functionality (Li *et al.* 2020a). Furthermore, in patients with multiple sclerosis, gut-derived B cells harbouring specific immunoglobulins for bacterial taxa increased in multiple sclerosis patients were observed trafficking to the central nervous system, though their role in mediating the disease is not fully understood (Probstel *et al.* 2020).

Microglia are the resident macrophage of the central nervous system and act as crucial gardeners of the brain, supporting neuronal growth, pruning synapses, and eliminating pathogenic threats via phagocytosis, releasing cytokines, or complement activation. Impairments in microglia function are associated with neurodevelopmental and neurodegenerative disorders, including autism spectrum disorder and aging-associated cognitive decline. In germ-free mice and mice treated with antibiotics, microglia are immature and present altered inflammatory gene expression profiles, metabolic features, and functionality, highlighting the importance of a healthy gut flora for the maintenance of a healthy brain immune system (Erny *et al.* 2015; Thion *et al.* 2018; Erny *et al.* 2021). Microglia reside within the central nervous system during healthy

states (Green *et al.* 2019), and thus would not have direct contact with intestinal microbes. Their regulation via gut microbiota-derived signals is not entirely understood, although recently the microbially-derived metabolite acetate was shown to traffic to the brain, where it is metabolized by resident microglia, among other cells (Erny *et al.* 2021). Supplementation of acetate to 5xFAD mice, a mouse model of Alzheimer's Diseases wherein the microglia are also disrupted, was able to rescue aspects of microglia maturation and functionality, including in the hippocampus, a brain region crucial for memory and cognition (Erny *et al.* 2021).

1.3.1.3 Microbial metabolites

The gut microbiota effectively acts as a collective of microscopic factories, synthesizing nutrients from the diet into other molecules. Gut microbes can produce a variety of neurotransmitters and neuromodulators (Cryan & Dinan 2012; Agirman & Hsiao 2021). Gamma-aminobutyric acid (GABA) can be produced by members of the *Bifidobacterium* and *Lactobacillus* genera; *Lactobacillus* genus can synthesize acetylcholine; dopamine and/or noradrenaline can be made by members of the genera *Bacillus*, *Escherichia* and *Saccharomyces*; and members of the genera *Candida*, *Enterococcus*, *Escherichia* and *Streptococcus* can create 5-hydroxytryptamine (5-HT) (Bravo *et al.* 2011a; Barrett *et al.* 2012; Nicholson *et al.* 2012; Lyte 2014; Wall *et al.* 2014; Agirman & Hsiao 2021). *Bifidobacteria infantis* supplementation also increased the circulating levels of serotonergic precursor, tryptophan, when administered to rats, while decreasing the kynurenine:tryptophan ratio (Desbonnet *et al.* 2008). Meanwhile, *Lactobacillus johnsonii* administration reduced the plasma concentration of kynurenine while increasing serotonin in rats (Valladares *et al.* 2013). When faecal microbiota from depressed patients was transplanted into healthy rats, the microbiota transfer resulted in dysregulated tryptophan metabolism and increased plasma and central kynurenine levels (Kelly *et al.* 2016), underscoring the gut microbiota's ability to influence host neurotransmitters. Studies involving germ-free rodents, anti-, pre-, and probiotics have also unmasked relationships between the gut microbiota and a plethora of gut peptides, including ghrelin, neuropeptide Y (NPY), peptide YY (PPY), glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), oxytocin, and neuropeptide

Y receptors, which has been reviewed extensively elsewhere (Lach *et al.* 2018; Agirman & Hsiao 2021).

Short-chain fatty acids (SCFAs) are perhaps the most well-studied microbial-derived metabolites (O'Riordan *et al.* 2022). This group of chemicals, primarily consisting of acetate, propionate, and butyrate, are mainly produced during microbial fermentation of host-indigestible fibres (Macfarlane & Macfarlane 2003). SCFAs can alter histone protein assembly through their ability to inhibit histone deacetylases and thereby modulate DNA methylation and regulate gene expression (Davie 2003; Cleophas *et al.* 2016), including in brain-derived astrocyte cell cultures (Spichak *et al.* 2021). Several host functions are affected by SCFAs including gastrointestinal homeostasis (Gill *et al.* 2018) and (neuro)immune function (Erny *et al.* 2017). SCFAs directly influence the release of hormones and neuropeptides from enteroendocrine cells, including GLP-1 and PPY (Rooks & Garrett 2016), which also have implications within the brain. Furthermore, SCFAs promote intestinal transit (Marathe *et al.* 2011) and influence the brain's immune system, as discussed previously. While SCFAs can penetrate the blood brain barrier (Tan *et al.* 2014), whether microbially-derived SCFAs play a role in directly influencing the brain remains largely unknown. Indeed, only recently was it discovered that the gut microbiota derived SCFA acetate can translocate to the mouse brain and influence the function of microglia (Erny *et al.* 2021).

The gut microbiota can also modulate circulating primary bile acids and produce secondary bile acids (Wahlstrom *et al.* 2016). Bile acids are important signalling hormones and facilitate digestive processes by supporting the absorption of fats and fat-soluble vitamins. Germ-free animals have a marked reduction in secondary bile acids (Joyce *et al.* 2014). Some intestinal bacteria such as *Bifidobacteria*, *Bacteroides, Lactobacilli,* and *Clostridium* can produce a bile salt hydrolase enzyme, stimulating deconjugation of glycine or taurine to conjugated bile acids *(Ridlon et al. 2006).* Bile acids are able to cross the blood brain barrier, although they may also be produced locally in the brain (Monteiro-Cardoso *et al.* 2021). *In vitro,* several bile acids have been shown to act as antagonists for NMDA and $GABA_A$ receptors (Schubring *et al.*

2012), which are linked to cognition (Collingridge *et al.* 2013) and anxiety (Sanders & Shekhar 1995) respectively. Furthermore, perturbances in bile acids have been observed in several neurological diseases, including Alzheimer's Disease, Parkinson's Disease, and stroke, although direct links between bile acids, the gut microbiota, and neuropathology have not yet been firmly established (Monteiro-Cardoso *et al.* 2021).

As stated previously, specific gut microbes are able to modulate the concentrations of tryptophan and its metabolites, including 5-HT, kynurenine, and indoles (Agus *et al.* 2018; Gheorghe *et al.* 2019), suggesting a further mechanism by which the gut microbiota can influence brain health. Indeed, alterations in the tryptophan metabolism pathway have been linked to stress-related psychiatric disorders, including depression (Gheorghe *et al.* 2019). The majority of 5-HT in the body is produced from dietary tryptophan in the gut by intestinal enterochromaffin cells and plays a vital role in regulating intestinal motility (Kendig & Grider 2015) While it is unclear as to whether gut-produced 5-HT can translocate to the brain to affect brain function, peripherally-produced 5-HT may interact with other mechanisms of gut-brain signalling, including vagal or immune pathways (Shajib *et al.* 2017; Berding *et al.* 2021b). Majority of dietary tryptophan is metabolized into kynurenine, which is able to cross the blood-brain barrier and be further metabolized into neuroactive products including kynurenic and quinolinic acids. Kynurenic acid acts as an antagonist to the N-methyl-d-aspartate (NMDA) receptor, allowing it to exert neuroprotective effects, while quinolinic acid is considered neurotoxic (Waclawikova & El Aidy 2018). Meanwhile, while indoles have beneficial properties including antioxidative effects, overproduction of indoles was sufficient to induce anxiety-like and depressive-like behaviour in rats (Jaglin *et al.* 2018).

1.3.2 Methodological approaches to studying the microbiota-gut-brain axis

Removing, replacing, or perturbing the gut microbiota of an organism allows for interrogation into the impacts of the gut microbiota on its host. Therefore, to study the microbiota-gut-brain axis, several key animal models and microbiota-targeted

techniques are commonly used, including germ-free rodents, antibiotic treatment, microbiota transplantation, and dietary perturbations including prebiotics.

1.3.2.1 Germ-free rodents

Germ-free (GF) rodents, which are devoid of all microbes and reared in sterile isolators, have been instrumental in understanding the impact of the gut microbiome on host function and health. Fascinatingly, multiple strains of GF mice present with similar behavioural phenotypes, including reduced depressive-like behaviours (De Palma *et al.* 2015; Campos *et al.* 2016), impaired memory and learning ability (Gareau *et al.* 2011; Hoban *et al.* 2018; Luk *et al.* 2018), and social deficits (Desbonnet *et al.* 2014; Buffington *et al.* 2016; Spichak *et al.* 2018; Stilling *et al.* 2018), although the impact of GF status on anxiety-like behaviours is conflicting (Nishino *et al.* 2013; Luk *et al.* 2018; Spichak *et al.* 2018). Due to their lack of microbially-driven immune priming, GF animals have dysfunctional and immature immune systems, including in gut mucosa immunity (Klaasen *et al.* 1993; Hapfelmeier *et al.* 2010), and in their microglia in the brain (Figure 1.6) (Erny *et al.* 2015; Thion *et al.* 2018; Erny *et al.* 2021). Furthermore, GF animals express differences in metabolite levels and gene expression in various regions of the brain (Sudo *et al.* 2004; Diaz Heijtz *et al.* 2011; Neufeld *et al.* 2011; Stilling *et al.* 2015; Chen *et al.* 2017), perhaps due to impairments in their blood brain barrier (Braniste *et al.* 2014). These features may underly alterations in hippocampal neuroplasticity (Ogbonnaya *et al.* 2015; Scott *et al.* 2020; Wei *et al.* 2021), neuronal long-term potentiation (Darch *et al.* 2021), and myelination (Hoban *et al.* 2016) that have been observed in GF brain (Figure 1.7).

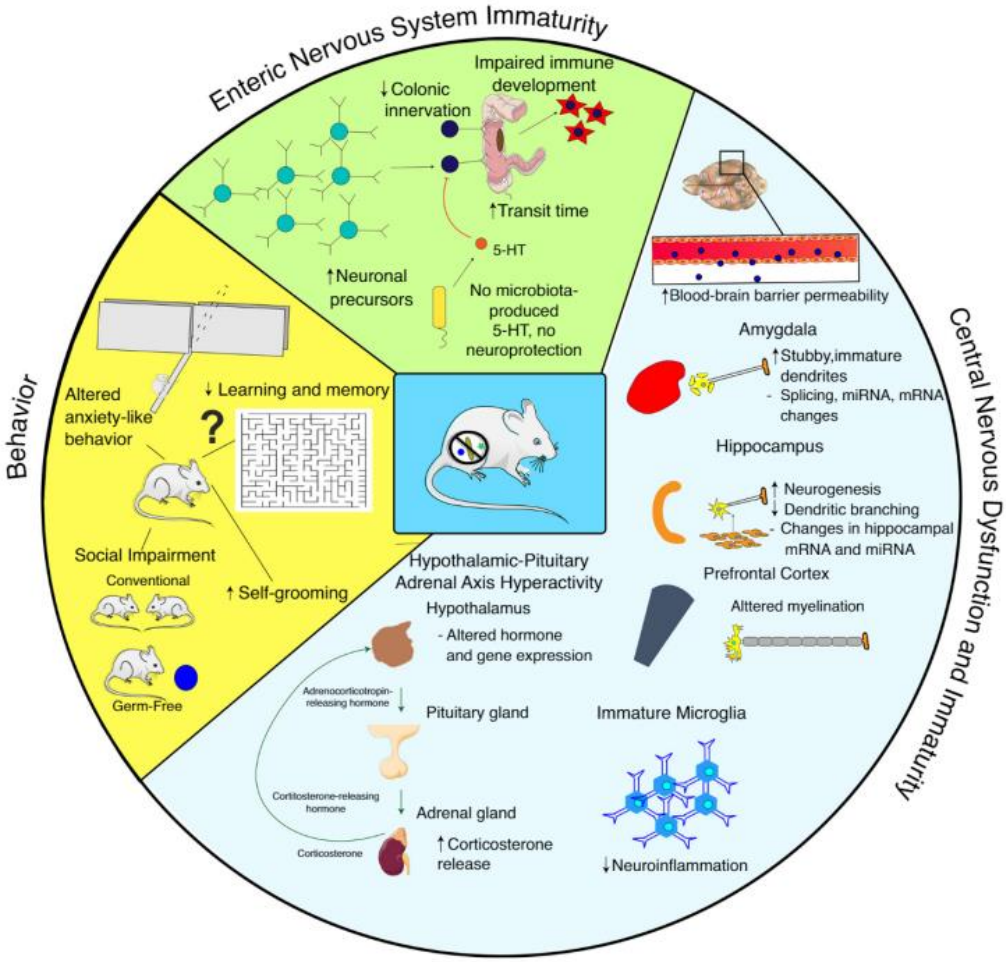


Figure 1.7. A summary of common findings regarding the phenotype of the GF rodent. GF rodents typically have reduced body weight, significantly enlarged caecums, gastrointestinal structural changes, reduced gut motility, altered liver metabolism and differences in bone density. Behaviourally, GF rodents display impaired learning and memory, social impairment, and altered anxiety-like behaviour. Immunity of GF rodents is severely compromised. The blood-brain barrier exhibits increased permeability, and various brain regions including the amygdala and hippocampus have altered genetic transcription, leading to immature neuronal morphology, altered adult hippocampal neurogenesis, and a hyperactive hypothalamic-pituitary-adrenal axis. Figure from (Spichak & Guzzetta et al. 2018).

While GF rodents offer a clean slate to study the impacts of microbes on their host, it is tedious and expensive to develop and maintain GF rodent colonies, and studies investigating the behaviour of GF rodents are limited by the space and manoeuvrability restrains of working within sterile isolators. Furthermore, GF animals are not the most translationally relevant model organism to understand the relationship(s) between specific microbes and the host due to their underdeveloped immune system, aberrant neurodevelopment, and gut functions (Spichak & Guzzetta et al. 2018). Colonization

of GF animals with individual microbes or known consortia of microbes can bypass some of these detriments. These 'gnotobiotic' rodents, while still maintained in costly sterile isolators, allow for investigations into the relationship between specific bacteria, or groups of bacteria, and the host. Furthermore, bacterial colonization of GF mice has been shown to ameliorate some behavioural and immune differences between GF and conventional rodents (Nishino *et al.* 2013; Buffington *et al.* 2016; Luk *et al.* 2018; Spichak *et al.* 2018).

1.3.2.2 Antibiotic deletion

Antibiotic-depletion models offer a trade-off between the insights gleaned from GF rodents and the costs, space, and translational restrictions of GF and gnotobiotic rodents by allowing for the time-sensitive depletion in gut microbes during a specific temporal window, such as early life, adolescence, or aging. The diversity of antibiotics as well as the ability to manipulate the administered dosage of antibiotics allow for a greater control over the microbial depletion. Some antibiotics are nonabsorbable, such as vancomycin and neomycin, meaning they only act directly within the gastrointestinal tract, allowing for clear interpretations of the impact of microbial depletion on the host (Tochitani *et al.* 2016). Other antibiotics, such as metronidazole, are not bound to the gastrointestinal tract and can cross from the gut into the periphery (Mellon *et al.* 2000) where they may act directly on the central nervous system, potentially altering the brain and behavioural processes.

1.3.2.3 Caesarean Section

In addition to depleting the gut microbiota through antibiotics and aseptic isolators, the caesarean section-born rodent is a translationally relevant preclinical model for early life perturbance of the gut microbiota. As discussed previously, caesarean section leaves remarkable scars on the gut microbiome which are apparent from birth and into early infancy (Dominguez-Bello *et al.* 2010; Bokulich *et al.* 2016). Furthermore, rodents born by caesarean section have impairments in social cognition, increased anxiety phenotype, and altered neurochemistry (Morais *et al.* 2020; Morais *et al.* 2021), some of which were rescuable by microbiota-targeted interventions, including

probiotics, prebiotics, and co-housing with vaginally-born mice (Morais *et al.* 2020). While this model is limited by the developing understanding of the microbiota-driven neurobiochemical consequences of caesarean section birth throughout various stages of life, the caesarean-section rodent model is a clinically relevant model for understanding the roles which the early life gut microbiota plays in host development.

1.3.2.4 Faecal Microbiota Transplantation

Faecal Microbiota Transplantation (FMT) is a convincing tool for understanding the impact of a specific microbial population on the host. Through sterile collection of faeces, anaerobic suspension, and filtration, it is possible to administer gut microbiota collected from healthy or diseased human or rodent donors into recipient rodents and understand the causative relationship between various microbiota and the health of their host. FMT was first documented in 1958 for its therapeutic role in treating pseudomembranous colitis in humans (Eiseman *et al.* 1958), and has since become renowned medically for its efficacy in treating refractory *Clostridium difficile* infection (Schwan *et al.* 1983; van Nood *et al.* 2013; Han *et al.* 2016; van Beurden *et al.* 2017). More recently, FMT has also proven to be an extremely valuable technique for researching how the gut microbiota influences its host. In 2016, using FMT, researchers first demonstrated that the gut microbiota plays a crucial role in regulating the host's behaviour; transfer of faecal microbiota from depressed humans into naïve rats induced depressive-like and anxiety-like phenotypes into the recipient rats, relative to rats who received FMT from healthy human donors (Kelly *et al.* 2016). Since then, FMT has helped elucidate the relationships between the gut microbiota and host immunity , metabolism (Zheng *et al.* 2016), and brain function in health and disease states (Gheorghe *et al.* 2021).

1.3.2.5 Dietary manipulations

Given the gut microbiota's strong sensitivity to the diet of the host, dietary manipulations and supplementations are also useful for understanding the relationships between specific microbial states and the host. Some dietary components, including polyphenols and fibres, can be utilized by the gut microbiota

to support the health of the host and thus are deemed prebiotics (Gibson *et al.* 2017). Prebiotics, including polyphenols and fibres, have been shown to improve cognition (Boehme *et al.* 2020) and dampen the impacts of stress on neurobiology (Donoso *et al.* 2020), likely through remodelling the gut microbiome. Probiotics, which are bacteria that benefit the host's health, can also be administered via diet, and can be used to better understand the microbiota-gut-brain axis. Probiotic administration has been found to modulate neurochemistry (Desbonnet *et al.* 2010; Bravo *et al.* 2011b) and improve cognition (Savignac *et al.* 2015), anxiety-like, and depressive-like behaviours (Abildgaard *et al.* 2017a; Abildgaard *et al.* 2017b; Tian *et al.* 2019a; Tian *et al.* 2019b; Abildgaard *et al.* 2021; Tian *et al.* 2021).

1.3.2.6 Additional perturbances to consider in studying the gut microbiome

Additional factors that influence the gut microbiota, including stress, exercise, weaning, and drugs, are also valuable tools for interrogating how specific changes to the gut microbiota relate to host phenotypes. However, the mechanistic role of the microbiota in contributing to behavioural or neurochemical changes that may also occur under these factors would require further elucidation using one or a combination of the previously described models (ie. FMT, GF, gnotobiotic, and antibiotic depleted models).

1.3.3 Diet and the microbiota-gut-brain axis

As previously covered, diet plays a profound role in shaping the microbial community within the gut (Johnson *et al.* 2019). Furthermore, specific dietary components including some fibres, polyphenols, and polyunsaturated fatty acids have shown beneficial effects on aspects of brain health and behaviour. Therefore, diet appears to be a potential tool for modulating the microbiota-gut-brain axis towards improved health (Berding *et al.* 2021b) (Figure 1.8).

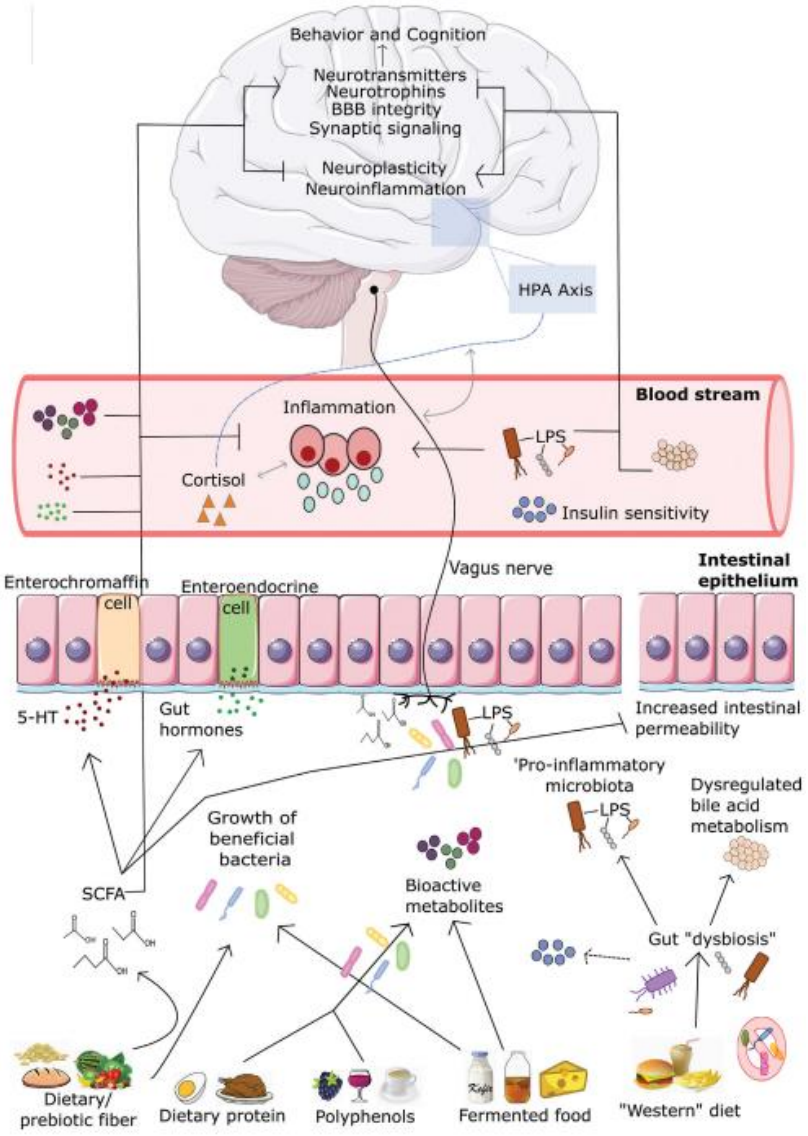


Figure 1.8. Aspects of microbiota-gut-brain communication sensitive to dietary modulation. Several components of the microbiota-gut-brain axis are potentially modulated by diet, including microbial production of metabolites, immune, endocrinal, metabolic, and neuronal pathways. Figure from (Berding et al. 2021b; Moloney et al. 2021)

1.3.3.1 Fibres

In addition to restructuring the gut microbiota, fermentable dietary fibres are able to be broken down by microbes to produce beneficial SCFAs (O'Riordan *et al.* 2022). As discussed previously, the SCFA acetate has been shown translocate from the gut to the brain to influence microglia function (Erny *et al.* 2021). Furthermore, supplementation with individual or combinations of dietary fibres, including galacto-oligosaccharide, fructo-oligosaccharide, and inulin, have been able to improve anxiolytic and

depressive-like behaviour as well as cognitive performance in preclinical models (Burokas *et al.* 2017; Boehme *et al.* 2020; O'Mahony *et al.* 2020), perhaps through their immunomodulatory effects, although this has yet to be mechanistically deciphered. Limited clinical evidence suggests that a high-fibre diets can also enhance cognitive performance (Berding *et al.* 2021a), as well as decreased depressive score in obese women (Uemura *et al.* 2019).

1.3.3.2 Polyphenols

The consumption of polyphenols has been associated with many clinical health benefits including improved cognition in both elderly (Valls-Pedret *et al.* 2012) and health adults (Philip *et al.* 2019), lower risk of depression (Chang *et al.* 2016; Godos *et al.* 2018). Likewise, orange juice rich in flavonoids decreased depressive symptoms and serum brain-derived neurotrophic factor (BDNF) levels compared with consumption of orange juice low in flavonoids, which was accompanied by an increase in the bacterial families *Bacteroidaceae, Bifidobacteriaceae,* and *Lachnospiraceae* in the faecal microbiome (Park *et al.* 2020). Preclinical studies have revealed that polyphenols can alleviate early life stress-induced depressive-like and anxiety-like behaviour, lower corticosterone levels, and restructure the gut microbiome and its predicted ability to communicate with the brain (Donoso *et al.* 2020). While the exact mechanisms by which polyphenols act on the microbiota-gut-brain axis to potentially promote cognition and reduce depressive-like and anxiety-like behaviour after stress are currently unclear, polyphenols have been shown to promote neuroplasticity and neurogenesis within the hippocampus of the brain, a region which plays critical roles in regulating these behaviours (Sarubbo *et al.* 2018).

1.3.3.3 Polyunsaturated fatty acids

The polyunsaturated fatty acid ω-3, which is highly abundant in fish oil, have been shown to improve cognitive performance and attention, enhance mood, regulate stress sensitivity, and reduce the risk of developing depression (Yehuda *et al.* 2000; Fontani *et al.* 2005; Vancassel *et al.* 2008; Denis *et al.* 2013; Firth *et al.* 2019). While the mechanism(s) by which polyunsaturated fatty acids act beneficially on the microbiota-

gut-brain axis is largely unknown, some studies suggest that their ability to increase the abundance of beneficial *Bifidobacteria* and reduce *Enterobacteria* abundance (Costantini *et al.* 2017; Watson *et al.* 2018), as well as modulate intestinal short-chain fatty acids (Egerton *et al.* 2020), may facilitate their promotion of anti-inflammatory responses which could in turn impact the brain (Berding *et al.* 2021b)

1.4 The hippocampus

Many of the reported impacts of the gut microbiome on the brain and behaviour are related to hippocampal-regulated processes, such as learning and memory, the hypothalamic–pituitary–adrenal axis, and anxiety. Furthermore, the hippocampus is a highly plastic structure, and markers of neuronal plasticity such neurogenesis, neurotrophic factors including BDNF, and receptors for neurotransmitters, such as GABA, and hormones, including glucocorticoid and mineralocorticoid receptors, which can influence hippocampal plasticity, have been shown to be altered by aging, stress, diet, and exercise (Tanapat *et al.* 1998; McEwen & Magarinos 2001; Mirescu & Gould 2006; Klempin & Kempermann 2007; Bercik *et al.* 2011a; Neufeld *et al.* 2011; Klempin *et al.* 2013; O'Leary *et al.* 2014; Cooper *et al.* 2018; Kozareva *et al.* 2019a), which are all also factors that influence the gut microbiota. Thus, the hippocampus is likely a key brain area that is influenced by the gut microbiota.

1.4.1 The structure of the hippocampus

The hippocampus, named appropriately for its resemblance to a seahorse (genus: *Hippocampus*) (Bir *et al.* 2015), is a large, curved region within the brain of humans and other vertebrates. The hippocampus can be divided segregated a long its longitudinal axis into anterior and posterior regions in humans, and dorsal and ventral subregions in rodents (Strange *et al.* 2014), and more recently the intermediate region between which spans between the ventral and dorsal regions (Levone *et al.* 2021a). The hippocampus is comprised of different regions, including the dentate gyrus (DG), Cornu Ammonis (CA; which is further subdivided into the CA1, CA2, CA3 and CA4) and the subiculum. These regions contain primarily excitatory pathways (Andersen *et*

al. 1969) and are interconnected to one another via the main hippocampal circuit, the trisynaptic loop (Knierim 2015).

The hippocampus is innervated and primarily receives signals from the entorhinal cortex, perirhinal cortex, postrhinal cortex, raphe nucleus, locus coeruleus, nucleus reuniens medial septum, and amygdala, while it projects outputs primarily to the nucleus accumbens, lateral septum, prefrontal cortex, and amygdala (Pitkanen *et al.* 2000; Kajiwara *et al.* 2003; Mello-Carpes & Izquierdo 2013; Gerlei *et al.* 2021). Considering its connectivity with these structures, it is no surprise that the hippocampus is important for learning, memory, and emotional behaviour. The dentate gyrus takes in most of the cues from the entorhinal cortex and provides input to the CA3 and CA1 through the mossy fiber pathway (Babcock *et al.* 2021). In doing so, dentate gyrus granule cells are able to relay information by projecting axons into the CA3, which can cascade by CA3 projections into the CA1 region via the Schaffer collateral pathway. The trisynaptic loop is then completed by projections extending from the CA1 back to the entorhinal cortex (Knierim 2015). The CA1 is the primary source of hippocampal outputs and is comprised by numerous neurons that project mainly to the entorhinal cortex and the subiculum (Szabo *et al.* 2017).

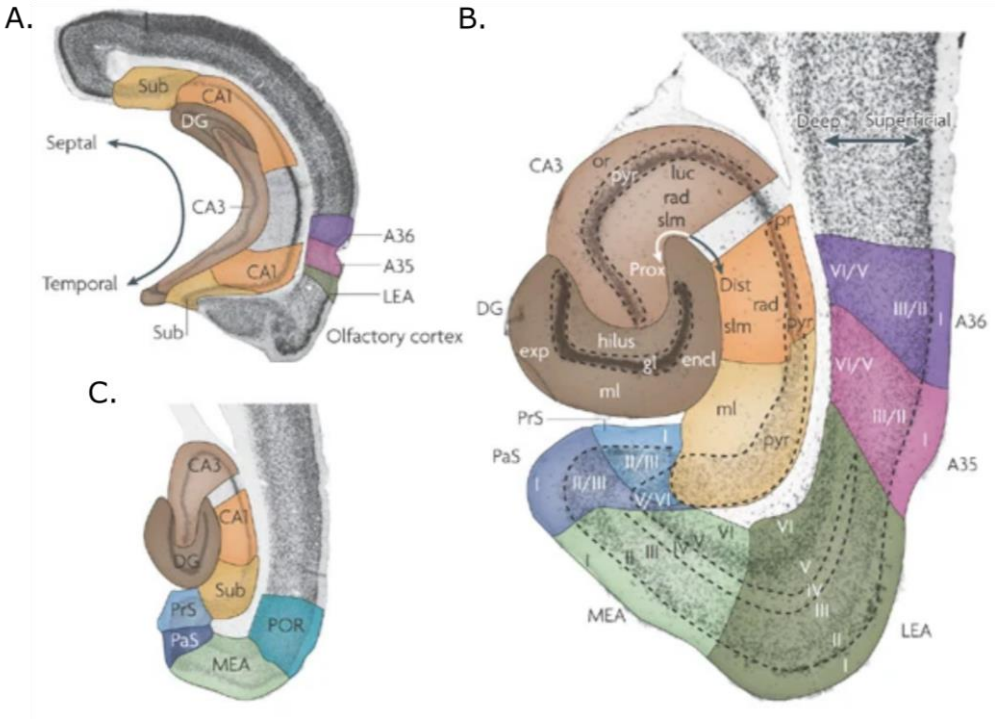


Figure 1.9. The anatomy of the hippocampus. Images taken of the rat hippocampus overlaid with regional identification. (A and B) Transverse sections. (B) Coronal section. Abbreviations: dentate gyrus (DG) cornu annomis (CA) 1, CA2, CA3, and CA4. Image adapted from (van Strien et al. 2009).

Three cellular layers exist within the dentate gyrus: the molecular layer, the granule cell layer, and the polymorphic cell layer, also known as the hilus (Knierim 2015). The molecular layer is occupied by few cell bodies, and instead is contains dendrites of dentate granule cells that are extending to receive input from the entorhinal cortex. The granule cell layer is the main layer of the dentate gyrus and is comprised of a high density of granule cells. The polymorphic layer, comprised primarily of mossy cells, resides within the granule cell layer, but can be distinguished and separated by dentate pyramidal basket cells which lay between these layers (Knierim 2015; English *et al.* 2017). The granule cell layer is also home to the subgranualar zone, where neural progenitor cells reside. Neuroprogenitor cells reside and constitutively produce new neurons throughout postnatal life via a process known as hippocampal neurogenesis.

1.4.2 Hippocampal functions

While the hippocampus has intrigued scientists for centuries (Bir *et al.* 2015), the functional importance of the hippocampus was not understood until 1957, when the

famous case of patient H.M. had their hippocampus lesioned as a treatment for epilepsy (Scoville & Milner 1957). As a consequence, H.M. lost their ability to generate new declarative memories, establishing the hippocampus as key to the formation and consolidation of memory (Scoville & Milner 1957). Declarative memory is the memory of information or events that can be stored and later retrieved and is subdivided into semantic (memory of facts and knowledge) and episodic memory (memory of experiences) (Eichenbaum 2001, 2004; Wixted *et al.* 2018).

Since then, the hippocampus has been shown as a critical contributor to the knowledge of one's location and spatial awareness through the discovery of the place cell. In rats, pyramidal cells within the CA1, CA2, CA3, and granule cells of the dentate gyrus were shown to selectively fire when the rat occupied specific locations within its environment. Some place fields seem to establish spatial understanding based on distances to specific objects or landmarks while others may use path integration computations to encode location (O'Keefe & Dostrovsky 1971; O'Keefe 1976; Morris *et al.* 1982; O'Keefe & Speakman 1987; Moser *et al.* 1995; O'Keefe *et al.* 1998; Moser *et al.* 2008; Knierim 2015).

Another type of memory that is widely under the regulation of the hippocampus is pattern separation. Pattern separation refers to the ability to distinguish between similar episodic memories and is mainly driven by the dentate gyrus (Kassab & Alexandre 2018). During aging, pattern separation it is one of the first types of memories to decline, and this decline correlated to aging-related hyperactivity in the CA3 and dentate gyrus (Yassa *et al.* 2011).

The hippocampus has also been implicated in the regulation of emotions, such as anxiety, depressive behaviour, and stress response. Lesioning the hippocampus is sufficient to alter emotional behaviours including anxiety (Bannerman *et al.* 2002; Kjelstrup *et al.* 2002; Bannerman *et al.* 2003). Furthermore, chronic stress has been shown to reduce the volume of the hippocampus (Lee *et al.* 2009). In parallel, patients diagnosed with depression consistently display reduced hippocampal volume (Campbell *et al.* 2004; Videbech & Ravnkilde 2004) and present with impaired spatial memory (Gould *et al.* 2007) compared with healthy, age-matched peers. The

responsiveness of the hippocampus to stress is in part due to its high concentration of glucocorticoid and mineralocorticoid receptors, which are reactive to stress-induced changes in cortisol (in humans) or corticosterone (in rodents) that occur with the activation of the hypothalamic-pituitary (HPA) axis (Jacobson & Sapolsky 1991). Furthermore, the activation of these markers within the hippocampus, along with signalling from the anterior pituitary and paraventricular nuclear of the hypothalamus, induces negative feedback on HPA axis and reduces the release of glucocorticoids post-stress (De Kloet *et al.* 1998).

1.4.3 Functional segregation of the hippocampus along its longitudinal axis

Precisely how the hippocampus contributes to functions as diverse as spatial memory and learning processes, or the regulation of anxiety is not yet fully understood. However, increasing evidence suggests that the hippocampus might be functionally segregated along its longitudinal axis into dorsal and ventral subregions in rodents, and anterior and posterior regions in humans (Strange *et al.* 2014) (Figure 1.10). More recently, it has been suggested the intermediate hippocampus might also be functionally distinct (Levone *et al.* 2021c).

For instance, the dorsal and ventral hippocampal regions exhibit unique genetic expression and responses to external cues (Dong *et al.* 2009; Levone *et al.* 2021a; Levone *et al.* 2021b). Studies involving lesions in specific regions of the hippocampus suggest that the dorsal and ventral hippocampus take on distinct functional roles. The dorsal hippocampus is widely believed to be involved in spatial learning and memory (Ferbinteanu & McDonald 2001; Pothuizen *et al.* 2004) and contextual fear retention (Kim & Fanselow 1992). Indeed, lesioning as little as 25% of the dorsal hippocampus in rodents was sufficient to impair spatial leaning in the Morris water maze task, while lesions to the ventral hippocampus had no effect on spatial learning or memory within the Morris water maze (Moser *et al.* 1995).

Meanwhile, the ventral hippocampus appears more critical for regulating stress responsivity and anxiety (Henke 1990; Kjelstrup *et al.* 2002; O'Leary & Cryan 2014;

Levone *et al.* 2021a; Levone *et al.* 2021b). For example, lesions to the ventral, but not dorsal, hippocampus increased the number of entries animals took into the open arms of the elevated plus maze, dampened corticosterone response to an acute stressor (Kjelstrup *et al.* 2002), and prevented the effects of the antidepressant fluoxetine on antidepressant-like behaviour in the tail suspension test and novelty-induced hyperphagia test (Levone *et al.* 2021c), indicating a reduction in anxiety-like behaviour. Therefore, alterations in neurogenesis in specific regions of the hippocampus may yield different functional consequences (O'Leary & Cryan 2014).

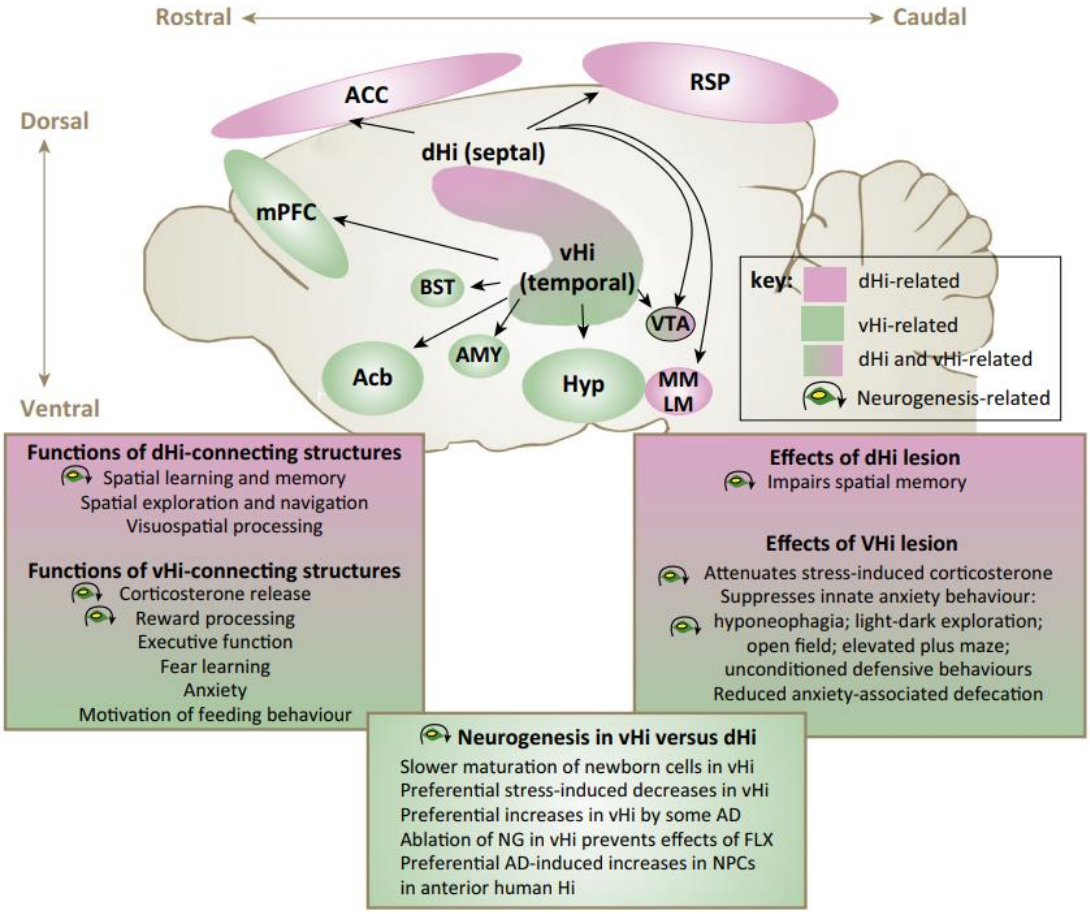


Figure 1.10. The functional segregation and connectivity of the rodent hippocampus along its dorsoventral axis. The dorsal hippocampus is indicated with pink while the ventral hippocampus is indicated with green. Abbreviations: anterior cingulate cortex (ACC); nucleus accumbens (Acb); antidepressant (AD); amygdala (AMY); bed nucleus of the stria terminalis (BST); the antidepressant fluoxetine (FLX); hippocampus (Hi); hypothalamus (Hyp); medial prefrontal cortex (mPFC); lateral mammillary nucleus (LM); medial mammillary nucleus (MM); neurogenesis (NG); neural progenitor cells (NPCs); retrosplenial cortex (RSP); ventral tegmental area (VTA). Figure from (O'Leary & Cryan 2014)

1.5 The microbiota-gut-hippocampus axis

The hippocampus is a brain region crucial for regulating cognitive functioning, including learning, memory, and emotional responses. Investigations involving germ-free rodents found that absence of a gut microbiota results in distinct alterations in the hippocampus, underlying that the gut microbiota plays a critical role in hippocampal function. For instance, the hippocampus of germ-free Swiss Webster mice displays a unique gene expression profile, including a reduction in the gene expression of *BDNF (Sudo et al. 2004; Gareau et al. 2011; Clarke et al. 2013) and* the *Nr2* glutamate receptor and increased expression of the dopamine receptor *Drd1a* (Diaz Heijtz *et al.* 2011). Meanwhile, serotonin is also increased in the hippocampus of male, but not female, germ-free Swiss Webster mice, an effect which was not rescued by colonization of these mice after weaning (Clarke *et al.* 2013). Furthermore, microglia in the germ-free hippocampus are underdeveloped and contain differences in their gene expression profile (Erny *et al.* 2015; Thion *et al.* 2018; Erny *et al.* 2021). The germ-free rodent hippocampus displays increased hippocampal neurogenesis and neuron survival (Ogbonnaya *et al.* 2015), which may be sex- and age-dependent (Scott *et al.* 2020). Such properties likely contribute to the differences observed between germ-free and conventional rodents in the long term potentiation of hippocampal neurons (Darch *et al.* 2021) and hippocampal neuronal activity in response to stress (Gareau *et al.* 2011), culminating in gut microbiota-dependent differences in hippocampal plasticity. Alterations in the hippocampal properties of germ-free mice may underly their impairments in hippocampus-associated behaviours such as memory and perturbed anxiety-like and depressive-like behaviour (Gareau *et al.* 2011; Neufeld *et al.* 2011; Clarke *et al.* 2013; Luk *et al.* 2018).

Research involving microbial manipulations, including faecal microbiota transplant, antibiotic depletion models, and microbiota-targeted dietary interventions, and have underscore the importance of the gut microbiota in maintaining hippocampal function. For instance, faecal microbiota transplantation has been shown to restructure the hippocampal gene expression, metabolome, and microglia in rodents, altering hippocampus-dependent cognitive performance (Kundu *et al.* 2019; Li *et al.* 2020c;

Boehme *et al.* 2021). Furthermore, the depletion of the gut microbiota through the administration of an antibiotic cocktail to healthy, adult female mice disrupted microbiota-sensitive Ly6C^{hi} monocytes, leading to reduced hippocampal neurogenesis and impaired spatial and object recognition ability (Mohle *et al.* 2016). Consumption of specific probiotics has induced remarkable effects of the gut microbiota on the hippocampus and hippocampus-dependent memory. Administration of *Bifidobacterium breve A1* to a mouse model of Alzheimer's disease reduced the expression of hippocampal genes related to neuroinflammation and improved working and long-term memory in mice (Kobayashi *et al.* 2017), as well as improving scores in the cognition-related Mini Mental State Examination (MMSE) assessment in patients with mild cognitive decline (Kobayashi *et al.* 2019a). Supplementation with *Costridium butyricum* was sufficient to rescue deficits in spatial learning and memory, increase hippocampal BDNF, and attenuate morphological differences in hippocampal granule cells in a mouse model of vascular dementia, potentially through the production of butyrate (Liu *et al.* 2015). Meanwhile, dietary consumption of the prebiotic quercetin increased the diversity of the gut diversity, reduced hippocampal amyloid β plaque concentration, upregulated hippocampal BDNF, and improved spatial learning and long-term memory function in the APP/PS1 mouse model of Alzheimer's disease (Lv *et al.* 2018). Alternatively, diets high in fat and sugar, which have deleterious consequences on the gut microbiome (Bisanz *et al.* 2019), disrupt hippocampal-dependent memory, alter hippocampal neuroinflammation gene expression, neurogenesis, and neural stem cell mitochondrial biogenesis (Cordner & Tamashiro 2015; Ribeiro *et al.* 2020).

Overall, the gut microbiome has been shown to orchestrate the neurochemistry and function of the hippocampus.

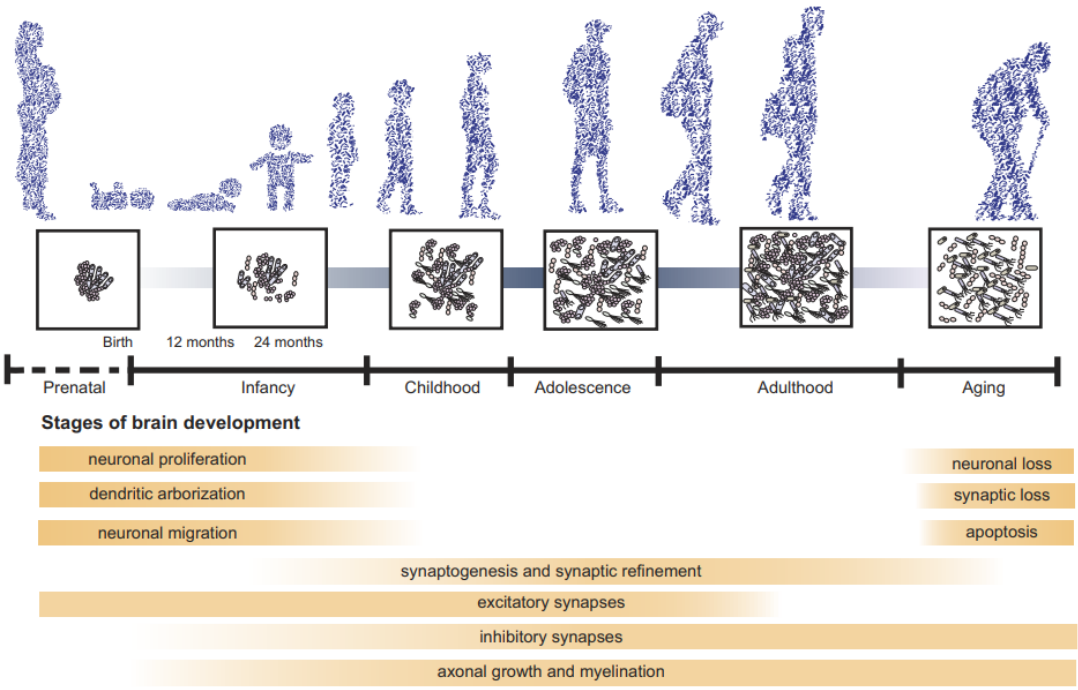


Figure 1.11. The dynamics of the gut microbiome across the lifespan overlap with changes in neural development and plasticity. Figure from (Cryan et al. 2019b).

1.6 Hippocampal neurogenesis

The hippocampus is just one of two brain areas where new neurons are constitutively throughout life via a process called hippocampal, neurogenesis. Hippocampal neurogenesis is believed to be an integral component in hippocampal neuroplasticity throughout life, in addition to other critical aspects of neuroplasticity that are also dynamic throughout life, such as synaptogenesis, synaptic pruning, long-term potentiation, and myelination (Bartsch & Wulff 2015). Neuroplasticity within the hippocampus is essential for memory and learning processes, as well as one's response and resiliency to stress, and impaired hippocampal neuroplasticity (including neurogenesis) is believed to contribute the onset of cognitive impairment, such as those observed in neurodegenerative conditions (Bartsch & Wulff 2015). Given the relationships observed preclinically between adult hippocampal neurogenesis, learning, and mood regulation (as discussed in section 1.6.3), it is important to understand the process of hippocampal neurogenesis, including the functions it enables, the factors that regulate it, and the natural trajectory of hippocampal neurogenesis throughout life.

In early life, the hippocampus is rapidly expanding, and neurogenesis, neuronal migration, myelination, and synaptogenesis are occurring at high rates. The production of new hippocampal neurons declines with age, a conserved feature observed in rodents, non-human primate, and post-mortem human tissue (Bayer 1980; Altman & Bayer 1990a; Jabes *et al.* 2010; Moreno-Jimenez *et al.* 2019). While the existence of adult neurogenesis particularly in later life remains controversial in humans due to several limitations, including the necessity to obtain brain tissue quickly *post-mortem* (Kempermann *et al.* 2018) which has recently been reported to interact with age in rat tissue (Terstege *et al.* 2022), increasing evidence supports the existence of neurogenesis in humans throughout life (Knoth *et al.* 2010; Spalding *et al.* 2013; Boldrini *et al.* 2018; Moreno-Jimenez *et al.* 2019; Tobin *et al.* 2019), which is well established to persist in rodents and other mammals (Eriksson *et al.* 1998). The trajectory of hippocampal neurogenesis during early life and aging is discussed in greater details in section 1.7.

1.6.1 The developmental stages of hippocampal neurons

Hippocampal neurogenesis (AHN) occurs in the neurogenic niche of the subgranular zone (SGZ) within the dentate gyrus (DG) of the mammalian adult hippocampus (Eriksson *et al.* 1998), where pluripotent neural stem cells (NSCs) proliferate and differentiate to produce new neurons (Kempermann & Kronenberg 2003). Majority of neural stem cells in the brain are not actively dividing and exist instead in a quiescent state (Babcock *et al.* 2021). Upon activation, these stem cells proliferate either symmetrically to form two additional quiescent stem cells, or asymmetrically into one neural stem cell along with a progenitor cell, which can differentiate into neurons or glia (Codega *et al.* 2014; Bond *et al.* 2015). Upon receiving GABAergic excitation, neuroblasts, or progenitors destined for a neuronal fate, develop further into immature neurons (Tozuka *et al.* 2005). Around half of these new neurons will undergo apoptosis and die during the first two weeks of life (Dayer *et al.* 2003), leaving the surviving newly born neurons to migrate to the granule cell layer (Kempermann *et al.* 2003). Here, during the early postmitotic maturation phase, and under the regulation of neuronal network signalling including GABAergic inputs (Piatti *et al.* 2011),

neurons begin to form synapses and dendritic spines while the axon elongates further (Sun *et al.* 2013). Finally, during the late maturation phase, neurons show signatures of mature granule cells, including a reduction in their long-term potentiation threshold, formations of glutamatergic synapses, and a change from the expression of calretinin to calbindin (Brandt *et al.* 2003; Schmidt-Hieber *et al.* 2004; Aimone *et al.* 2006), completing their integration into existing circuits (Castilla-Ortega *et al.* 2011). Therefore, AHN involves at least four specific stages: cell proliferation, neuronal differentiation and survival, and neuronal maturation (Christie & Cameron 2006).

Various endogenous factors such as BDNF (which is has been shown to be altered in GF mice) (Bercik *et al.* 2011a; Bercik *et al.* 2011b; Neufeld *et al.* 2011), and exogenous factors such as stress, diet, and exercise, which also influence the gut microbiome (Kang *et al.* 2014; Ribeiro *et al.* 2020; Bastiaanssen *et al.* 2021), have also been shown to regulate the proliferation, maturation and/or survival of the new hippocampal neurons, as discussed further below.

1.6.2 Methodological approaches to measuring hippocampal neurogenesis

Early research investigating hippocampal neurogenesis uncovered the existence of new-born granule cells in the adult dentate gyrus using thymidine-H^3 tagging (Altman & Das 1965; Cameron *et al.* 1993). Later research combined the incorporation of thymidine analogue with NeuN, a marker of mature neurons, to confirm that neural stem cells proliferate and differentiate into mature granular neurons in the dentate gyrus (Kuhn *et al.* 1996). Since then, various other protein markers have been identified as being expressed throughout the stages of neurogenesis, from the birth, development, maturation, and eventual death of neurons (Figure 1.12) (Kozareva *et al.* 2019a; Babcock *et al.* 2021). Immunohistochemistry is currently the most widely used method to directly analyse hippocampal neurogenesis, as it allows for the identification and staining of specific proteins, or antigens, expressed during specific stages of the neurogenesis process.

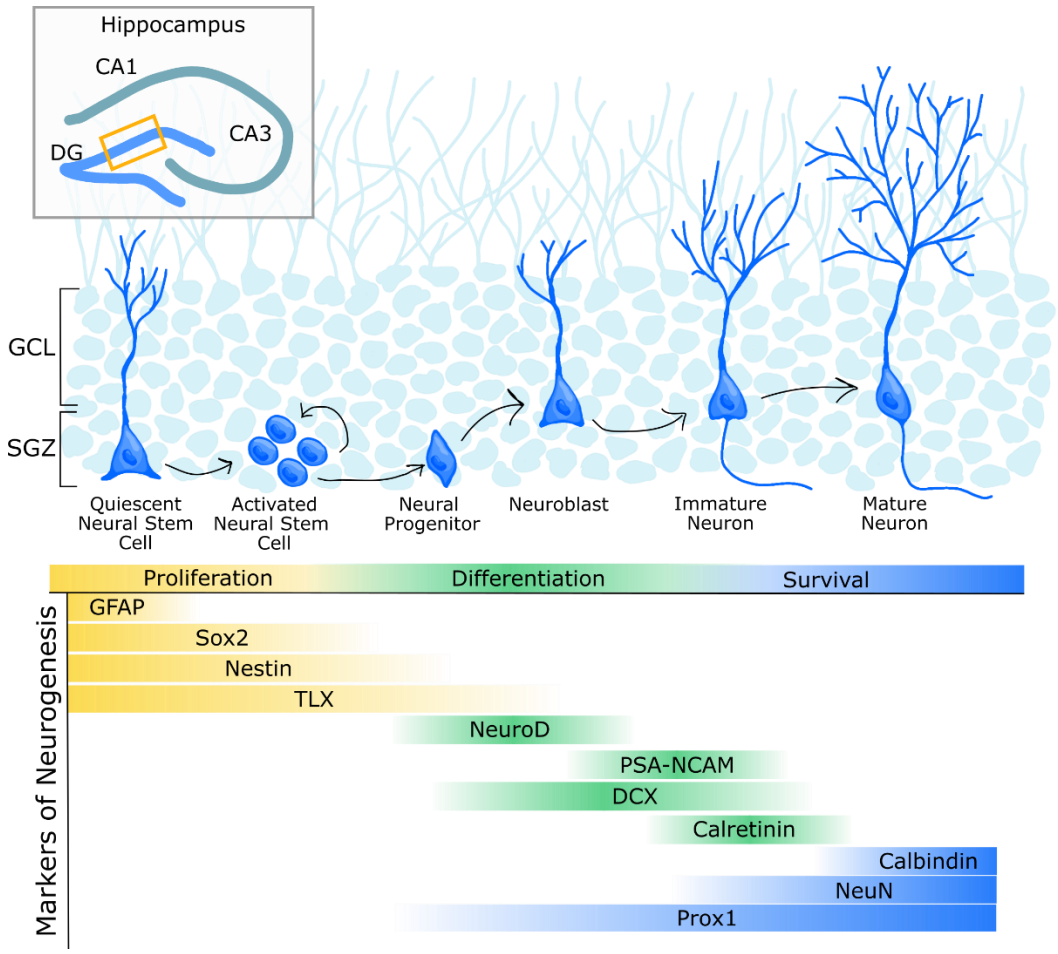


Figure 1.12. Common markers for various stages of hippocampal neurogenesis. Schematic representation of hippocampal neurogenesis in the mouse dentate gyrus (DG). Quiescent neural stem cells in the subgranular zone (SGZ) undergo activation yielding daughter cells that can self-renew the pool of neural stem cells or differentiate to generate neural progenitor cells and eventually neurons. Neuroblasts that survive subsequent phases of maturation will migrate into the granule cell layer (GCL) and turn into granule neurons. Finally, these cells will incorporate into hippocampal circuitry. Various commonly used markers expressed at different stages of hippocampal neurogenesis are indicated. Figure adapted from (Babcock et al. 2021)

While thymidine-H^3 tagging is no longer popular for assessing hippocampal neurogenesis, the thymidine analogue bromodeoxyuridine (BrdU) has become a widely used method to answer multiple questions around hippocampal neurogenesis. Upon administration via injection, BrdU incorporates into the DNA during the S-phase of the cell cycle, when cells are undergoing division (Taupin 2007). BrdU does not specifically label cells fated to become neurons, and thus must be used with additional neuronal markers, such as DCX, which is expressed by immature neurons, or NeuN, which is expressed by mature neurons (Taupin 2007). BrdU can thus be used to

measure either neuronal proliferation or survival, depending on when it was administered in relation to the intervention and tissue collection, and can later be analysed via immunohistochemistry. Similarly, the endogenous marker for cellular proliferation, Ki67, can be used in combination with immature neuronal-specific markers, such as DCX, to detect proliferation of cells during neurogenesis (Kee *et al.* 2002).

While immunohistochemistry currently offers the best clarity for understanding hippocampal neurogenesis due to its ability to inform about location and maturity of individual hippocampal neurons, gene expression analysis can also provide information about hippocampal neurogenesis. The relative expression of genes that are related to neurogenesis can be measured in hippocampal tissue via polymerase chain reaction or RNA sequencing and may be used to infer the relative level of hippocampal neurogenesis. The expression of endogenous genes that have been implicated in hippocampal neurogenesis can also be used to infer the potential state of hippocampal plasticity within tissue. Some genes that may be assessed include Glutamate Ionotropic Receptor NMDA Type Subunit 1 (Grin1), which supports synaptic plasticity and neurodevelopment (Platzer *et al.* 1993; Hasan *et al.* 2013), glucocorticoid receptor nr3c1 which can inhibit the cell cycle to regulate neurogenesis (Shin *et al.* 2015), or neurotrophic factors like (BDNF), which is an important regulator of hippocampal progenitor cell proliferation, maturation and survival (Vicario-Abejon *et al.* 1995; Choi *et al.* 2009). Nonetheless, gene expression analysis is often limited by the lack of single cell resolution that immunohistochemistry provides.

1.6.3 Functions of hippocampal neurogenesis

1.6.3.1 Learning and memory

Hippocampal neurogenesis is highly sensitive to external events, including cognitive tasks. Evidence that hippocampal neurogenesis is responsive to learning was first demonstrated in 1999 when male rats were administered BrdU one week prior to some of these rats being subjected to hippocampal-dependent spatial learning in the Morris

water maze task (Gould *et al.* 1999). In this task, a large circular pool is filled with water and a platform is hidden below the water's surface. Rodents are trained over several days to locate the hidden platform using spatial cues around the testing room, then finally the hidden platform is removed, and rodents are tested on their latency to enter the quadrant of the maze where the platform was previously located. The dentate gyrus of rats that underwent Morris water maze learning showed around double the amount of new-born neurons (Gould *et al.* 1999). Several additional studies have since supported the findings that spatial learning increased the survival of immature hippocampal neurons (Ambrogini *et al.* 2000; Hairston *et al.* 2005; Epp *et al.* 2007; Epp *et al.* 2010; Epp *et al.* 2011). However, some research has since revealed contradictory findings, demonstrating reduced survival of immature neurons following spatial learning (Dobrossy *et al.* 2003; Ambrogini *et al.* 2004; Mohapel *et al.* 2006), or no effect of spatial learning on hippocampal neurogenesis (Ehninger & Kempermann 2006; Mohapel *et al.* 2006), which suggests that multiple factors, such as learning mode, task difficulty, strain and sex differences, stress differences, and/or differences in method of assessment may underlie differences in observations (Chow *et al.* 2013; Epp *et al.* 2013). Nonetheless, this research highlights the possibility of a functional role for adult hippocampal neurogenesis in spatial learning processes.

Since then, numerous studies have implicated adult hippocampal neurogenesis in cognitive performance, including learning and memory (Imayoshi *et al.* 2008; Sahay *et al.* 2011). For instance, roughly twenty years ago, Shors and colleagues demonstrated that the generation of hippocampal neurons in adulthood was critical for trace conditioning memory (Shors *et al.* 2001). Furthermore, performance in the Morris water maze task, a behavioural test used to assess long-term spatial memory and learning in rodents, is strongly linked to hippocampal neurogenesis (Garthe & Kempermann 2013). Ablating hippocampal neurogenesis via inhibiting WNT signalling or the inducible transgenic regulation of the pro-apoptotic protein Bax in neural precursor cells impaired the ability of rodents to locate the hidden platform (Dupret *et al.* 2008; Jessberger *et al.* 2009). Furthermore, the performance of aged rats in the Morris water maze was sufficient to predict the level of neurogenesis in the hippocampus (Drapeau *et al.* 2003). This, coupled with the fact that adult hippocampal

neurogenesis declines with aging (Klempin & Kempermann 2007), has implicated the aging-related reduction in adult hippocampal neurogenesis as a potential contributor to aging-related cognitive decline .

Hippocampal neurogenesis also supports pattern separation ability and novel object recognition. Pattern separation is the ability to discriminate between overlapping contextual representations. Ablating adult hippocampal neurogenesis impairs pattern separation ability (Tronel *et al.* 2012), including spatial pattern separation and spatial relational memory in rodents (Clelland *et al.* 2009). Meanwhile, mice with genetically increased hippocampal neurogenesis showed improved pattern separation (Sahay *et al.* 2011). Reducing neurogenesis in the dentate gyrus also led to impaired object recognition in adult rats (Jessberger *et al.* 2009).

While hippocampal neurogenesis in humans is difficult to quantify due to the technical limitations around securing, collecting, and processing human *post mortem* tissue in a timely manner, accumulating evidence demonstrates that hippocampal neurogenesis is reduced in patients with clinically diagnosed cognitive disruptions, such as Alzheimer's Disease (Diaz-Moreno *et al.* 2018). Fascinatingly, the severity of cognitive decline in Alzheimer's Disease was linked with a progressive reduction in the quantity and maturity of hippocampal neurons (Moreno-Jimenez *et al.* 2019). Studies involving rodent models of Alzheimer's disease and mild cognitive impairment also display disrupted hippocampal neurogenesis (Haughey *et al.* 2002; Babcock *et al.* 2021). Furthermore, drug treatments for Alzheimer's Disease, including tacrine, memantine, and galantamine, are known to increase the proliferation of neuronal cells in naïve male mice (Jin *et al.* 2006), although whether increased hippocampal neurogenesis is required for their pro-cognitive effects is uncertain.

1.6.3.2 Stress-related psychiatric disorders

Stress, such as acute or psychological stress, is the physiological response that occurs in response to a perceived threat and alters the homeostasis in an organism. Stress is a risk factor for depression and anxiety disorders, and is hallmarked for altering an

organism's homeostasis, triggering the hypothalamic–pituitary–adrenal (HPA) axis and initiating a release of cortisol, or corticosterone in rodents.

Animal models of stress consistently demonstrate that stress during adulthood decreases hippocampal neurogenesis, reducing neuron proliferation and survival (Tanapat *et al.* 1998; Gould & Tanapat 1999; Naninck *et al.* 2015). Interestingly, adult hippocampal neurogenesis has also been shown to buffer an individual's behavioural and biological response to stress (Snyder *et al.* 2011; Levone *et al.* 2015). The exact relationship between neurogenesis and stress resilience remains unclear; contradictory evidence has shown that ablating hippocampal neurogenesis inhibits social avoidance following social defeat stress (Lagace *et al.* 2010), and conversely increases stress susceptibility to stress-induced depression-like behaviour (Mateus-Pinheiro *et al.* 2013). Nonetheless, promoting adult hippocampal neurogenesis in mice following exposure to unpredictable chronic mild stress was sufficient to normalize the hypothalamic-pituitary-adrenal axis stress response and produce some behavioural antidepressant-like effects (Culig *et al.* 2017). As previously discussed, there is evidence that the hippocampus is functionally segregated along its longitudinal axis, including in its response to stress and antidepressant action (O'Leary & Cryan 2014; Levone *et al.* 2021b). For instance, inhibiting adult hippocampal neurogenesis in the ventral, but not dorsal hippocampus prevented the anxiolytic effects of the antidepressant fluoxetine (Wu & Hen 2014). The effects of stress on regulating hippocampal neurogenesis in a region-dependent manner may be dependent on differences in neural progenitor cell sensitivity to glucocorticoids (Levone *et al.* 2021a).

Common antidepressants, including fluoxetine, promote hippocampal neurogenesis (Duman *et al.* 2001). These pro-neurogenic properties of antidepressants are required for the beneficial effects of antidepressant drugs (Hill *et al.* 2015). For instance, disrupting antidepressant-induced hippocampal neurogenesis was sufficient to inhibit rodent anxiolytic behavioural response to fluoxetine (Santarelli *et al.* 2003). Additionally, increasing hippocampal neurogenesis via deletion of the Bax gene in neural stem cells of adult mice led to reduced anxiety-like and depressive-like

behaviours (Hill *et al.* 2015), while impairing adult hippocampal neurogenesis promoted anxiety-like behaviour (Revest *et al.* 2009). Ultimately, concrete evidence suggests that adult hippocampal neurogenesis plays an important role in regulating the stress response as well as depressive-like and anxiety-like behaviour.

1.7 Hippocampal neurogenesis throughout the lifespan

Due to the importance of hippocampal neurogenesis in regulating cognition, emotion, and stress-related psychiatric diseases, it is increasingly important to understand the factors influencing hippocampal neurogenesis (Figure 1.13), and how they may potentially be utilized to improve current disease treatment options.

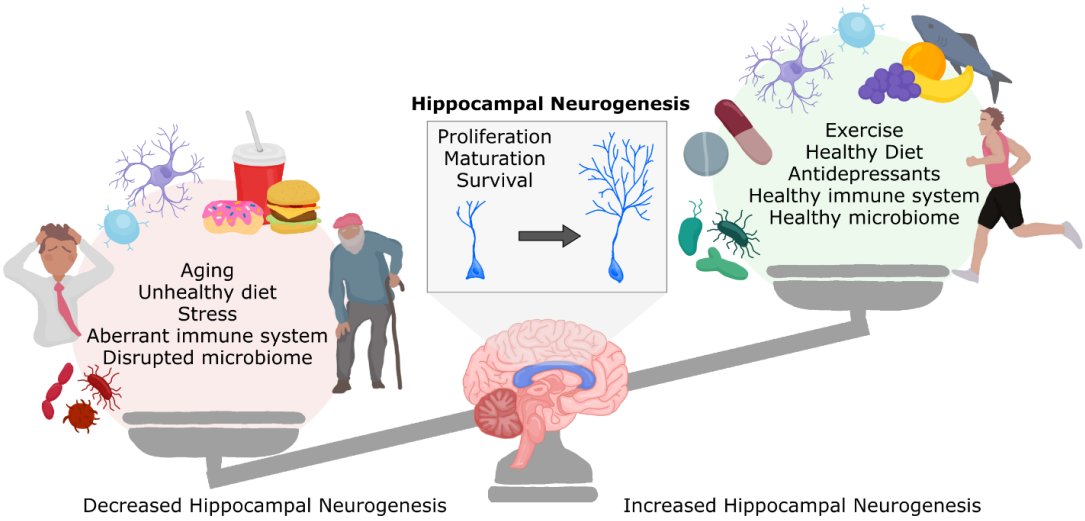


Figure 1.13. Factors influencing adult hippocampal neurogenesis. Adult hippocampal neurogenesis is increased following exercise, antidepressant drug consumption, a healthy diet, neurogenic immune signaling, and a healthy gut microbiome. However, other factors can be detrimental to adult hippocampal neurogenesis, including aging, diets high in fat and sugar, stress, an aberrant immune system, and disruption of the gut microbiome. These factors can influence the proliferation, maturation and survival of neurons in the adult hippocampus. Figure adapted from (Cruz-Pereira et al. 2020)

1.7.1 Hippocampal neurogenesis in early life

The hippocampus is rapidly developing throughout the perinatal period. The dentate gyrus begins to form during late embryogenesis, when granule cells originating from the neuroepithelium migrate from the waning secondary dentate matrix towards the subpial zone and begin to form the outer shell of the granular layer (Altman & Bayer 1990a; Li *et al.* 2009). Following birth, the hilus forms a new zone of proliferating

neural stem cells, along with a glial scaffold that supports the migration of neurons as well as acting as a source of precursor cells for neurogenesis and gliogenesis (Brunne *et al.* 2013; Lajud & Torner 2015). In rodents, around 85% of dentate granule cells are generated after birth, with granule cell proliferation peaking around postnatal day (P) 10 (Bayer 1980; Rakic & Nowakowski 1981; Altman & Bayer 1990a; Gould & Cameron 1996; Huang 2014). Neural stems cells are largely abundant in rodents until P14, after whence their quantity gradually begins to decline (Malatesta *et al.* 2000), though synaptogenesis, another important contributor to hippocampal plasticity, continues regularly for several weeks (Seress *et al.* 2001). Around the third and fourth weeks of rodent life, overlapping the timeframe of weaning from breastmilk to solid food, the tertiary dentate matrix recedes, and the subgranular zone of the dentate gyrus becomes the primary neurogenic niche in the hippocampus for the remainder of life (Altman & Bayer 1990b). Unfortunately, due to difficulties in obtaining and assessing neurogenesis in human tissue, the developmental trajectory of hippocampal neurogenesis, especially during early life, is largely unstudied. Research on macaque monkey neurodevelopment suggests that primates have a larger relative proportion of granule cells at birth than rodents, with 40% of all dentate gyrus granule cells added postnatally in 5–10-year-old macaques, and hippocampal neurogenesis peaking within 3 months of birth (Jabes *et al.* 2010). Therefore, hippocampal neurogenesis within a 3-month-old macaque may most closely relate to the age of P10 in rodents. The exact age which this is resembled in humans is yet to be determined, though literature suggests a steady decline in human hippocampal cell proliferation and immature neuronal populations post-birth with few immature hippocampal neurons detected beyond infancy (1 year of age) (Sorrells *et al.* 2018).

Early life hippocampal neurogenesis appears critical for priming an individual for success in adulthood. Disrupting hippocampal neurogenesis during the adolescent window of P21 to P50 via radiation exposure led to reduced hippocampal neuronal proliferation and survival in adulthood, coinciding with impaired fear-conditioning and worsened spatial cognition in the Morris water maze (Rola *et al.* 2004; Achanta *et al.* 2009). Hippocampal neurogenesis is also disrupted in preclinical models of various disorders that affect cognition or emotional behaviour and commonly present during

late adolescence or early adulthood, including stress-inducible depressive-behaviour and antidepressant action (Sahay & Hen 2007), schizophrenia (Kempermann *et al.* 2008), and attention-deficit hyperactivity disorder (Diaz-Granados *et al.* 1994; Lee *et al.* 2012), suggesting that perturbances to early life hippocampal neurogenesis may underlie these conditions, although further research into the neurobehavioral consequences of perturbed early life hippocampal neurogenesis needs to be conducted.

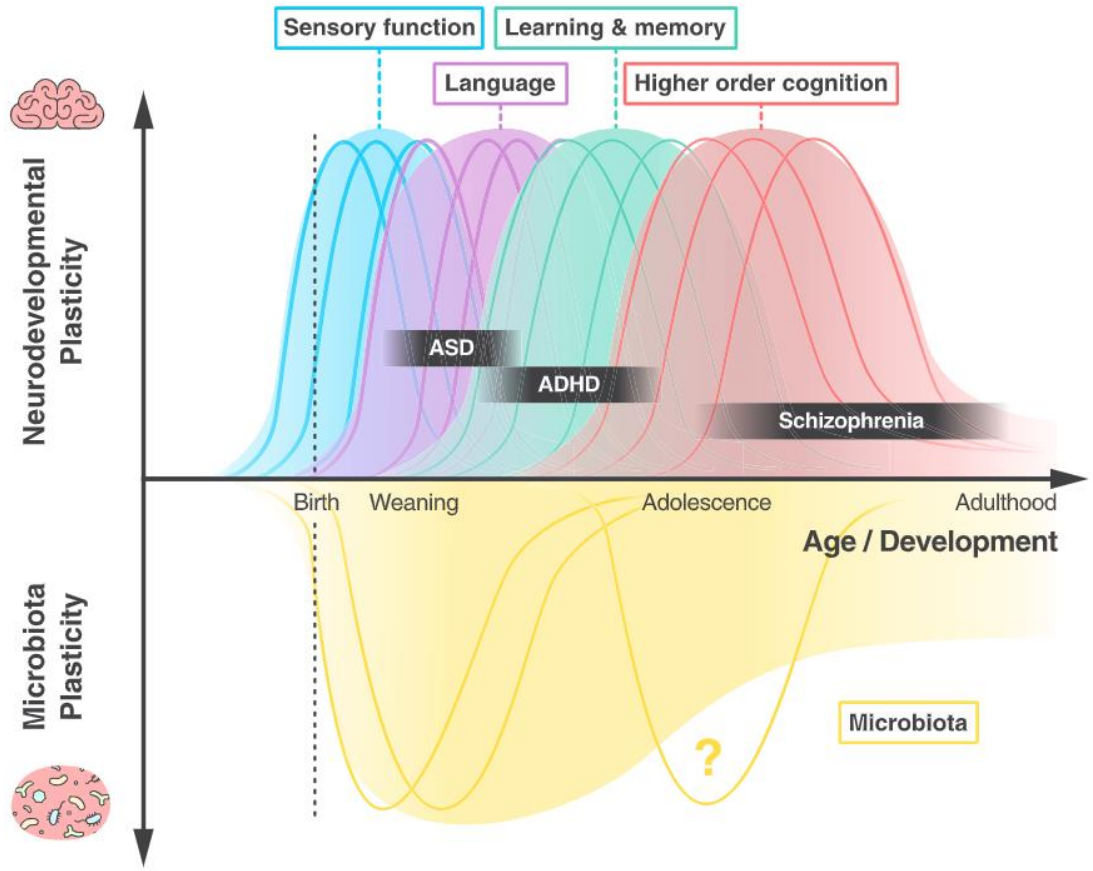


Figure 1.14. Critical windows of neurodevelopment and plasticity in the brain overlap with timeframes of increased microbiota plasticity. Figure from (Cowan et al. 2020)

1.7.2 Hippocampal neurogenesis and aging

Hippocampal neurogenesis dramatically declines during physiological aging in rodents, primates, and humans (Kuhn *et al.* 1996). Aged rodents (20-24 months) have a substantial loss in dentate gyrus progenitor proliferation, leading to a reduction in the production of new-born granule cells (Kempermann *et al.* 1998; Klempin &

Kempermann 2007). Furthermore, senescence of the hippocampal neurogenic niche reduces the proportion of neural stem cells that are actively dividing (Diaz-Moreno *et al.* 2018; Kalamakis *et al.* 2019), perhaps due to a depletion in the quantity of neural stem cells (Encinas *et al.* 2011).

This reduction in neurogenesis is noticeable in mice as young as 6 months of age, and correlates to impaired cognition (Imayoshi *et al.* 2008) as well as the progression of cognitive decline in Alzheimer's Disease in rodent models and human *post mortem* tissue (Kozareva *et al.* 2019a; Moreno-Jimenez *et al.* 2019). Fascinatingly, rejuvenating the aged neuronal niche has been shown to rescue aging-associated cognitive deficits (Navarro Negredo *et al.* 2020). Furthermore, lifestyle factors such as exercise and cognitive stimulation promote hippocampal neurogenesis and may be protective against neuropathologies including cognitive decline (Kempermann *et al.* 1997; Kramer *et al.* 1999; Kramer *et al.* 2006). This evidence suggests that targeting the aging-associated decline in hippocampal neurogenesis may be an important strategy for developing novel therapeutics for treating declining cognition in aging.

1.7.3 Factors influencing hippocampal neurogenesis

In addition to aging, environmental factors and lifestyle choices including stress, antidepressants, diet, exercise, and environmental enrichment contribute to hippocampal neurogenesis. The impact of these factors on hippocampal neurogenesis are briefly discussed below and highlighted in Figure 1.13.

1.7.3.1 Stress and antidepressants

The relationships between stress, antidepressant treatment, and adult hippocampal neurogenesis, including the functional importance of adult hippocampal neurogenesis for regulating stress response and antidepressant action, has been discussed previously. Nonetheless, it is important to recall that stress has been shown to decrease adult hippocampal neurogenesis by reducing neuron proliferation and survival (Tanapat *et al.* 1998; Gould & Tanapat 1999; Naninck *et al.* 2015). Meanwhile, antidepressant treatments have the ability to increase adult hippocampal neurogenesis

(Duman *et al.* 2001; Santarelli *et al.* 2003; Surget *et al.* 2011). Additionally, as discussed previously, both stress (O'Mahony *et al.* 2020; Bastiaanssen *et al.* 2021) and antidepressants (Cussotto *et al.* 2019) can alter the gut microbiome, which may relate to their ability to influence neurogenesis. Still, the relevance of hippocampal neurogenesis in clinical patients has been difficult to assess due to the limitations in acquiring and researching human *post-mortem* tissue.

Furthermore, stress that occurs earlier in life may yield different effects on hippocampal neurogenesis which persist into adulthood in a sex-specific manner. In females, early life stress, such as maternal separation stress, impairs hippocampal neurogenesis prior to puberty, although this effect normalizes in adulthood (Loi *et al.* 2014). Counterintuitively, male rats that were exposed to maternal separation stress expressed a short-term enhancement of hippocampal neurogenesis prior to puberty, although this consequentially impaired neurogenesis in adulthood (Loi *et al.* 2014). This data was supported by additional findings that juvenile stress between P25-P27 decreased ventral hippocampus neuronal proliferation in male, but not female, adult rats (Brydges *et al.* 2018). Separately, prepubertal juvenile stress was not found to induce changes in hippocampal neuron survival or maturation in neither male nor female, indicating this model of juvenile stress specifically alter to neuronal proliferation (Harris *et al.* 2022).

1.7.3.2 Environmental enrichment and exercise

Environmental enrichment, including exercise, is an established method for increasing adult hippocampal neurogenesis in healthy adult rodents (van Praag *et al.* 2005). The addition of environmental stimuli increases the number of hippocampal neurons in adult mice (Kempermann *et al.* 1997). Through the promotion of hippocampal neurogenesis, environmental enrichment is able to promote the recovery from psychosocial stress (Schloesser *et al.* 2010). Exercise is another form of environmental enrichment that promotes the production and survival of neurons in the adult hippocampus. Although the mechanisms by which exercise promote hippocampal neurogenesis are not fully established, the exercise-induced release of serotonin in the central nervous system is key for the promotion of neuronal

proliferation (Klempin *et al.* 2013). Additionally, the peripheral blockade of vascular endothelial growth factor (VEGF) prevented the effects of exercise on hippocampal neurogenesis (Fabel *et al.* 2003), indicating that both central and periphery factors may be key for the pro-proliferative effects of exercise. In addition to voluntary exercise and environmental enrichment, training rodents with tasks that involve hippocampal-dependent learning and memory increases gyrus granule cells (Gould *et al.* 1999; Aimone *et al.* 2006). As discussed previously, exercise also impacts the gut microbiome (Clarke *et al.* 2014; Bressa *et al.* 2017), and some of the mediators believed to be involved in the mechanism of exercise-induced increases in hippocampal neurogenesis have also been shown to be regulated by the gut microbiota (Kang *et al.* 2014; Cavallucci *et al.* 2020). Recently, the gut microbiota from mice that experienced environmental enrichment was shown to promote neuronal plasticity in the visual cortex, potentially via modulation of short-chain fatty acids (Lupori *et al.* 2022), though whether the gut microbiota drives similar pro-plasticity occurrences in the hippocampus is currently unknown.

1.7.3.3 Diet

Preclinical dietary manipulations have revealed the importance of diet in promoting and maintaining adult hippocampal neurogenesis. Diets high in fat and sugar decrease hippocampal cell proliferation, neuron survival and hippocampal-dependent behaviour (Lindqvist *et al.* 2006; Ribeiro *et al.* 2020; Robison *et al.* 2020) potentially through the regulating neuronal mitochondria biogenesis (Ribeiro *et al.* 2020) and/or the increase in circulating corticosterone (Lindqvist *et al.* 2006). Meanwhile, long-term running exercise was able to counteract the consequences of a high-fat on new-born neuron survival in the dentate gyrus and promote spatial learning and flexible memory performance in the Morris water maze task (Klein *et al.* 2016). Alternatively, caloric restriction promotes hippocampal neurogenesis, likely through the upregulation of brain-derived neurotrophic factor (Lee *et al.* 2002a; Lee *et al.* 2002b).

Dietary supplementation with prebiotics and probiotics, which influence the gut microbiome, have also been shown to alter hippocampal neurogenesis. Diets enriched in prebiotic polyphenols can promote hippocampal neuron proliferation and survival

(Casadesus *et al.* 2004; An *et al.* 2008; Sarubbo *et al.* 2018) and ameliorate early life stress-induced impairments to behaviour (Donoso *et al.* 2020). Meanwhile, supplementation of specific bacteria can increase hippocampal neurogenesis (Ait-Belgnaoui *et al.* 2014; Petrella *et al.* 2021) and mitigate neuroinflammatory responses to lipopolysaccharide injection (Petrella *et al.* 2021).

1.7.3.4 Biological sex

Despite the issue that sex-related differences in biology have been widely historically ignored in research, biological sex has been shown to determine the rate of proliferation and differentiation of cells into neurons within the hippocampus. In rats administered BrdU, new-born male rats showed increased cellular proliferation in the hippocampus compared to age-matched females (Bowers *et al.* 2010). Interestingly, treatment with the sex-dependent hormone oestradiol was able to elevate cell proliferation in the hippocampus of female, but not male, rats, and many of these cells matured into neurons in the CA1 region (Bowers *et al.* 2010). These effects are time-sensitive, persisting until 3 weeks of age. Moreover, the administration of tamoxifen, an oestrogen receptor antagonist, significantly reduced hippocampal cell proliferation in male, but not female, rats (Bowers *et al.* 2010), strongly implicating hormonally mediated pathways in the sex-dependent differences of early life hippocampal neurogenesis. Within the hippocampus, androgen and oestrogen receptors are also expressed in a region-specific and sex-specific manor; the CA3 and CA4 regions of adult female rats have more β oestrogen receptors than male rats (Zhang *et al.* 2002), while male rats have greater androgen receptors in the CA1 and dentate gyrus, depending on the phase of oestrus cycle (Feng *et al.* 2010). Interestingly, the female rodent oestrus cycle is able to influence cellular proliferation in the dentate gyrus. During proestrus, female Sprague Dawley rats had increased cell proliferation than non-proestrus female rats and also male rats (Tanapat *et al.* 1999). Contrastingly, male Wistar rats have been shown to have increased numbers of immature neurons in their dentate gyrus compared to females (Hillerer *et al.* 2013).

While sex-dependent differences in hippocampal neurogenesis are apparent under normal conditions, further disparities arise between males and females under states of

stress or learning and are also age-specific. The male hippocampus responds more reductively to stress, with several studies finding that male, but not female, rodents had reduced cellular proliferation and/or neurogenesis when exposed to chronic or acute stressors (Falconer & Galea 2003; Hillerer *et al.* 2013; Naninck *et al.* 2015). Interestingly, chronic stress during adolescence has shown the opposite effect, whereby female mice subjected to restraint stress during adolescence had reduced hippocampal neurogenesis in adulthood, but hippocampal neurogenesis was increased in male mice subjected to the same conditions (Barha *et al.* 2011). However, altered hippocampal neurogenesis was not observed in male or female rats subjected to juvenile stress (Harris *et al.* 2022). Meanwhile, memory training in the Morris water maze task increased hippocampal neurogenesis in males, but not female, Sprague Dawley rats (Chow *et al.* 2013), indicating that hippocampal neurogenesis responds uniquely to environmental stimuli depending on the sex of the host. The ability for sex to influence hippocampal neurogenesis may therefore underly or drive disparities in the susceptibility of males and females to diseases linked to hippocampal neurogenesis, such as Alzheimer's Disease and depression, although the limitations of assessing hippocampal neurogenesis in humans makes this difficult to assess.

1.8 Gut and microbially derived factors influencing hippocampal neurogenesis

Multiple gut-derived factors can influence the birth, development, and survival of new neurons in the hippocampus. Research involving vagotomy, germ-free mice and microbiota perturbation techniques has allowed for increased understandings of the mechanisms by which the gut microbiota-gut-brain axis can impact host hippocampal neurogenesis.

1.8.1 Vagus nerve signalling

Vagus nerve stimulation is approved therapy for difficult to treat depression and like many other antidepressant treatments VNS has been shown to increase some aspects of adult hippocampal neurogenesis. Stimulation of the vagus nerve has been reported to trigger increased progenitor cell proliferation, numbers of maturing neurons, and

BDNF protein expression in the hippocampus of rodents (Revesz *et al.* 2008; Biggio *et al.* 2009). Chronic simulation of the vagus nerve also promotes dendritic branching on immature hippocampal neurons (Biggio *et al.* 2009). Meanwhile, severing the vagus nerve via subdiaphragmatic vagotomy was sufficient to reduce hippocampal cell proliferation, neuronal differentiation, and survival, and BDNF mRNA expression in the adult mouse hippocampus (O'Leary *et al.* 2018).

Through virus-based tracing techniques, it was possible to determine that vagal signals derived from the gut control hippocampal function and hippocampus-dependent episodic and spatial memory (Suarez *et al.* 2018) though whether these signals were derived specifically from microbiota-vagus nerve interactions was not determined. Nonetheless, specific gut bacteria, such as *Lactobacillus rhamnosus,* demonstrate the ability to alter depressive-like behaviour, hippocampal microglia density, and specific hippocampal protein expression through the vagus nerve in preclinical research (Bravo *et al.* 2011b; Liu *et al.* 2021). The ability for oral *Lactobacillus rhamnosus JB1* treatment to alleviate the impact of stress on behaviour may be related to its ability to decrease the expression of the hippocampal $GABA_{B1b}$ gene (Bravo *et al.* 2011b), a receptor subunit that plays a crucial role in mediating stress resilience and the production of new hippocampal neurons (O'Leary *et al.* 2014).

1.8.2 Immune system and neuroinflammation

The gut microbiota regulates several aspects of the host immune system, including in the brain, allowing the gut microbiota to influence hippocampal neurogenesis via immune signalling pathways. For instance, germ-free mice have defected hippocampal microglia, as well is a large increase in the density of microglia present (Erny *et al.* 2015). The regulation of microglia by the gut microbiota appears to be at least partially controlled by microbially produced acetate, which can translocate from the gut to influence the maturity and function of microglia in the brain (Erny *et al.* 2021). Microglia contribute to the balance of neuronal cell birth and death in the subgranular zone through apoptosis-coupled phagocytosis (Sierra *et al.* 2010), allowing them to clear debris from destroyed cells. In response to phagocytosis, the microglia secretome is altered towards substrates that have been shown to support new neuron proliferation

(Sierra *et al.* 2010; Diaz-Aparicio *et al.* 2020). Furthermore, upon activation, microglia produce various cytokines to induce an immune response to dangerous threats. Neuroinflammation, mediated by proinflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor α (TNFα), and IL-6, has detrimental consequences to the proliferation and development of newly born hippocampal neuronal cells (Ekdahl *et al.* 2003; Monje *et al.* 2003; Green & Nolan 2012; Pawley *et al.* 2020). By regulating the function and maturity of hippocampal microglia, the gut microbiota may be able to reshape hippocampal neurogenesis, although this direct mechanistic link is yet to be established.

In addition to impacting the function of microglia, the gut microbiota contributes to the systemic priming of immune cells that may traffic to the brain. Through the use of genetic knockout mice and antibody depletion, Ly6C^{hi} monocytes were shown to play a pivotal role in promoting hippocampal neurogenesis (Mohle *et al.* 2016). Ly6C^{hi} monocytes traffic throughout the body and are highly sensitive to gut microbiota-derived signals. Following antibiotic treatment, mice had lower numbers of Ly6C^{hi} monocytes in the brain, blood, and bone marrow, and displayed deficits in hippocampal neurogenesis and behaviour (Mohle *et al.* 2016). Adaptive transfer of Ly6C^{hi} monocytes to antibiotic-treated mice was able to restore cellular proliferation and neuron survival in the dentate gyrus (Mohle *et al.* 2016). Fascinatingly, supplementation with an 8-strain probiotic cocktail was also able to rescue antibiotic-induced deficits in hippocampal neurogenesis and Ly6C^{hi} monocyte levels, providing further evidence that the gut microbiota can regulate hippocampal neurogenesis through Ly6C^{hi} monocytes (Mohle *et al.* 2016).

Specific gut microbes also play a role in instigating T cell mediated depressive-like behaviours, although the involvement of hippocampal neurogenesis in this pathway has yet to be explored. T cells are essential regulators of hippocampal neurogenesis. After homing to regions of damage, T cells activate and produce cytokines which program microglia to support neuronal survival and renewal (Butovsky *et al.* 2005; Shaked *et al.* 2005). Furthermore, CD8+ T cells are required for the beneficial effects of environmental enrichment on hippocampal neurogenesis, synaptic plasticity, and

behaviour including anxiety-like behaviour and spatial learning in mice (Zarif *et al.* 2018). Meanwhile, depletion of CD4+ T lymphocytes significantly reduced hippocampal neurogenesis, while neuronal precursor cell proliferation was increased in $Rag2^{-/-}$ mice following repopulation of CD4+ cells (Wolf *et al.* 2009). Another subset of T cells, T helper 17 (Th17) cells, can also produce toxic IL-17 (Langrish *et al.* 2005), which inhibits adult hippocampal neurogenesis (Liu *et al.* 2014), and can promote susceptibility to depression (Beurel *et al.* 2013). The gut microbiome has been shown to modulate the function of various types of T cells, including CD8+ T cells (Gonzalez-Perez & Lamouse-Smith 2017; Yu *et al.* 2020), CD4+ T cells (Dillon *et al.* 2016; Edelblum *et al.* 2017), and Th17 cells (Ivanov *et al.* 2009; Medina-Rodriguez *et al.* 2020), suggesting potential T cell-dependent mechanisms by which the gut microbiota might influence hippocampal neurogenesis.

By microbial crosstalk with microglia, monocytes, and T cells, the gut microbiota demonstrates the ability to influence hippocampal neurogenesis in adult rodents. More research needs to be conducted to fully understand the cellular pathways and scope of capability of microbiota-immune signalling on influencing hippocampal neurogenesis.

1.8.3 Microbial Metabolites

Due to the difficulties in tracing the origins of metabolites within a living organism, it is difficult to conclude that specific gut microbiota-derived molecules can translocate from the gut to the brain where they may hinder or support neuronal birth, maturation, and survival. As such, few radioactive tracing studies exist that examine gut microbiota-derived metabolites within the brain. Recently, using radioactive labelling, gut-derived short chain fatty acid acetate was proven to translocate from the gut microbiota into microglia in the mouse brain, where it influenced microglia metabolism and function (Erny *et al.* 2021). While it is possible to speculate that alteration in the functionality of microglia would be able to alter hippocampal neurogenesis, this study did not examine hippocampal neurogenesis. Nonetheless, short chain fatty acids, including acetate, butyrate, and propionate, have been shown to promote neurogenic differentiation in neural stem cells when applied in physiologically relevant levels *in vitro* (Ribeiro *et al.* 2020; Yang *et al.* 2020).

Furthermore, short chain fatty acid supplementation was sufficient to improve spatial learning and memory performance, prevent cognitive impairment, and increase BDNF protein expression in neurodegenerative disease models (Dash *et al.* 2009; Barichello *et al.* 2015; Stilling *et al.* 2016), although hippocampal neurogenesis has not yet been assessed.

The gut microbiota plays a crucial role in producing and manipulating neurotransmitter levels in the gut. Of interest, tryptophan appears as a critical microbiota-modulable metabolite which can impact host cognitive function and hippocampal neurogenesis. Recent research has found that indoles produced by gut bacteria harbouring the ability to metabolize tryptophan can modulate hippocampal neuroplasticity and promote the differentiation of neural progenitor cells through the activation of the aryl hydrocarbon receptor (Wei *et al.* 2021). The gut microbiota can also regulate the production of serotonin in the gut (Reigstad *et al.* 2015). Germ-free mice have increased hippocampal serotonin (Clarke *et al.* 2013), and display increased adult hippocampal neurogenesis (Ogbonnaya *et al.* 2015; Scott *et al.* 2020). However, whether gut-derived serotonin can translocate to the brain to promote hippocampal neurogenesis is currently unknown.

1.9 Hypothesis and overall objectives

The gut microbiota is emerging as a critical regulator of hippocampus-associated processes including cognition and hippocampal neurogenesis. However, little is known about how specific alterations to the gut microbiota may perturb hippocampal neurogenesis and neuroplasticity. Therefore, the overall goal of this thesis was to characterize how various gut microbiota manipulations impact host hippocampus-associated cognitive function and hippocampal neurogenesis throughout the lifespan, with the hypothesis that that perturbance to the gut microbiota will induce distinct alterations in hippocampal neurogenesis, neuroplasticity, and cognitive behaviour. This hypothesis was addressed via the following experimental questions:

1.9.1 Aim 1: To determine whether the gut microbiota are causative factors in aging-induced deficits in cognition, immunity, and hippocampal neurogenesis by transferring the gut microbiota from young individuals into aged mice

Aging has been previously shown to alter the gut microbiota. Furthermore, hippocampal neurogenesis and cognitive processes decline during the aging process. Previous research has demonstrated that microbiota transferred from aged to young mice triggers changes in host hippocampal neurogenesis and immunity. However, no study had sought to address whether rejuvenating effects could occur when microbiota were transferred from young to aged mice. Therefore, in this study (**Chapter 2)** we investigated whether the transfer of a young microbiota into aged mice was able to rescue these aging-induced deficits in cognitive behaviour, hippocampal neurogenesis, and immunity.

1.9.2 Aim 2: To determine whether specific bacterial consortia associated with aging-related cognitive decline can alter host cognitive behaviour and when transplanted into young naïve rats

Previous research has found that elderly patients diagnosed with mild cognitive impairment harbour distinct gut microbiota and intestinal metrics compared to cognitively healthy elderly. In this experiment **(Chapter 3)** we investigated whether consortia of microbes identified as being more abundant in healthy or cognitively impaired elderly patients were able to induce similar cognitive states when transplanted into naïve adult rats.

1.9.3 Aim 3: To determine whether microbiota-targeted dietary intervention with prebiotic supplementation can rescue early life stress-induced detriments in the gut microbiota and hippocampus

Early life stress, including maternal separation stress, disrupts the gut microbiome and leaves lasting impacts on hippocampal neurogenesis and behaviours. Prebiotics, including polyphenols and fish oil, have previously been shown to alter the gut microbiome and ameliorate some long-term behavioural and physiological deficits

induced by early life stress. However, whether these prebiotics may act through the gut microbiota to induce changes in the hippocampus has not yet been elucidated. In this study **(Chapter 4),** we examined how early life stress and multiple prebiotic treatments impact the functional ability of the gut microbiota, and whether this led to alterations in neuroplasticity-related hippocampal gene expression.

1.9.4 Aim 4: To determine whether birth mode impacts hippocampal neurogenesis in male and female offspring

Caesarean section birth leads to well-characterized distinctions in the gut microbiome that last until around the period of weaning. Furthermore, caesarean section birth is often accompanied with prophylactic antibiotic administration, which has unknown consequences on the offspring's gut microbiota or brain development. Mice born by caesarean section show increased anxiety-like behaviour and deficits in sociability that are ameliorated by targeting the early life gut microbiota. However, whether birth mode impacts hippocampal neurogenesis at any stage of life has never been investigated. Therefore, **in Chapter 5** we investigated whether birth mode altered hippocampal neurogenesis in male and female adolescent mice, including when accompanied by the translationally relevant administration of maternal antibiotics.

Chapter 2

Microbiota from young mice counteracts selective age-associated behavioural deficits

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2.1 Abstract

The gut microbiota is increasingly recognized as an important regulator of host immunity and brain health. The aging process yields dramatic alterations in the microbiota, which is linked to poorer health and frailty in elderly populations. However, there is limited evidence for a mechanistic role of the gut microbiota in brain health and neuroimmunity during aging processes. Therefore, we conducted faecal microbiota transplantation from either young (3–4 months) or old (19–20 months) donor mice into aged recipient mice (19–20 months). Transplant of a microbiota from young donors reversed aging-associated differences in peripheral and brain immunity, as well as the hippocampal metabolome and transcriptome of aging recipient mice. Finally, the young donor-derived microbiota attenuated selective age-associated impairments in cognitive behaviour when transplanted into an aged host. Our results reveal that the microbiome may be a suitable therapeutic target to promote healthy aging.

2.2 Introduction

Aging triggers metabolic and immune alterations that lead to perturbation of brain function and behaviour, including impairments in hippocampal-associated cognitive behaviour (Bettio *et al.* 2017). Notably, the gut microbiota, encompassing the population of trillions of microorganisms, undergoes a parallel community shift, which has been correlated to changes in host frailty and cognition (Claesson *et al.* 2012b; Cryan *et al.* 2019b).

Animal models have shown specific roles for the microbiota in shaping hallmarks of aging in the gut (Stebegg *et al.* 2019; Donaldson *et al.* 2020). Moreover, the consequences of an elderly-associated microbiota on a young host involve alterations in host immunity, neurogenesis, and cognition (Fransen *et al.* 2017; Kundu *et al.* 2019; D'Amato *et al.* 2020; Li *et al.* 2020c). Notably, transferring microbiota from young fish (African turquoise killifish) into middle-aged fish improves lifespan and motor behaviour (Smith *et al.* 2017). However, it is completely unknown whether microbiota from young donors can restore aging-associated impairments in mammals.

To determine whether faecal microbiota transplantation (FMT) from young mice can ameliorate aging-induced neurocognitive and immune impairments, we collected faecal microbiota from naive young mice (3–4 months) and transplanted this into aged mice (‘aged yFMT’, 19–20 months). A separate group of aged mice received faecal microbiota from naive old mice to control for handling during FMT administration (‘aged oFMT’, 19–20 months). To allow aging-associated comparisons, naive young mice received the same yFMT mixture (‘young yFMT’). We found aging-associated differences in microbiota (Fig. 2.1 and Supplementary Tables 2.1 and 2.2), immunity (Fig. 2.2 and Extended Data Figs. 2.2 and 2.3), hippocampal neurogenesis (Extended Data Fig. 2.2), hippocampal metabolomics (Fig. 2.3, Extended Data Fig. 2.7 and Supplementary Table 2.3) and transcriptomics (Fig. 2 and Extended Data Fig. 2.7), and behaviour (Fig. 2.4 and Extended Data Fig. 2.5); some, but not all, of which were attenuated by microbiota transplantation from a young mouse into an aged host. Our research offers the possibility that a microbiota from a young individual may have beneficial effects when given to an aged host.

2.3 Methods

2.3.1 Animals

Male young adult C57BL/6 mice (n = 25; Envigo; 10–12 weeks) and aged C57BL/6 mice (n = 50; Charles River; 19–20 months) were used in this study. All experiments were performed in accordance with European guidelines following approval by University College Cork Animal Ethics Experimentation Committee (AE19130/P052). Animals were habituated to the animal facility for at least 4 weeks before experiments started and were kept under a 12-h light–dark cycle at 21 ± 1 °C and humidity of 55 ± 10%. Food (ENVIGO Teklad Global 18% Protein Rodent Diet (Sterilizable)) and water were given ad libitum.

2.3.2 Faecal collection and faecal microbiota transplant

For all faecal material collection, mice were placed in an empty plastic cage, free of bedding and sterilized with 70% ethanol, until defecation. For future microbiome analysis, faecal pellets were immediately collected and snap-frozen on dry ice, then stored at −80 °C until sequencing.

To collect material for FMT, a separate cohort of naive young adult C57BL/6 mice (n = 40, Envigo; 12–15 weeks at collection, group-housed) and the same aged mice defined above, supplemented with additional naive old mice to ensure enough collection volume (n = 83, 18.8–20 months at collection, group-housed), were used, before the start of the experimental timeline. Faecal pellets were collected fresh and immediately transferred to an anaerobic hood, where they were pooled per group to ensure enough volume. Faecal material was homogenized in reduced sterile phosphate-buffered saline (PBS) with 20% glycerol (w/v) and filtered through a 70-µm strainer to remove large particles before being aliquoted and frozen at −80 °C. To ensure enough volume of FMT material without risking potential cross contamination, collection was spaced out over six to nine collection days per group, one group at a time. before FMT, equal aliquots from every collection day were pooled together to consistently generate enough volume.

FMT was performed by oral gavage biweekly (100 µl of 100 mg ml−1 homogenized faecal slurry). Briefly, mice were gently scruffed and faecal slurry was administered through an oral gavage needle (23 G). FMT occurred once per day for the first three days to encourage microbiota engraftment, then twice per week thereafter.

2.3.3 Study design and experimental timeline

Aged mice were randomly delegated into one of the two groups, balanced by weight: aged mice receiving FMT from young donor mice (aged yFMT) and to control for handling, aged mice receiving FMT from old donor mice (aged oFMT). Young mice were given FMT from young mice (young yFMT) to allow for inter-age comparisons. To ensure appropriate engraftment of microbiota and to allow for potential microbiota-driven neurocognitive and behavioural effects, mice were orally gavaged with FMT once per day for the first 3 days, then twice weekly thereafter in line with previous research (Bruce-Keller *et al.* 2015).

Baseline faecal samples were collected before the start of the study and after 4 weeks of FMT inoculation to assess the effects of FMT on recipient faecal microbiota before the start of the behavioural battery. Following 4 weeks of FMT, blood was collected and flow cytometry was run to assess markers of mesenteric and peripheral immunity accompanied with cytokines measurements (see sections on flow cytometry methodology and cytokine assessment). Mice then underwent a series of behavioural tests designed to assess a variety of locomotor, social, cognitive and anxiety-like parameters, including the following: Y-maze, novel object recognition test (NOR), elevated plus maze (EPM), three-chamber social interaction test (SIT) (sociability and social memory) and the Morris water maze (MWM). Finally, mice were killed via decapitation, and blood and tissues were immediately collected for further analysis (Fig. 2.1a).

Aged and young mice were monitored throughout the study through a dedicated observation battery that comprises different aspects of health, including general appearance, physical characteristics and sensorimotor reflexes (Roux *et al.* 2005; O'Leary *et al.* 2016) (Supplementary Table 7).

An additional study was performed following a similar design to allow for visualization and further investigation of potential mechanisms of action, including microglia and adult hippocampal neurogenesis. Herein, mice were treated once per day with 150 mg kg−1 body weight BrdU (Sigma-Aldrich, cat. no. 59-14-3) diluted to 20 mg ml−1 in sterile saline via intraperitoneal injection during the first three FMT inoculation days to label proliferating cells. Following 4 weeks of FMT inoculation, the novelty-induced hypophagia (NIH) test was performed. Finally, mice were anesthetized with 90 mg kg−1 pentobarbital and tissues were fixed via transcardial perfusion

with chilled sterile saline for 2 min, followed by 8 min of 4% paraformaldehyde (PFA). Brains were collected and transferred into 4% PFA overnight, then transferred to 15% sucrose overnight and finally 30% sucrose for 24 h before being snap-frozen in isopentane and stored at −80 °C.

2.3.4 DNA extraction for 16S rRNA amplicon microbiota analysis

DNA was extracted using the QiaAMP Power Faecal Pro kit (QIAGEN) according to the manufacturer's instructions. DNA concentration was normalized and 16S rRNA amplicon libraries were prepared using primers to amplify the V3–V4 region of the bacterial 16S rRNA gene, with Illumina adapters incorporated as described in the Illumina 16S rRNA Metagenomic Library Preparation guide, with the exception that 30 amplification cycles were used. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. The pooled libraries were assessed using an Agilent high sensitivity DNA kit and examined by quantitative PCR (qPCR) using the Kapa Quantification kit for Illumina (Kapa Biosystems) according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced (2 × 300 bp) on the Illumina MiSeq platform.

2.3.5 16S microbiota analysis and bioinformatics

Paired-end reads were pre-filtered on the basis of a quality score threshold of >28 and trimmed, filtered for quality and chimeras using the DADA2 library in R (v.3.6.3) (Callahan *et al.* 2016). Quality control was performed using the fastqc program in Ubuntu v.18.04. Samples with fewer than 10,000 reads after filtering were discarded. Taxonomy was assigned with DADA2 against the SILVA SSURef database release v.138 (Quast *et al.* 2013). Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. Amplicon sequence variants were aggregated at genus level. Those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as nonzero in 10% or fewer of total samples. After filtering, 86 different genera were left in total over all microbiome samples.

Microbiota bioinformatics were performed in R (v.3.6.3) with the Rstudio GUI (v.1.2.5033). Principal component analysis was performed on CLR-transformed values using the ALDEx2 library (Fernandes *et al.* 2014). The number of permutations was always set to 1,000. The iNEXT

library was used to calculate α-diversity using the Chao1, Shannon and Simpson indices. β-diversity was assessed in terms of Aitchison distance and visualized via principal-component analysis (Hsieh *et al.* 2016). Wd* (Hamidi *et al.* 2019) followed by a pairwise post hoc and Bonferroni test was used to find structural differences between treatments on a compositional level. The degree of change per mouse through time was calculated in terms of Aitchison distance, using one-way ANOVA followed by Tukey's test. Degree of change in the microbiome after treatment or volatility was calculated as Aitchison distance (Bastiaanssen *et al.* 2021). The Piphillin web server was used to infer the functional metagenome in terms of Kos (Iwai *et al.* 2016). Using these KOs, GBMs were calculated using the omixerRpm R library (Valles-Colomer *et al.* 2019). Differential abundance of microbial genera as well as functional modules was calculated using the Wilcoxon signed-rank test implementation in the ALDEx2 library. To correct for multiple testing (FDR) in tests involving microbiota features, the internal ALDEx2 implementation of the Benjamini–Hochberg post hoc procedure was performed with a q-value of 0.1 as a cutoff. All custom R scripts are available online at https://github.com/thomazbastiaanssen/Tjazi. To assess gain and/or loss of genus events, we tallied all occurrences of a genus being present in one of the two measured time points but not in the other, respectively. Microbiota figures were generated using ggplot2.

2.3.6 Flow cytometry

To assess immunity following 4 weeks of intervention with FMT, before mice were subjected to behavioural assessments, blood sample preparation was performed as previously described with minor modifications (van de Wouw *et al.* 2020a). See Supplementary Methods for detailed description and Supplementary Table 2.8 for antibodies used. Data were analysed using FlowJo (v.10). Target populations were normalized to the number of live cells or the respective parent population. Gating strategy is depicted in Extended Data Fig. 2.4.

2.3.7 Hippocampal metabolomics

Mice were decapitated at the end of Study 1, and the hippocampus was carefully dissected and immediately snap-frozen on dry ice and stored at −80 °C until use. For metabolomics, the left hemisphere of the hippocampus was used. Hippocampal metabolomics were conducted at

Metabolon Inc. using UHPLC–MS–MS. Supplementary Methods contain a detailed description of sample preparation.

Peaks were quantified using AUC. Biostatistics were run in R (v.3.6.3) with Rstudio GUI (v.1.2.5033). Missing values were imputed by taking 95% of the minimum observed abundance per metabolite. Metabolites that were detected in fewer than 10% of samples were dropped from the analysis. Principal-component analysis was performed on CLR-transformed values using the ALDEx2 library (Fernandes *et al.* 2014). The number of permutations was set to 1,000. PERMANOVA followed by a pairwise PERMANOVA was used to find structural differences between treatments on a compositional level. To find metabolites that were altered by aging and restored by yFMT, we first performed a Mann–Whitney U-test between all yFMT samples and the aged oFMT group, identifying metabolites where the aged oFMT group was different from the two yFMT groups. Then we ran pairwise Mann–Whitney U-tests in between both individual yFMT groups and the aged oFMT group, to verify that both yFMT groups were indeed different from the aged oFMT group levels individually. To correct for multiple testing in tests involving metabolomics features, Storey's q-value post hoc procedure was performed with a q-value of 0.2 as a cutoff. Metabolites that were found to be altered in aging and restored by yFMT were mapped to their corresponding Human Metabolome Database identifier and subjected to pathway analysis using the MetaboAnalyst online pipeline, choosing the murine KEGG library as a reference. Custom R scripts are available online at https://github.com/thomazbastiaanssen/Tjazi. Metabolomics figures were generated using ggplot2.

2.3.7.1 Linking functional microbiome and hippocampal metabolomics

To assess whether changes in the hippocampal metabolome could be linked to changes in the functional microbiome, we converted the Human Metabolome Database IDs of the 35 metabolites found to be altered by aging and restored in the aged yFMT group to their respective KEGG compound IDs. We then proceeded to test for correlations between KOs in the microbiome that were inferred by Piphillin that were linked to these KEGG compound IDs using Pearson's correlation coefficient. P values were corrected using the Benjamini–Hochberg procedure, using $q = 0.1$ as a cutoff. In all cases, both compounds and KOs were CLR-transformed before any testing.

2.3.8 Hippocampal transcriptomics

2.3.8.1 Sample preparation and sequencing

Mice were decapitated at the end of Study 1, and the hippocampus was carefully dissected and immediately snap-frozen on dry ice and stored at −80 °C until use. For transcriptomics the right hemisphere of the hippocampus was used and total RNA was extracted using the RNeasy Plus Universal Mini kit (QIAGEN, cat. no. 73404) according to the manufacturer's instructions. For tissue dissociation, 2-ml SC Micro Tubes PCR-PT (Sarstedt, cat. no. 72.694.406) were filled with 800 mg ± 100 mg Zirconia/Silica beads (BioSpec Products, cat. no. 11079101z, 0.1 mm in diameter). RNA concentration and quality were determined using a Nanodrop 1000 (Thermo Fisher Scientific), followed by Bioanalyzer (Agilent) to measure the RNA integrity number. Samples with an RNA integrity number >8.5 were considered for sequencing. Library preparation and sequencing as well as Fastq-file generation was conducted by Edinburgh Genomics using paired-end sequencing on an Illumina NovaSeq 6000. Supplementary Methods contain details on RNA-seq pre-processing.

2.3.8.2 RNA-seq bioinformatics analysis

Biostatistics were run in R (v.3.6.3) with the Rstudio GUI (v.1.2.5033). First, genes were filtered by variance using the genefilter library in R. Gene counts were CLR-transformed using the ALDEx2 library. To find genes that were altered by aging and restored by yFMT, we focused as a first step on genes that have been linked to the microglia sensome (Hickman *et al.* 2013). For this, we performed a Mann–Whitney U-test between all yFMT samples and the aged oFMT group, identifying genes where the aged oFMT group was different from the two yFMT groups. Then we conducted pairwise Mann–Whitney U-tests in between both individual yFMT groups and the aged oFMT group to verify that both yFMT groups were indeed different from the aged oFMT group levels individually (Supplementary Table 2.5). Additionally, for our untargeted analysis, in the subset of genes that were altered by aging (as defined by young FMT versus aged oFMT), we tested for genes that were differentially abundant between aged oFMT and aged yFMT, with the intent of finding genes that were altered by aging and restored to young levels by yFMT. We used two-sample Student's t-tests to assess differential expression on CLR-transformed gene counts. In

all cases, the Benjamini–Hochberg procedure was used to control the FDR, using $q < 0.2$ as a cutoff. All R scripts are available online at https://github.com/thomazbastiaanssen/FMT_Aging.

2.3.9 Behaviour

All behavioural tests were performed and scored by experimenters blinded to the treatment groups.

2.3.9.1 Spontaneous alternation in the Y-maze

To assess short-term working memory, spontaneous alternation behaviour in the Y-maze tests, hippocampal-dependent spatial memory and exploration and was carried out and analysed as previously described (Scott *et al.* 2017). Briefly, mice were habituated to the room 30 min before testing and behaviour recorded for 5 min using a ceiling-mounted camera.

2.3.9.2 Novel object recognition

To assess short-term recognition memory, mice were subjected to the NOR task as previously described with some modifications (Golubeva *et al.* 2017). Animals were habituated to the room 60 min before the test. On the pre-trial day, mice were habituated to the empty, open arena (40 × 45 × 45 cm, L × W × H) in two habituation phases (10 min each, with 3 h time in between). One the following day, the test mouse was exposed for 10 min to two identical objects placed in the corners of the arena (acquisition phase). At 3 h later, one of the familiar objects was substituted with a novel object and the animal was allowed to explore the objects for 10 min (retention phase). The test was conducted under dimmed lighting (15 lux). Animal behaviour was video recorded; time spent in exploration of the objects was blindly scored in Ethovision v.15 (Noldus). Exploration behaviour was defined as orienting the nose towards the object at a distance <2 cm or direct contact with the object. Discrimination index was calculated according to the formula: $(t(novel) - t(familiar)) / (t(novel) + t(familiar))$. Mice that interacted <12 s in total with either the novel or the familiar object, were excluded from the analysis.

2.3.9.3 Open field

The open field is a widely used assay to assess approach avoidance behaviour, locomotor activity and the behavioural response to a novel context and was conducted as previously described with some modifications (van de Wouw *et al.* 2020b). Briefly, mice were placed in an open arena

(40 × 45 × 45 cm, L × W × H) and allowed to explore the arena for 10 min, which represented the first habituation phase for NOR testing. Animals were habituated to the room 60 min before the test. Testing was performed under dim light (15 lux). The open field test box was cleaned with 70% ethanol in between animals. Experiments were videotaped using a ceiling-mounted camera and were analysed for time spent in the virtual centre zone (33% of the total area) and total distance travelled using Ethovision v. 15.

2.3.9.4 Elevated plus maze (EPM)

The EPM test was used to assess anxiety-like behaviour and was conducted as previously described (van de Wouw *et al.* 2020b). Briefly, mice were allowed to explore the maze, which was elevated 1 m above the ground and consisted of a grey cross-shaped maze with two open arms and two closed arms (50 × 5 cm with 15-cm walls in the closed arms and 1 cm walls in the open arms) for 5 min. Mice were habituated to the room 30 min before the test. Experiments were conducted in red light (5 lux). The EPM apparatus was cleaned with 70% ethanol in between animals. Experiments were videotaped using a ceiling-mounted camera and time spent as well as the number of entries in the open arms, which was defined as all paws in the open arm, was measured.

2.3.9.5 Three-chamber social interaction test

Sociability and social novelty were assessed in a three-chamber apparatus as previously described (van de Wouw *et al.* 2020b) and scored by hand using BORIS v. 7.9.7 (Friard & Gamba 2016). The test consisted of three sequential 10-min trials: (1) habituation; (2) sociability (analysis of time animals spent in the chamber with the conspecific mouse or with the object); and (3) social novelty preference (analysis of time animals spent in the chamber with the novel mouse or in the chamber with the familiar mouse).

2.3.9.6 Morris water maze (MWM)

The MWM represents a test for spatial learning and memory (Vorhees & Williams 2006) and was conducted as previously described (Boehme *et al.* 2020). Briefly, mice were trained over 5 d, with four trials per day each lasting 2 min to spatially allocate the submerged platform. To evaluate learning performance, the latency to find the hidden platform, mean distance from platform, average path length and velocity were analysed by comparing the average of four trials across each

training day. Data were analysed via one-way repeated measurement ANOVA post hoc Holm–Sidak. AUC was calculated for each learning parameter by integrating each training curve and by one-way ANOVA post hoc Holm–Sidak. In addition, the use of hippocampal-dependent over hippocampal-independent search strategies (direct swim, rotating, focused search, circling, scanning and thigmotaxis) were assessed per trial as previously described (Higaki *et al.* 2018). On day six, the platform was removed and a probe trial lasting 60 s was conducted. To assess spatial memory, latency to target quadrant and time spent in the platform quadrant were analysed. Latency to the hidden platform was scored by hand. All other metrics were scored using Ethovision v. 15.

2.3.9.7 Novelty-induced hypophagia (NIH)

The NIH test is used to assess hippocampal neurogenesis-dependent hypophagia in a novel, aversive environment (Dulawa & Hen 2005). Briefly, mice were single housed for 2 days prior to the test. From Day 3 until Day 5, mice were trained to drink a diluted sweetened condensed milk (Carnation; diluted 1:3 milk:water) from serological pipettes presented in their home cage for 30 minutes. On Day 6, mice were presented with the same mixture in their home cage under dim lighting conditions (50 lux), and their latency to drink and volume consumed was recorded every 5 minutes over a test period of 20 minutes. On Day 7, mice were placed in a novel cage void of shavings and bedding, under bright lighting conditions (approximates 1,200 lux) and presented the condensed milk mixture in serological pipettes for 20 minutes. Their latency to drink and volume consumed was recorded every 5 minutes over a test period of 20 minutes. The difference in latency and volume consumed between the home change and novel cage testing day were calculated as a measurement of novelty-induced hypophagia.

2.3.10 Plasma collection and corticosterone assay

Using lithium–heparin-coated capillaries, trunk blood was collected at the terminal time point of the study. Blood was centrifuged at 3,500g at 4 °C for 15 min. Plasma was aspirated and stored at −80 °C. Plasma corticosterone was measured as previously described (Scott *et al.* 2017) by ELISA, following vendor instructions (ENZO Corticosterone ELISA, cat. no. ADI-900-097). Concentration is expressed in ng ml−1.

2.3.11 Blood cytokine assay

Cytokine secretion in blood was assessed using the Proinflammatory Panel 1 (mouse) V-PLEX Kit (Meso Scale Discovery, cat. no. K15048D-1). Samples were run in duplicates. Reading and analyses were performed using the MESO QuickPlex SQ 120, SECTOR Imager 2400. Values under the curve fit and detection range were excluded. Concentration is expressed in pg ml−1.

2.3.12 Microglia analysis

For morphological characterization of microglia, ionized calcium-binding adaptor molecule (Iba1) staining was performed with modifications as previously described (Boehme *et al.* 2014). Brains were sliced on a cryostat at −20 °C in 30-µm sections into antifreeze solution. Iba1 is a Ca-dependent cytosolic protein that is expressed in the brain in microglia (Ito *et al.* 1998).

Iba1 staining was carried out using the 3,3′-diaminobenzidine method as described previously (Boehme *et al.* 2014). The focus was on the cornu ammonis (CA1) region of the hippocampus as it has been widely related to spatial memory (Tsien *et al.* 1996) and is described in detail in Supplementary Methods.

Microglia soma area was determined by hand-tracing microglia soma in ImageJ. The morphological complexity of microglia was assessed using the ImageJ-based 'Sholl Analysis Tool' (Ferreira *et al.* 2014), a common technique used to evaluate branching of cells including analysis of microglia morphology, conducted blindly following previously described guidelines (Gonzalez Ibanez *et al.* 2019) and described in detail under Supplementary Methods.

2.3.13 Analysis of neurogenesis (BrdU+/NeuN+ staining, immunohistochemistry)

The same brains used in microglia analysis were used to assess the survival of newly born hippocampal neurons. Washing steps involved washing tissue in PBS on a gentle plate mixer three times for 5 min each unless otherwise indicated. Free-floating sections were washed and then incubated in 2N HCl at 37 °C for 15 min. Sections were rinsed twice in a sodium tetraborate buffer for 5 min, then washed in PBS. Blocking solution (10% normal donkey serum (NDS), 0.3% PBS-T) was added and sections were set onto a gentle shaking plate for 1 h at room temperature (RT).

Sections were incubated in anti-rat BrdU (Abcam, cat. no. AB6326) in 1% NDS, 0.1% PBS-T, 1:100 dilution at 4 °C overnight. Following BrdU antibody retrieval, sections were washed. All subsequent steps were carried out in darkness. Sections were incubated in secondary antibody (donkey anti-rat 594, 1:200 dilution; Thermo Fisher Scientific, cat. no. A-21209) for 90 min at RT in 1% NDS, 0.1% PBS-T in PBS, then washed. For NeuN staining, samples were blocked again for 30 min in blocking solution, then incubated in mouse anti-NeuN (Merck, cat. no. MAB377) in 1% normal goat serum (Abcam, cat. no. ab7481), 0.1% Triton-X 100, in PBS, 1:100 dilution at 4 °C overnight. Sections were washed and placed in the second secondary antibody (donkey anti-mouse 488, 1:200 dilution; Thermo Fisher Scientific, cat. no. A-21202) for 90 min at RT in 1% NDS, 0.1% PBS-T. Finally, sections were washed, mounted onto slides and cover-slipped using PVB DABCO and stored at 4 °C until imaging. Stained slices were imaged under dim light at RT using an Olympus BX53 upright research microscope. Hippocampal BrdU+/NeuN+ cells were quantified as cells that were distinguishably co-stained for both BrdU and NeuN fluorescent markers.

2.3.14 Intestinal motility assay

Gastrointestinal motility was assessed as previously described (Golubeva *et al.* 2017). Briefly, mice were single-housed at 8.00 a.m. with ad libitum access to food and drinking water. Three hours later, 0.2 ml of nonabsorbable 6% carmine red in 0.5% methylcellulose dissolved in sterile PBS was administered by oral gavage, after which, drinking water was removed. The latency for the excretion of the first red-coloured faecal pellet was subsequently timed as a measure of gastrointestinal motility.

2.3.15 Assessment of faecal water content and weight

Mice were singly housed for 1 h during which faecal pellets were collected (five per animal). Pellets were subsequently weighed, dried at 50 °C for 24 h and weighed again. The average weight per pellet and percentage of faecal water content was calculated.

2.3.16 Telomere detection

Telomere attrition is a hallmark of aging (Lopez-Otin *et al.* 2013). To measure telomere length, DNA was extracted from whole blood samples that were collected at the end of the study, using

the High Pure PCR Template Preparation kit (Roche, cat. no. 11796828001) according to the manufacturer's instructions. Subsequently, the average telomere length was determined and calculated using the Absolute Mouse Telomere Length Quantification qPCR Assay kit (ScienCell, cat. no. M8918) following the manufacturer's instructions.

2.3.17 Statistical analysis

Statistical analyses were conducted using SPSS v.25 (IBM) and GraphPad Prism v.8.3.0 (GraphPad Software). Before statistical analysis, data were analysed for normality using the Shapiro–Wilk test and for equality of variances using the Levene's test. If not otherwise indicated, nonparametric data were analysed by Kruskal–Wallis post hoc Dunn's, parametric data by one-way ANOVA post hoc Holm–Sidak and is shown as mean ± s.e.m. All tests were two-sided. Outliers were excluded using the ROUT method67 with q = 1%. Statistical significance was set at $P < 0.05$.

2.3.18 Statistics and probability

Where possible experiments were repeated at least twice with independent biological replicates and all replications revealed similar results. These analyses included blood cytokine multiplex ELISAs and plasma corticosterone ELISAs that were run in duplicate and qPCR, which was performed in triplicate.

2.3.19 Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article on the Nature Aging article page.

2.3.19.1 Data availability

Sequencing data have been deposited in the Sequence Read Archive. Microbiota sequencing data are available at http://www.ncbi.nlm.nih.gov/bioproject/737691 (accession PRJNA737691) and hippocampal RNA-seq data are available at http://www.ncbi.nlm.nih.gov/bioproject/738160 (accession PRJNA738160). Source data are provided with this paper. Other data that support the findings of this study are available from the corresponding author upon request.

2.3.19.2 Code availability

Custom R scripts are available online at https://github.com/thomazbastiaanssen/Tjazi. All R scripts are available online at https://github.com/thomazbastiaanssen/FMT_Aging.

2.4 Results

2.4.1 Age-associated shifts in gut microbiota following FMT

To investigate the impact of aging on faecal microbiota, we analysed baseline faecal microbiomes of young and aged mice using 16S rRNA amplicon sequencing. We reassessed composition and diversity after 4 weeks of FMT to examine engraftment and colonization dynamics of the FMT (Fig. 2.1a). Notably, we detected no differences between the number of taxa gained or lost between the groups (Extended Data Fig. 2.6). Pre-FMT, we found clear differences between young and aged mice in β-diversity. However, following FMT, differences in β-diversity were no longer significant (Fig. 2.1b). No temporal or intergroup α-diversity changes were significant using Simpson or Shannon indices. The fact that only richness, measured by Chao1, dropped significantly following FMT in all groups (Fig. 2.1c), indicates that evenness was retained among the reduced number of taxa post-FMT. Twenty genera were significantly changed following FMT (Fig. 2.1d). Some of these temporal differences seemed congruent between aged mice regardless of treatment, indicating potential age-dependent responses regardless of the content of the FMT. On the other hand, certain genera, including Enterococcus, were altered in aged mice, but transitioned towards young mouse abundance following yFMT exclusively (Fig. 2.1e). Further studies should tease out the dynamics and interactions of these specific genera with the aged host.

We next inferred the functional capacity of the microbiome in terms of KEGG orthologs (KOs) using the Piphillin framework (Iwai *et al.* 2016), followed by presence or absence of gut–brain modules (GBMs), functional modules that are involved in gut–brain communication (Valles-Colomer *et al.* 2019). Functionally, we found significant changes in nine GBMs following FMT (Fig. 2.1f). Notably, propionate synthesis III and acetate degradation were reduced in aged yFMT, but not in aged oFMT mice, suggesting differences in the metabolism of short-chain fatty acids, which are involved in regulation of immune cell function, gut–brain communication and aging (Dalile *et al.* 2019). We found that aged oFMT microbiota composition had shifted significantly more than young yFMT in terms of Aitchison distance post-FMT, while no significant shift was observed between aged yFMT and young yFMT (Fig. 2.1g).

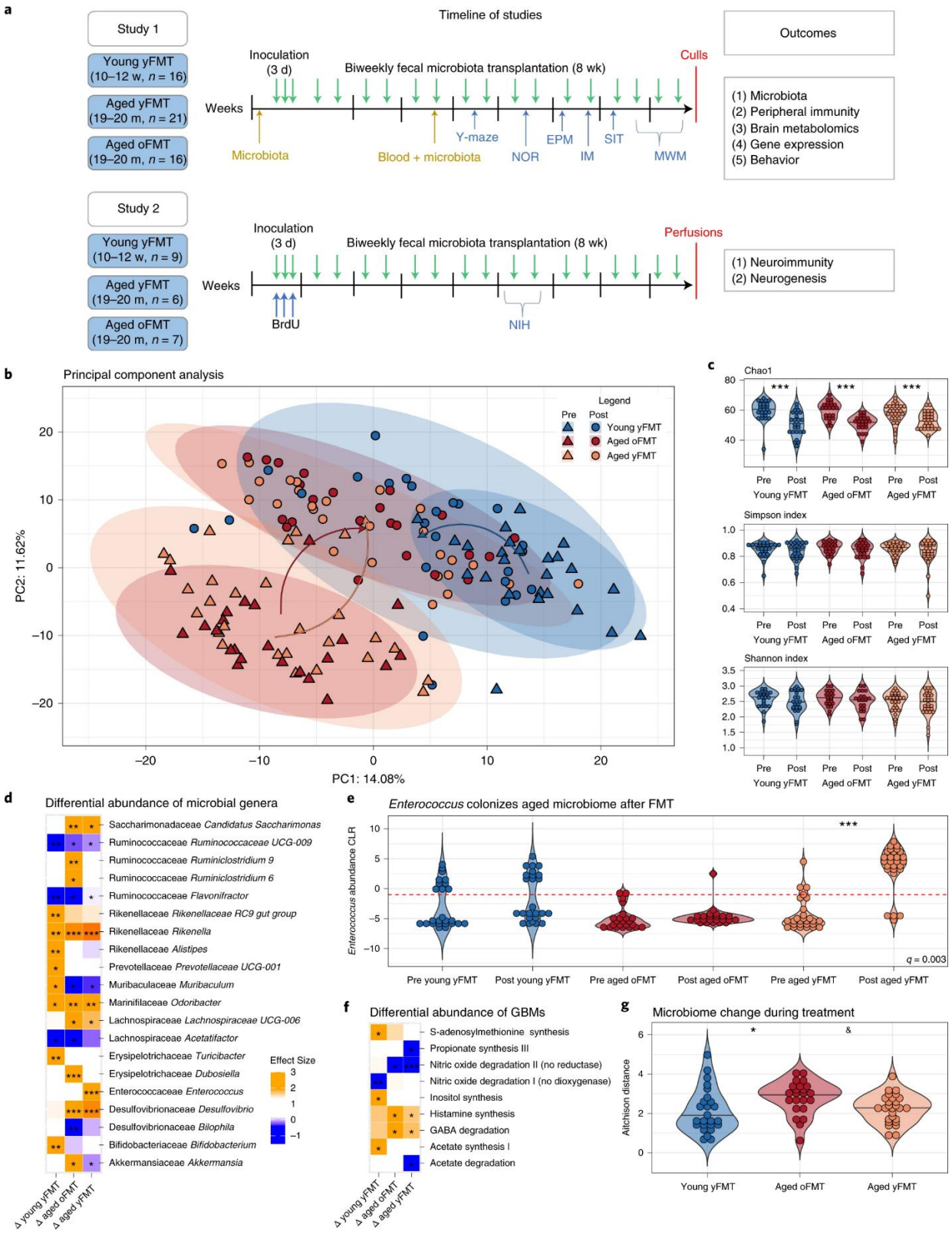


Figure 2.1 (A) Overview of the experimental design and study timeline. Vertical bars represent 1 week. Behaviors conducted include Y-maze, novel object recognition (NOR), elevated plus maze (EPM), intestinal motility (IM), three-chamber social interaction test (SIT), Morris water maze (MWM), and, in Study 2, the novelty-induced hypophagia

test (NIH). BrdU (bromodeoxyuridine) indicates intraperitoneal injection of bromodeoxyuridine. (B) Principal component analysis showing the effects of FMT on fecal microbiome in young and aged mice in terms of β-diversity as measured in Aitchison distance. Arrows indicate the trajectory per group following its respective treatment. Ellipses indicate 95% confidence intervals per group. Wd-test, P < 0.001. (C) Violin plots displaying the effects of FMT on young and aged mice in terms of α-diversity. Black horizontal lines in violin plots depict medians. The Chao1 index decreased following FMT; two-way analysis of variance (ANOVA), (F(1,144) = 50.1, P < 0.001). ***P < 0.001. No effects were found in the Shannon or Simpson index. (D) Heat map showing genera differentially altered by FMT. Color depicts effect size, with blue (negative) indicating higher abundances pre-treatment and red (positive) indicating higher abundances post-treatment. *q < 0.1, **q < 0.01, ***q < 0001. Comprehensive statistical results can be found in Supplementary Table 2.1.(E) Violin plots showing Enterococcus abundance is restored to young levels in old mice after yFMT but not after oFMT. Black horizontal lines in violin plots depict medians. The y axis shows center log-ratio (CLR)-transformed abundance. Dashed horizontal red line indicate a threshold for estimated abundance of 0 (Mann–Whitney U-test (two-sided), ***P < 0.001, q < 0.001, e = 1.48). (F) Heat map showing GBMs differentially altered by FMT. Color depicts effect size, with blue (negative) indicating higher abundances pre-treatment and red (positive) indicating higher abundances post-treatment. *q < 0.1, **q < 0.01, ***q < 0.001. Comprehensive statistical results are in Supplementary Table 2.2. (G) Violin plots displaying the differences in Aitchison distance traveled per microbiome after treatment. Black horizontal lines in violin plots depict medians. Aged oFMT group changed more than young yFMT (F(72) = 3.82, P = 0.026). Tukey's honestly significant difference (HSD) (two-sided) test was used. Young yFMT versus aged oFMT, P = 0.03; young yFMT versus aged yFMT, P = 0.88; aged yFMT versus aged oFMT, P = 0.082. *P < 0.05 and &P < 0.1. n = 25 (young yFMT), 23 (aged oFMT), 27 (aged yFMT) for all figures except g, where n = 25 (young yFMT), 27 (aged oFMT) and 23 (aged yFMT) independent mice are included.*

2.4.2 yFMT modulates age-induced peripheral and hippocampal immunity

The gut microbiota is a key regulator of host immunity especially in aging (Fransen *et al.* 2017; Thevaranjan *et al.* 2017). Moreover, the immune system influences hippocampal-associated cognitive behaviour (Mohle *et al.* 2016). Thus, the immune system may be an important link between alterations in the gut microbiota and potential effects on the brain in aging.

To determine whether aging-invoked immune alterations can be ameliorated by FMT from a young donor, we characterized innate and adaptive immunity in the gut-associated mesenteric lymph nodes (MLNs) and in the circulation. Aging triggered a substantial increase in early activated CD8+ T cells in MLNs, which was reversed by yFMT (Fig. 2.2a). In contrast, memory CD8+ T cells (CD44hi) were unaffected (Extended Data Fig. 2.3a), perhaps due to the low turnover rate of memory CD8+ T cells (Baliu-Pique *et al.* 2018). Furthermore, CD103+ dendritic cells, which are tightly linked to CD8+ T-cell activation and sensitive to changes in the microbiome (Tan *et al.* 2016), were reduced in aged yFMT mice compared to aged oFMT mice (Fig. 2.2a). yFMT into aged mice decreased the proportion of F4/80+ macrophages in MLNs and rescued the decrease of the activation marker CD11b on Ly6Chi monocytes (Fig. 2.2a). Despite strong effects of aging on innate and adaptive immunity in the circulation (Extended Data Fig. 2.3c,d), yFMT only trended towards decreasing the aging-associated rise of CD11b expression on Ly6G+ neutrophils (Fig.

2.2b)(Uhl *et al.* 2016) while rescuing the aging-associated elevation in peripheral interleukin (IL)-10 (Fig. 2.2b). However, yFMT did not reverse aging-associated reductions in interferon-γ and IL-5, which are both predominantly produced by T-cell populations, implying a stage of immunosenescence (Fig. 2.2b). This indicates that yFMT into an aged host may selectively modulate peripheral immunity. The MLN immune profile was particularly sensitive to FMT-induced changes in the aged host.

Given the relationship between the gut microbiota, neuroinflammatory processes and brain plasticity (Erny *et al.* 2015; Mohle *et al.* 2016; van de Wouw *et al.* 2019), we characterized microglia, the brain's resident macrophages, in the hippocampus, a brain region that is critical for cognition. Microglia are essential for regulating cellular aspects of cognition, supporting neuroplasticity (Elmore *et al.* 2018) and responding to various signals, including cytokines. Higher populations of activated microglia, distinguished by enlarged somas, are prominent in neurodegenerative conditions. Aged oFMT mice showed substantial enlargement in microglia cell soma size, which was further pronounced when microglia somas were classified as large or small (Fig. 2.2c)(Kozareva *et al.* 2019b). This phenotype was reversed by yFMT (Fig. 2.2c). However, we did not observe changes in microglia complexity, or maximum or average branch length (Extended Data Fig. 2.2b). We further investigated whether yFMT could alter genes linked to the microglia sensome using targeted RNA-seq (Hickman *et al.* 2013). Among these we found six genes that were substantially altered by aging and restored by yFMT after false discovery rate (FDR) correction (Fig. 2.2c and Extended Data Fig. 2.7b). Specifically, we found changes in the immunoregulatory factors Trem2 and Dap12, an adaptor that regulates Trem2 signalling (Hickman *et al.* 2013). Dap12 appears as a key regulator of the microglial sensing function through direct or indirect interaction with various sensome proteins (Hickman *et al.* 2013). Notably, the Trem2–Dap12 complex plays a central role in physiological brain aging and in age-associated neurodegenerative diseases (Mecca *et al.* 2018). yFMT also restored the aging-induced transcriptional elevation in expression of the complement factor C1qb. C1qb plays a role in synaptic pruning (Schafer *et al.* 2012) and represents a part of the heterotrimer C1q, which has been linked to cognitive impairment, with C1q-deficient mice showing less cognitive decline (Stephan *et al.* 2013). Further, yFMT drove expression restoration in Gpr84, which can trigger inflammatory signalling in myeloid cells and is reported to be elevated in mouse models of multiple sclerosis and Alzheimer's disease (AD) (Recio *et al.* 2018) and in Fcgr2b, which is linked

to memory impairment in a model of AD (Kam *et al.* 2013). These data highlight that yFMT is capable of restoring distinct age-associated changes in microglia activation, a hallmark of cognitive decline.

In addition to supporting brain immunity, microglia regulate hippocampal neurogenesis (Gemma *et al.* 2010), a key process for learning and memory, which is hindered in aging (Kundu *et al.* 2019). Intestinal microbiota can alter hippocampal neurogenesis, potentially contributing to age-associated disruptions in neurocognition (Ogbonnaya *et al.* 2015; Kundu *et al.* 2019). We confirmed a decrease in the number of surviving newly born hippocampal neurons in aged mice (Extended Data Fig. 2.2a); however, this was not rescuable with yFMT.

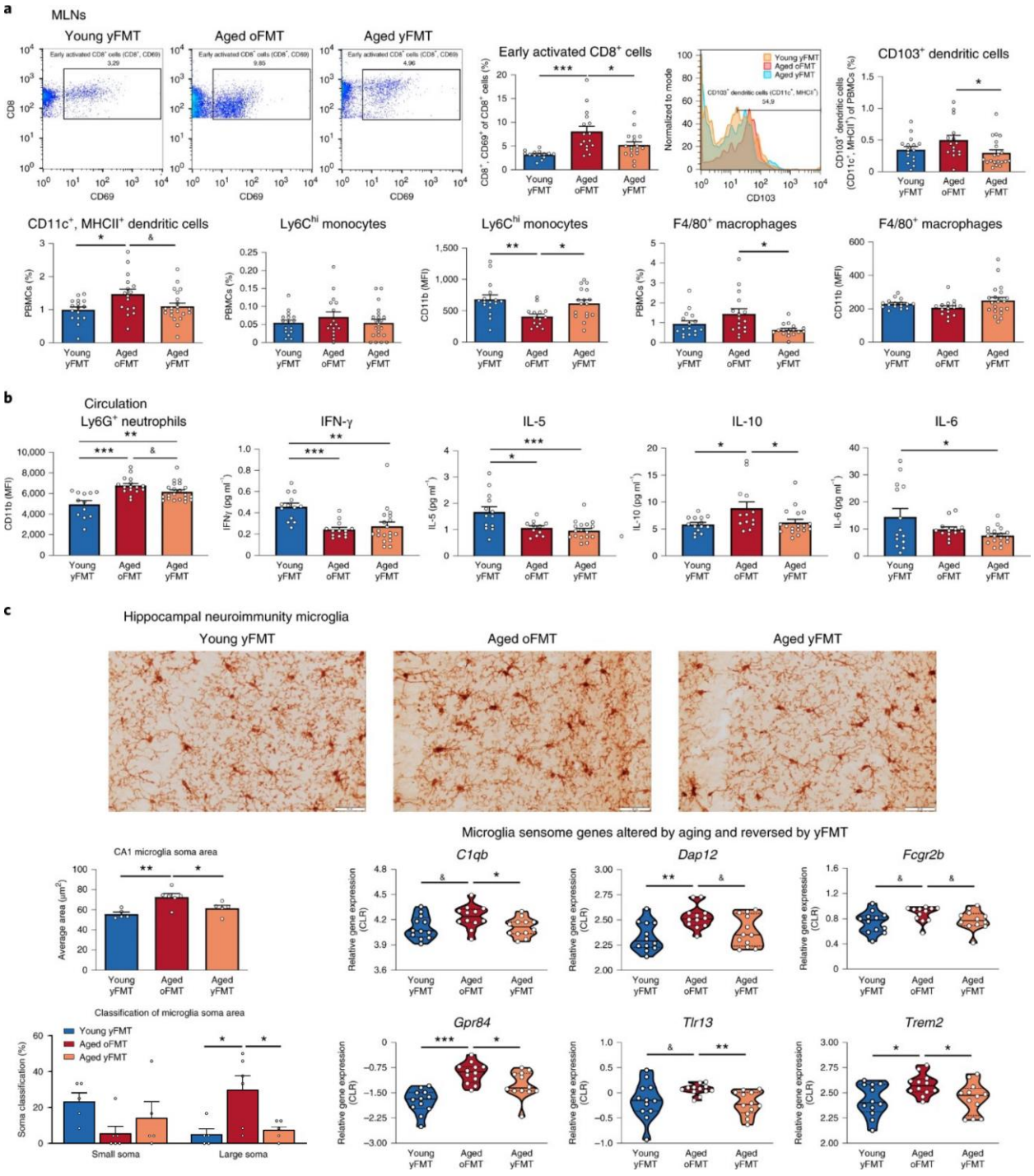


Figure 2.2. yFMT reshapes peripheral- and brain-associated immunity. (A) Immune profile in MLNs. Differences were detected in both early activated CD8+ T cells (CD69+) ($F(2,44) = 9.418$, $P < 0.001$) and CD103+ dendritic cells (DCs) ($H(3) = 7.836$, $P = 0.02$, Kruskal–Wallis post hoc Dunn's. Notaby, yFMT reversed ($P = 0.022$) the age-associated increase ($P < 0.001$) in early activated CD8+ T cells, whereas aged yFMT showed reduced levels of CD103+ DCs compared to aged oFMT ($P = 0.023$). n = 14–16 (young yFMT), 14–16 (aged oFMT), 17–21 (aged yFMT). (B) Immune profile in circulation before behavioral assessment. Distinct marker expression change, assessed by median fluorescence intensity (MFI), for the activation marker CD11b on Ly6G+ neutrophils ($F(2,46) = 12.53$, $P < 0.001$), were found with an increased expression in aged oFMT (CD11b, $P < 0.001$) which tended to be rescued in aged yFMT (CD11b, $P = 0.0645$). Both interferon (IFN)-γ and IL-5 decreased in both age groups ($F(2,42) = 9.331$,

*P < 0.001 and (F(2,39) = 8.758, P < 0.001), respectively) while an age-associated increase in IL-10 in aged oFMT F(2,42) = 4.357, P < 0.001, post hoc, P = 0.03) was found, which was counteracted in aged yFMT (P = 0.033). Aged yFMT exhibited decreased IL-6 compared to young yFMT (F(2,42) = 3.768, P = 0.031, post hoc: P = 0.027). n = 12–14 (young yFMT), 13–16 (aged oFMT), 18–21 (aged yFMT). (C) Hippocampal neuroimmunity was assessed by examining microglia soma area (F(2,13) = 8.418. P = 0.005). Scale bar, 50 µm. In aged oFMT mice an increase in microglia soma size was found compared to young yFMT mice (P = 0.005), which was reversed in aged yFMT mice compared to aged oFMT (P = 0.042). When microglia were further classified as having a 'large' or 'small' soma (F(2,13) = 6.328, P = 0.012), aged oFMT mice had significantly more microglia classified as 'large' compared to young yFMT (P = 0.022) and aged yFMT (P = 0.264). n = 5 (young yFMT), 6 (aged oFMT), 5 (young yFMT). Hippocampal transcriptomics using RNA-seq was performed to investigate whether yFMT alters genes of the microglia sensome23. Violin plots represent the six genes of the microglia sensome that were significantly altered by aging and restored by yFMT. The y axis shows CLR-transformed relative gene expression. n = 12 per group. Mann–Whitney U-test followed by Storey's q-value post hoc tests: *P < 0.05, **P < 0.01, ***P < 0.001, &P < 0.1. Statistical results can be found in Supplementary Table 2.5. If not otherwise indicated data were analyzed by one-way ANOVA post hoc Holm–Sidak test (a–c). All tests were conducted as two-sided. Each data point represents one independent mouse. Mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 and &P < 0.1.*

2.4.3 The aged hippocampal metabolome is sensitive to yFMT

Given the aging-associated changes in the brain and the influence of gut microbiota on hippocampal function (Mohle *et al.* 2016), we examined whether FMT from young to aged mice shaped the hippocampal metabolome.

Aging induced significant differences in hippocampal metabolites, with aged and young mice clustering separately (Fig. 2.3a,c). Notably, 35 metabolites altered in aging were restored towards pre-aged levels by yFMT (Fig. 2.3b,d and Supplementary Table 2.3), resulting in aged yFMT mouse hippocampal metabolomes clustering between aged and young individuals (Fig. 2.3c). In addition to numerous amino acids, which play critical roles in neurotransmission and aging (Canfield & Bradshaw 2019), we found that retinol (vitamin A) was altered by aging and restored by yFMT. Given our previous findings that vitamin A alone (Biyong *et al.* 2021) or in combination with omega-3 (Provensi *et al.* 2019) can modify gut microbiota composition and that the age-related reduction in retinoid signalling can be restored by supplementation, which helps to prevent age-related memory deficits (Touyarot *et al.* 2013), amelioration of vitamin A levels in aging may be a critical factor underlying benefits of yFMT. GABA and N-glycolylneuraminate were also among the metabolites restored by yFMT. Both metabolites play critical roles in cognition and brain plasticity and altered GABAergic signalling is closely linked to aging and cognitive decline (McQuail *et al.* 2015). While the role of N-glycolylneuraminate in the aged rodent brain remains largely unclear, as a sialic acid, N-glycolylneuraminate may have a protective role in microglial phagocytosis and neuroinflammation in the aged mouse brain (Puigdellivol *et al.* 2020). In line with previous research (Hunsberger *et al.* 2020), arginine increased with aging and is linked to the

nitric oxide pathway and neurodegeneration (Malinski 2007); its reversal by yFMT into aged mice proposes possible neuroprotective effects of yFMT on the aged brain. Functionally, these restored metabolites are enriched in six metabolic pathways (Fig. 2.3e), predominantly related to amino acid metabolism, which is critical for healthy cognition in aging (Canfield & Bradshaw 2019) and aminoacyl transfer RNA biosynthesis, which is crucial for proper functioning of the brain (Schaffer *et al.* 2019).

Using the KEGG database as a framework, we found that numerous KOs from the gut microbiome that require or produce one of the 35 restored metabolites, correlated with the levels of these metabolites in the hippocampus (Extended Data Fig. 2.7a and Supplementary Table 2.4). A large proportion of these metabolites can cross the blood–brain barrier (Supplementary Table 2.6) and may therefore translocate from the gut to the brain to influence brain health. Together, this suggests that the levels of these metabolites are affected by metabolic activity in the gut microbiome.

By employing an untargeted hippocampal RNA-seq approach we found that yFMT restores the age-associated elevation in glul expression, which encodes glutamine synthetase (Extended Data Fig. 2.7b). This finding mirrors our hippocampal metabolomics data and correlates with glutamine-related metrics in the gut microbiome-predicted functions (Extended Data Fig. 2.7a). Notably, glutamine synthetase is linked to inflammatory response in microglia suggesting a possible link between metabolomic changes and reactivity of microglia (Palmieri *et al.* 2017) and altered levels of glutamine-related metabolites are implicated in behavioural responses (Olson *et al.* 2018). Overall, our findings indicate that yFMT remodels the microbiome to restore aspects of the aged hippocampal metabolome, with glutamine as a potential driver.

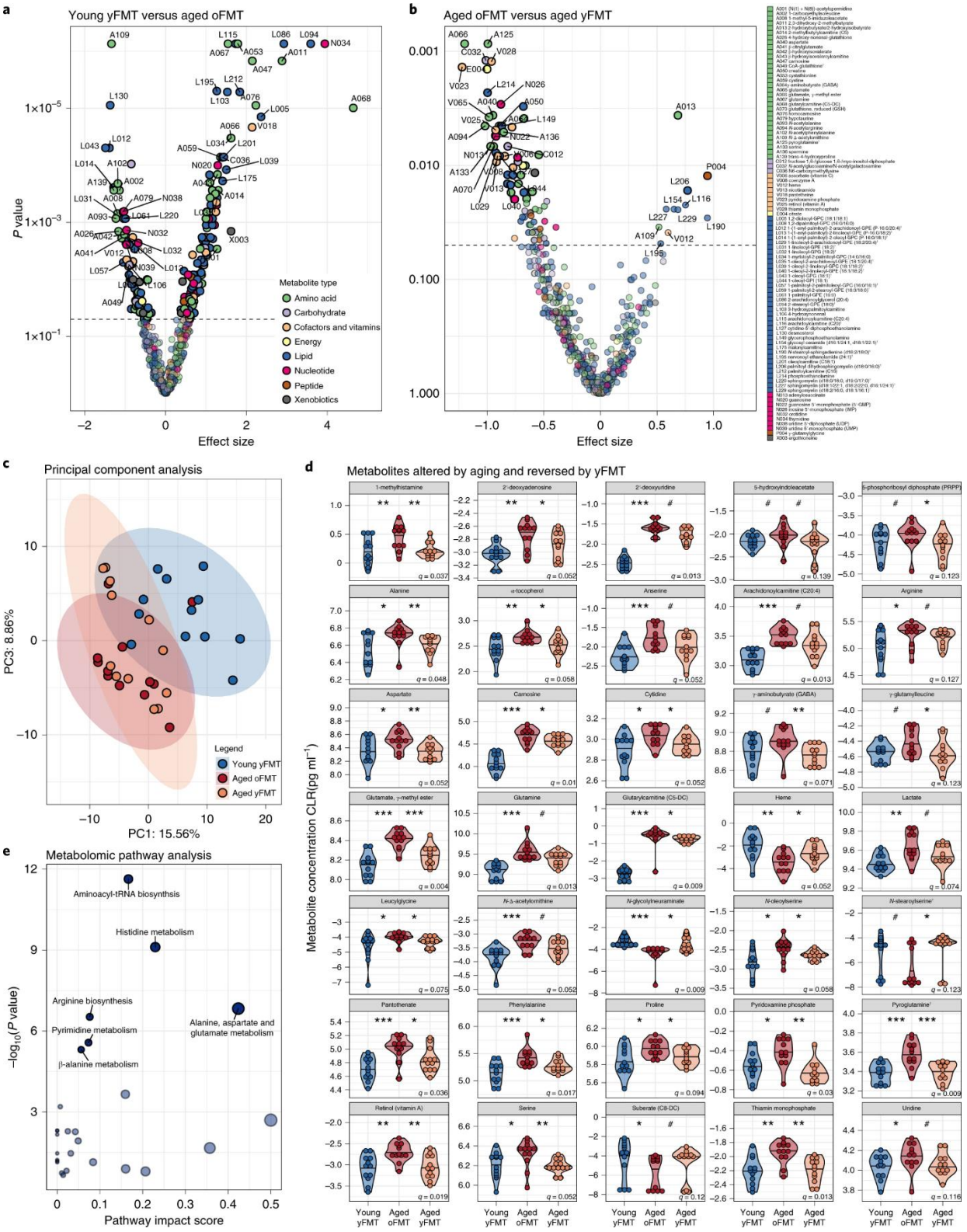


Figure 2.3. yFMT rescues aging-induced alterations in the aging hippocampal metabolome (A, B) Volcano plots depicting the effect of aging (A) and the difference between oFMT and yFMT (B) in the hippocampal metabolome. Metabolites on the 5% extremes of effect sizes of differential abundance are labeled. Effect sizes represents the median

*of the ratio of the between-group difference and the larger of the variance within groups. Horizontal dashed lines represent P = 0.05. Comparisons with P > 0.05 or q > 0.2 are depicted as smaller and more transparent. Comprehensive statistical results, including the full legend for the abbreviations, are in Supplementary Table 2.2. (C) Principal component analysis showing the effects of FMT on the hippocampal metabolome in young and aged mice in terms of Aitchison distance. Ellipses show the 95% confidence intervals per group. PERMANOVA, P < 0.001. (D) Violin plots representing the 35 metabolites that were significantly altered by aging and restored towards young levels after yFMT. Black horizontal lines in violin plots depict the medians. The y axis shows CLR-transformed metabolite concentrations. Q-values can be seen in the bottom-right corners of every figure. Mann–Whitney U-test (two-sided) post hoc tests, *P < 0.05; **P < 0.01; ***P < 0.001; #P < 0.1. n = 12 per group. Statistical results are in Supplementary Table 2.3. (E) Scatter-plot showing results of MetaboAnalyst Pathway analysis. Labeled, dark-blue points depict the pathways that withstood FDR at a 0.1 threshold. A dagger symbol (†) following the name of a biochemical compound indicates that the identity has been inferred rather than confirmed by a standard.*

2.4.4 yFMT offsets hippocampal-related behavioural changes in aging

Alterations in the gut microbiome have been widely linked to changes in behaviour, including hippocampal-dependent cognition (Cryan *et al.* 2019b), which declines during the aging process (Bettio *et al.* 2017). Thus, we examined whether yFMT rescued aging-induced, hippocampus-associated behavioural abnormalities in aged mice in a series of cognitive tasks. Consistent with extant literature, aging was associated with impaired learning (Fig. 2.4a) and an increase in the latency to enter the target quadrant in the Morris water maze probe trial. Notably, this was attenuated in aged mice that received FMT from a young donor (Fig. 2.4c), indicating that yFMT may improve aging-associated impairments in long-term spatial memory. Notably, there were no differences in locomotor activity between groups (Extended Data Fig. 2.5f). In addition to alterations in long-term memory, we found that yFMT into aged mice restored age-associated impairments during learning. Aged yFMT mice showed attenuations in mean distance from the platform on training days 4 and 5 and paralleled young yFMT mice in average path length and average velocity (Fig. 2.4a). These improvements were more apparent when data was visualized as a percent change from training day 1 (Extended Data Fig. 2.5c).

We also assessed short-term working memory and short-term recognition memory using the Y-maze and novel object recognition (NOR) tests. While there were no significant aging-induced impairments in either spontaneous alternation behaviour in the Y-maze or the novel object recognition index (Extended Data Fig. 2.5a,b), aged oFMT mice tended to interact less with objects in the NOR test (Extended Data Fig. 2.5b). Furthermore, in the three-chamber social interaction

test, aged oFMT mice spent significantly less time in the chamber with a social partner than their young yFMT counterparts (Extended Data Fig. 2.5d). Both aging-associated deficits in environmental interaction were rescued by FMT from young donors (Extended Data Fig. 2.5b,d). Additionally, while we did not observe an age effect on anxiety-like behaviour, yFMT dramatically increased the time aged mice spent in the open arms of the elevated plus maze (Fig. 2.4d). This could indicate a potential anxiety-alleviating therapeutic effect of yFMT in an aged host. Overall, these data suggest that yFMT can rescue specific aspects of aging-induced impairments in behaviour.

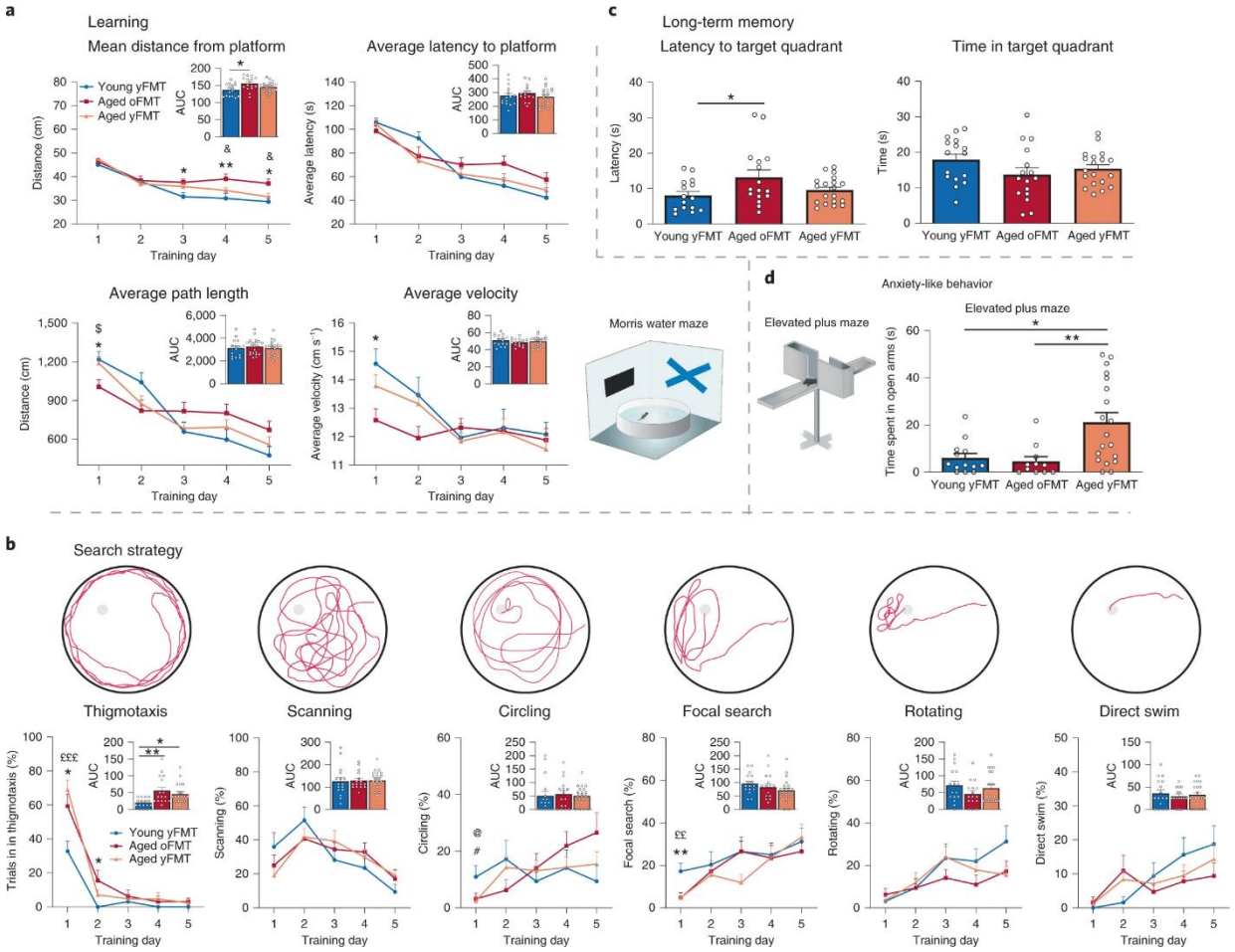


Figure 3.4. yFMT improves aspects of long-term spatial memory assessed via MWM. (A) Learning ability. Area under the curve (AUC), mean ± s.e.m., is inlayed on respective graphs. Treatment significantly impacted the mean distance from platform (F(1.387,5.548) = 6.620, P = 0.040); Young yFMT mice spent more time closer to the platform than aged oFMT mice (day 3: F(2,50) = 3.539, P = 0.037, post hoc P = 0.039; day 4: F(2,50)5.336, P = 0.008 post hoc P = 0.006; day 5: F(2,50) = 3.985, P = 0.025, post hoc P = 0.028) and AUC (F(2,50) = 4.795, P = 0.012; aged oFMT versus young yFMT (P = 0.0097). Aged yFMT mice were nearly significantly different from aged oFMT on days 4

*and 5 (P = 0.091, P = 0.076, respectively). While there were no overall differences in latency to platform (F(1.249,4.997) = 0.715, P = 0.469), path length (F(1.141,4.564) = 0.077, P = 0.824) or average velocity (F(1.082,4.329) = 2.023, P = 0.225), aging had clear effects on day 1; aged oFMT showed reduced average path length and average velocity compared to young yFMT (average path length F(2,50) = 3.947, P = 0.026, post hoc P = 0.040; average velocity F(2,50) = 4.798, P = 0.012, post hoc P = 0.010). Aged yFMT mice were not significantly different from young yFMT mice on any day of any training parameter. Similar to young yFMT mice, aged yFMT displayed significantly increased average path length on day 1 compared to aged oFMT (P = 0.045). n = 16 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). (B) Search strategy. Age substantially impacted search strategy on training day 1, where aged oFMT and aged yFMT spent significantly more trials in thigmotaxis than young yFMT mice (F(2,50) = 8.964, P < 0.001; post hoc P = 0.012 and P < 0.001, respectively). Aged oFMT mice remained distinguishable from young yFMT mice on day 2 (F(2,50) = 3.911, P = 0.026, post hoc P = 0.022). Considering AUC, aged oFMT was significantly different from young yFMT F(2,49) = 6.306, P = 0.004, post hoc P = 0.004). Aged yFMT displayed a similar difference from young yFMT (P = 0.026). Age also impacted focal search on day 1 (F(2,50) = 6.240, P = 0.004, post hoc P = 0.008), irrespective of yFMT in aged mice (P = 0.008). Furthermore, aging impacted circling strategy on training day 1 (F(2,50) = 3.211, P = 0.049), though post hoc only revealed trends between the aged oFMT versus young yFMT (P = 0.094) and aged yFMT versus young yFMT (P = 0.064). n = 16 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). (C) Long-term memory. The probe trial revealed an age-associated impairment in latency to target quadrant (F(2,47) = 3.353, P = 0.044), wherein aged oFMT mice were significantly slower to the target quadrant compared to young yFMT mice (P = 0.047). Aged yFMT mice were indistinguishable from young yFMT mice. n = 16 (young yFMT), 16 (aged oFMT), 20 (aged yFMT). (D) Treatment significantly altered time spent in open arms (H(3)=11.54, P = 0.003, Kruskal–Wallis). Post hoc analysis showed that aged yFMT exhibited an increase in time spent in open arms compared to aged oFMT (P = 0.007) and young yFMT (P = 0.025). n = 14 (young yFMT), 14 (aged oFMT), 19 (aged yFMT). If not otherwise indicated, data were analyzed by one-way ANOVA post hoc Holm–Sidak. Each data point represents one independent mouse. Mean ± s.e.m. For AUC, *P < 0.05, **P < 0.01. For all other intergroup comparisons, young yFMT versus aged oFMT: #P < 0.10, *P < 0.05, **P < 0.01; aged yFMT versus aged oFMT: &P < 0.10, $P < 0.05, $$P < 0.01; young yFMT versus aged yFMT: @P < 0.1, ££P < 0.01, £££P < 0.001.*

2.5 Discussion

Here, we demonstrate that transplanting microbiota from healthy, young mice into aged male mice can attenuate aging-associated deficits in behaviour. Further studies should acknowledge the impact of sex-specific factors that may shape the response to FMT in the aged female brain and subsequent behavioural effects (Jaggar *et al.* 2020; Darch *et al.* 2021). Upon investigating potential mechanisms for how intestinal microbiota may orchestrate these improvements, we found several GBMs and predicted microbiome functions that were altered following yFMT. Furthermore, specific aspects of gut-associated, circulating and hippocampal immunity were restored following yFMT into aged mice, suggesting that yFMT drove restorations in immune functions which may underlie ameliorations in age-associated cognitive deficits. Moreover, we uncovered restorative effects of yFMT on the aging hippocampal metabolome and transcriptome, which coincided with the improvements in behaviour. We hypothesize that gut microbiota-derived metabolites may play a role in the alteration in hippocampal metabolomics through indirect or direct mechanisms, as several of the metabolites we uncovered are able to translocate across the blood–brain barrier. Future studies should further elucidate the direct mechanistic underpinnings of how specific gut microbes drive these changes in rejuvenating the aging brain. While specific aging-associated deficits in behaviour, immunity and neurogenesis were not restored by yFMT, this research provides fundamental evidence that the gut microbiota should be considered as a potential therapeutic target for treating aspects of aging-associated decline in hippocampus-related function.

2.6 Supplemental information

2.6.1 Supplementary methods

2.6.1.1 Flow cytometry

Briefly, blood was collected by tail tipping using Eppendorf tubes containing 2.5 µL 3% EDTA solution to prevent blood clotting. Blood was resuspended in each 10 mL home-made red blood cell lysis buffer (15.5 mM NH4Cl, 1.2 mM NaHCO3, 0.01 mM tetrasodium EDTA diluted in deionized water) for three min. Blood samples were subsequently centrifuged (1500×g, 5 min), plasma taken for cytokine analysis (see 3.11 for full details), and cells resuspended in PBS containing 1:1000 FVS780 (BD Biosciences, cat. no. 565388) and incubated for 15 min at RT to distinguish live from dead cells. Subsequently, samples were centrifuged (1500×g, 5 min) and each aliquot (two per sample) resuspended in 50 µl BV staining buffer (BD Biosciences, cat. no. 563794). All subsequent procedures were conducted on ice / at 4°C. For staining, 5 µl of FcR blocking reagent (Miltenyi, cat. no. 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies for extracellular staining to investigate cells of the (a) innate and (b) the adaptive immune system (see Supplementary Table 2.8), and incubated for 30 min on ice. Samples of panel a were washed in staining buffer and fixed in 4% PFA for 30 min on ice, whilst samples for panel b underwent intracellular staining using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific, cat. no. 00-5523-00), according to the manufacturer's' instructions, together with FoxP3-APC (Thermo Fisher Scientific, cat. no. 17-5773-82). Fixed samples were subsequently resuspended in staining buffer and analyzed the following day using the BD FACSCelesta and BD FACSDiva (version 8.0).

To assess gut-associated immunity at the end of the study, mesenteric lymph nodes (MLNs) were carefully dissected and processed for Flow Cytometry as previously described66. Briefly, MLNs were transferred onto a 70 µm strainer and disassembled using the plunger of a 1-mL syringe. The strainer was subsequently washed with 10 mL media (RPMI-1640 medium with L-glutamine and sodium bicarbonate, supplemented with 10% FBS (Sigma, cat. no. F7524l) and 1% Pen/strep (Sigma, cat. no. P4333), centrifuged and 3×106 cells were resuspended in 90 µl staining buffer and split into three aliquots for the staining procedure. Samples were always kept on ice. For the staining procedure, 5 µl of FcR blocking reagent (Miltenyi, cat. no. 130-092-575) was added to

each sample. Samples were subsequently incubated with a mix of antibodies (see Supplementary Table 2.8) for 30 min on ice followed by a washing step and final fixation using 4% PFA for 30 min on ice. Samples were analyzed the following day using the BD FACSCalibur and BD CellQuest Pro (version 5.2). Data was analyzed using FlowJo (version 10). The investigated cell populations were normalized to the number of obtained peripheral blood mononuclear cells (PBMCs) or the respective parent population.

2.6.1.2 Hippocampal metabolomics

Samples were prepared using the automated MicroLab STAR® system (from Hamilton Company). Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Several controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

For analysis, a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution was utilized. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z.

Raw data files are archived and extracted as described below. Raw data was extracted, peak-identified and QC processed using Metabolon's hard- and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities.

2.6.1.3 RNAseq pre-processing

Trimming Reads were trimmed using Cutadapt (version cutadapt-1.9.dev2). Reads were trimmed for quality at the 3' end using a quality threshold of 30 and for adapter sequences of the TruSeq stranded mRNA kit (AGATCGGAAGAGC). Reads after trimming were required to have a minimum length of 50. The reference used for mapping was the Mus musculus genome (build GRCm38) from Ensembl release 84. The annotation used for counting was the standard GTF-

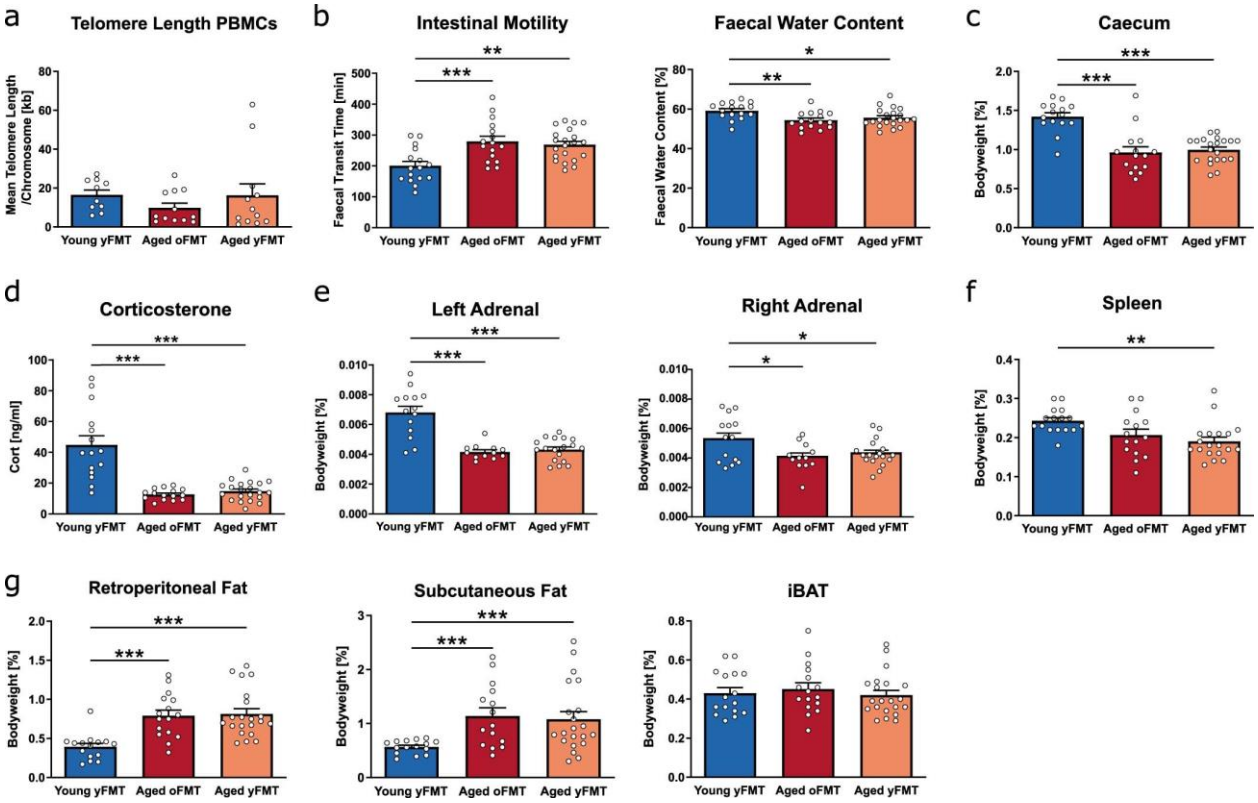
format annotation for that reference (annotation version 84). Reads were aligned to the reference genome using STAR2 (version 2.5.2b) specifying paired-end reads and the option --outSAMtype BAM Unsorted. All other parameters were left at default. Reads were assigned to features of type 'exon' in the input annotation grouped by gene_id in the reference genome using featureCounts. featureCounts assigns counts on a 'fragment' basis as opposed to individual reads such that a fragment is counted where one or both of its reads are aligned and associated with the specified features. Strandedness was set to 'reverse' and a minimum alignment quality of 10 was specified. In addition to the counts matrix used in downstream differential analysis, a matrix of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) values was generated, using the rpkm() function of edgeR (version 3.28.1) and normalized effective library sizes. Gene lengths for the FPKM calculation were the number of bases in the exons of each gene (only counting bases once where they occur in multiple exon annotations). Gene names and other fields were derived from input annotation and added to the count/expression matrices.

2.6.1.4 Microglia analysis

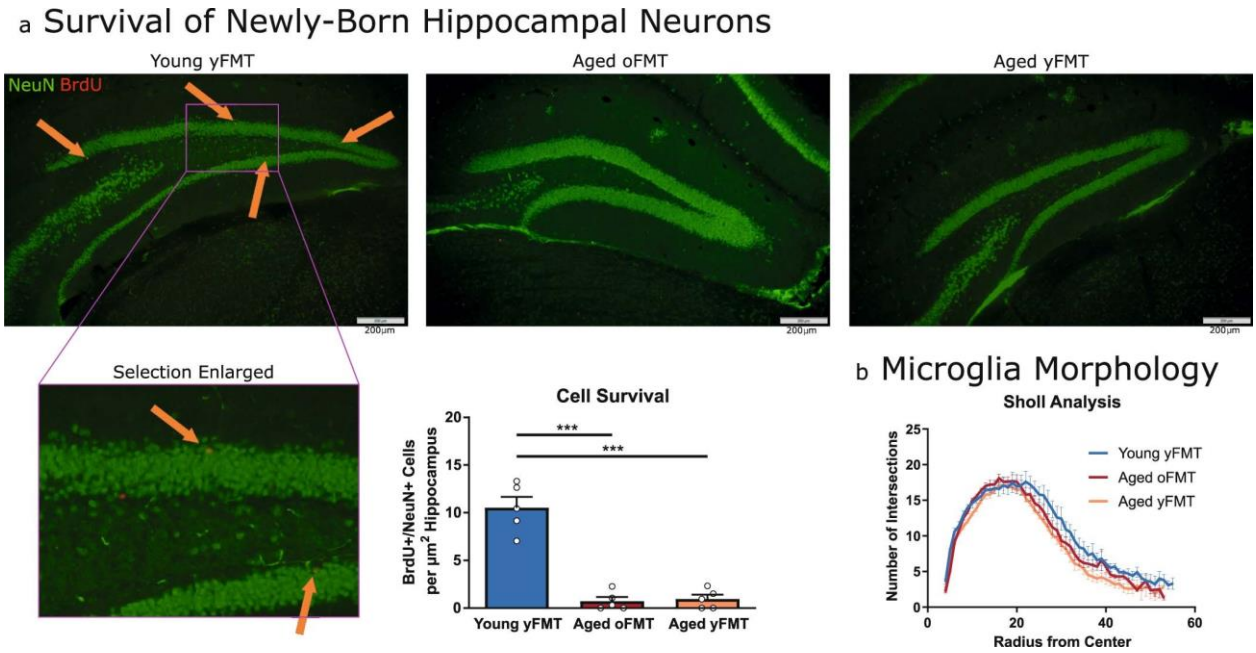
Briefly, slices (free-floating) were washed six times for 5 min in PBS, before sections were incubated for 30 min in 0.24% H2O2 (in PBS) and then washed four times for 5 min each in PBS-T (PBS with 0.2% Triton). After blocking in PBS-T serum (0.2% Triton, 3% NDS) for 1 hour, incubation was carried out overnight at 4°C with the primary antibody (rabbit anti-Iba1, 1:2000 in PBS-T, #019-19741; Wako Pure Chemical Industries, Osaka, Japan). On day two, slices were washed four times for 5 minutes in PBS-T and blocked with 3% PBS-T serum for 15 minutes, followed by a two-hour incubation with the biotin- conjugated secondary antibody (Biotin-Sp conjugated donkey anti-rabbit IgG, 1: 500 in PBS-T; Jackson ImmunoResearch, cat. no. 711-065-152). After washing four times for 5 min in PBS-T, sections were transferred to Vectastain ABC peroxidase kit (Vector Laboratories, cat. no. PK-6100) for 1 h. Following a four-fold five-minute wash with PBS-T, the DAB reaction was started. Using the SIGMAFAST™ DAB Tablets (Sigma, cat. no. D4293), slices were put in DAB and the reaction started by adding 1:1 H2O2 urea, slices were subsequently incubated for ten minutes. After four times wash with PBS for five minutes, sections were mounted using DPX (Sigma, cat. no. 06522) as a mounting media and stored for analysis at room temperature.

Altered morphology of the microglia, e.g. as a result of a lipopolysaccharide application, can be used to characterize whether the microglial cells are in the ramified or in the activated state. As a result of activation, microglial cells retract their processes into the cell body, thereby increasing its size. The Sholl analysis is a common technique used to evaluate and describe branching of cells, and is frequently used to analyse microglia morphology. The algorithm places concentric rings from a previously defined point and with a defined span and radius over the corresponding cell and counts the intersections of the structures with these rings to automatically detect the number of branches. The "Advanced Sholl Analysis Tool" version 3.6.12 (https://imagej.net/Sholl) was used. Images were taken on the Olympus BX53 Upright Microscope with a 40x objective in Tagged Image File Format (.tif), to prevent losing any image information. The resolution was 2880x1800 pixels. The pictures were converted to 8-bit. Twelve cells per hemisphere per animal were evaluated. Cells were selected according to the following criteria: the entire cell had to be in focus, the cell had to be as clear as possible from its surrounding neighbouring cells separable (i.e., the extensions should be as clearly as possible in the case of overlap), the cell was allowed on average not oblique be cut and it had to be always a single cell - duplications or superimpositions of several microglial cells in the area of Somas and/or the extensions were excluded. Care was taken that the analysed cell within the frame was cropped according to the extent of branches that were clearly connected to the cell body of interest and fully separable from crossing branches of other cells. The evaluation of specifically selected microglial cells was performed as follows: First, the soma of the corresponding cell was hand-traced using the ImageJ Polygon Selection tool. Within each mouse brain, 24 representative microglia cells (12 from either hemisphere) were selected within the region of interest, and whose entire cell soma and branches were fully present within the image. Thus, the parameters important for the further course could be grasped: the centre of gravity of the cell (as starting point for the Sholl analysis), the area of the cell and the pixel coordinates, in order to cut out the soma from the later obtained skeleton. The cell with the highlighted soma was subsequently cut out of the corresponding image using the Freehand Selection tool. Then a binary image was created. This binary image was skeletonized in the next step. Subsequently, the soma was cut out. The focus point of the analysed soma was marked and the Sholl analysis performed. The chosen radius was 2 to 60 µm to detect the complete cell. The distance of the concentric rings placed over each cell was 1 µm. On average, the first intersection was registered at 4 µm. Finally, the number of intersections per radius, the average maximum length of the projections and the soma size were evaluated per microglia.

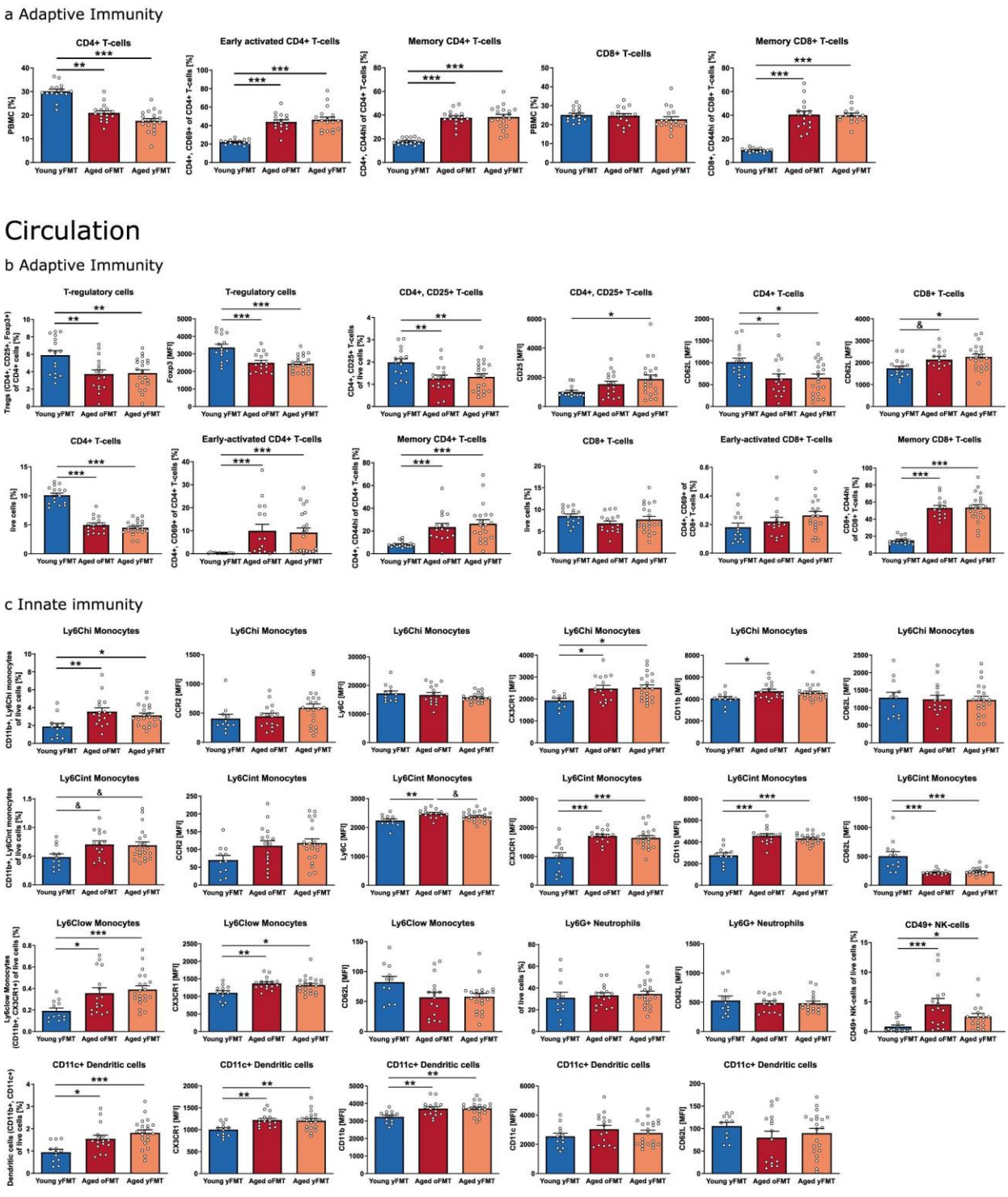
2.6.2 Extended data figures



*Extended data figure 2.1. Aging impacts on physiological parameters. a, No differences were found in telomere length amongst groups. n = 10 (young yFMT), 12 (aged oFMT), 12 (aged yFMT). b, Aged mice exhibited a decrease in intestinal motility independent of the donor FMT ($F(2,50)=9.055$, $p < 0.001$). Post-hoc analysis indicated decreased intestinal transit time in aged oFMT ($p < 0.001$) and aged yFMT ($p = 0.002$) compared to young yFMT. Aging decreased fecal water content independent of the donor FMT, respectively ($F(2,50)=5.464$, $p = 0.007$) with decreased fecal water content in both aged oFMT ($p = 0.007$) and aged yFMT ($p = 0.041$) compared to young yFMT. n = 16 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). c, Aged mice exhibited decreased cecum weight ($F(2,48)=22.78$, $p < 0.001$; post-hoc: $p < 0.001$) which was unaltered by FMT from young to aged mice. n = 15 (young yFMT), 15 (aged oFMT), 20 (aged yFMT). d, Aged mice showed decreased plasma corticosterone levels independent of the donor FMT ($F(2,48)=28.78$, $p < 0.001$) with young yFMT compared to aged oFMT ($p < 0.001$) and aged yFMT ($p < 0.001$), respectively. n = 15 (young yFMT), 15 (aged oFMT), 20 (aged yFMT). e, Concomitant with the decreased corticosterone levels, both left and right adrenal weight were decreased by age. Organ weight is depicted as % relative to body weight. n = 14 (young yFMT), 12 (aged oFMT), 18 (aged yFMT). f, Treatment significantly altered spleen weight ($H(3)=12.93$, $p = 0.016$). Post-hoc analysis (Dunn's) indicates reduced spleen weight in aged yFMT compared to young yFMT ($p = 0.001$). n = 16 (young yFMT), 15 (aged oFMT), 19 (aged yFMT). g, Aged mice, independent of the donor FMT, showed an accumulation of fat depots, subcutaneous ($H(3)=12.84$, $p = 0.002$, Kruskal-Wallis post-hoc Dunn's) and retroperitoneal ($H(3)=21.24$, $p < 0.001$, Kruskal-Wallis post-hoc Dunn's) while inter scapular brown fat (iBAT) remained unaltered. n = 15 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). (a-g) If not otherwise indicated: data was analyzed by one-way ANOVA post-hoc Holm–Sidak. All statistical tests were conducted two-sided. Each datapoint represents one independent mouse. Mean ± s.e.m., *p < 0.05, **p < 0.01, ***p < 0.001.*

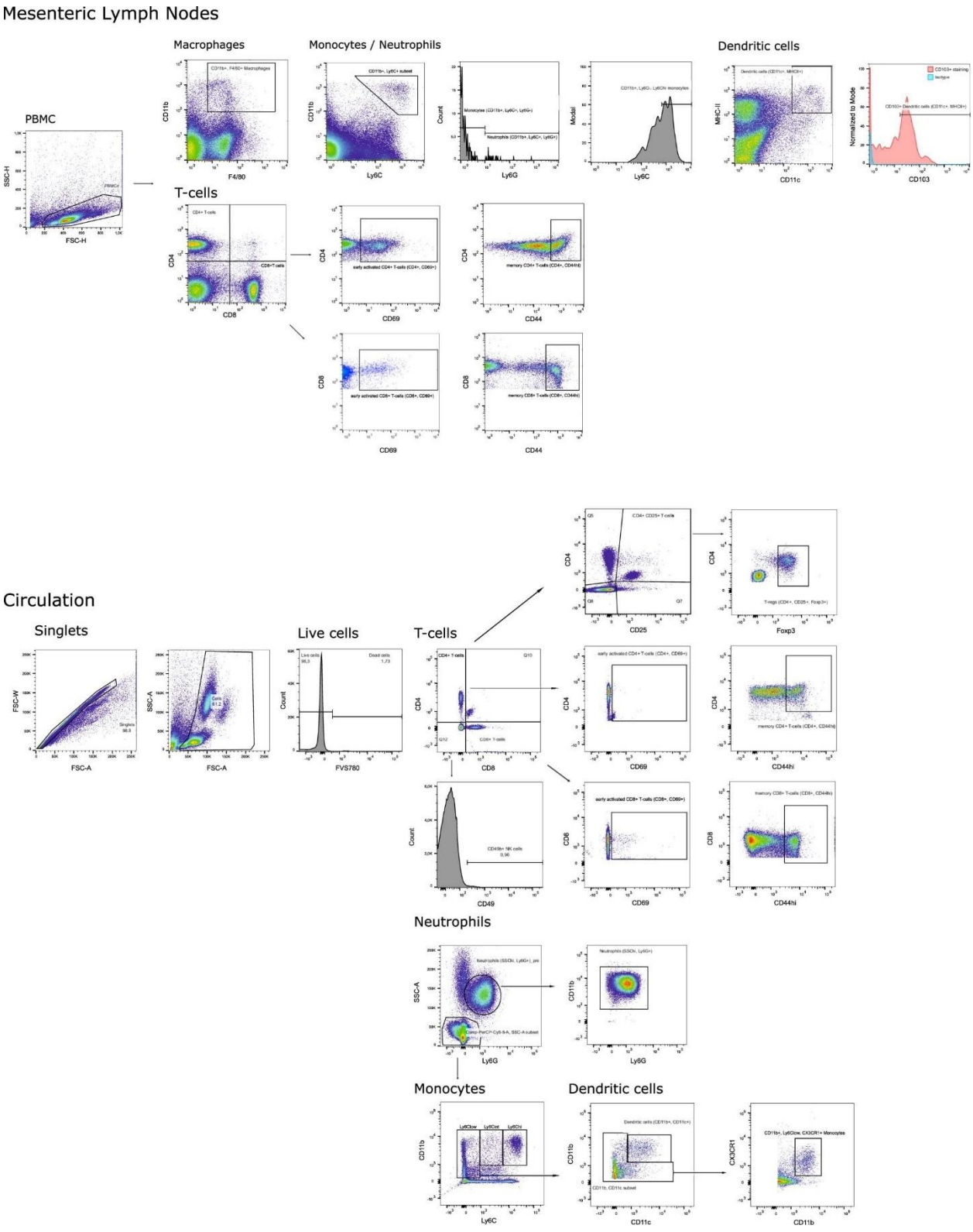


Extended data figure 2.2. yFMT did not improve the aging-associated reduction in survival of newborn hippocampal neurons or alter microglia morphology. a, Aging induced dramatic declines in the number of surviving new-born neurons, labelled with BrdU + /NeuN + ($F(2,12)=55.23$, $p < 0.001$; young yFMT vs aged oFMT ($p < 0.001$). However, there were no differences in neuron survival between aged oFMT and aged yFMT mice ($p = 0.832$). One-way ANOVA post-hoc Holm–Sidak. Mean ± s.e.m. n = 5 independent mice per group. b, There were no significant differences in microglia branching morphology revealed by Sholl analysis. Mean (number of intersections per µm radius from center) ± s.e.m. n = 5 independent mice per group. Scale bar = 200 µm.



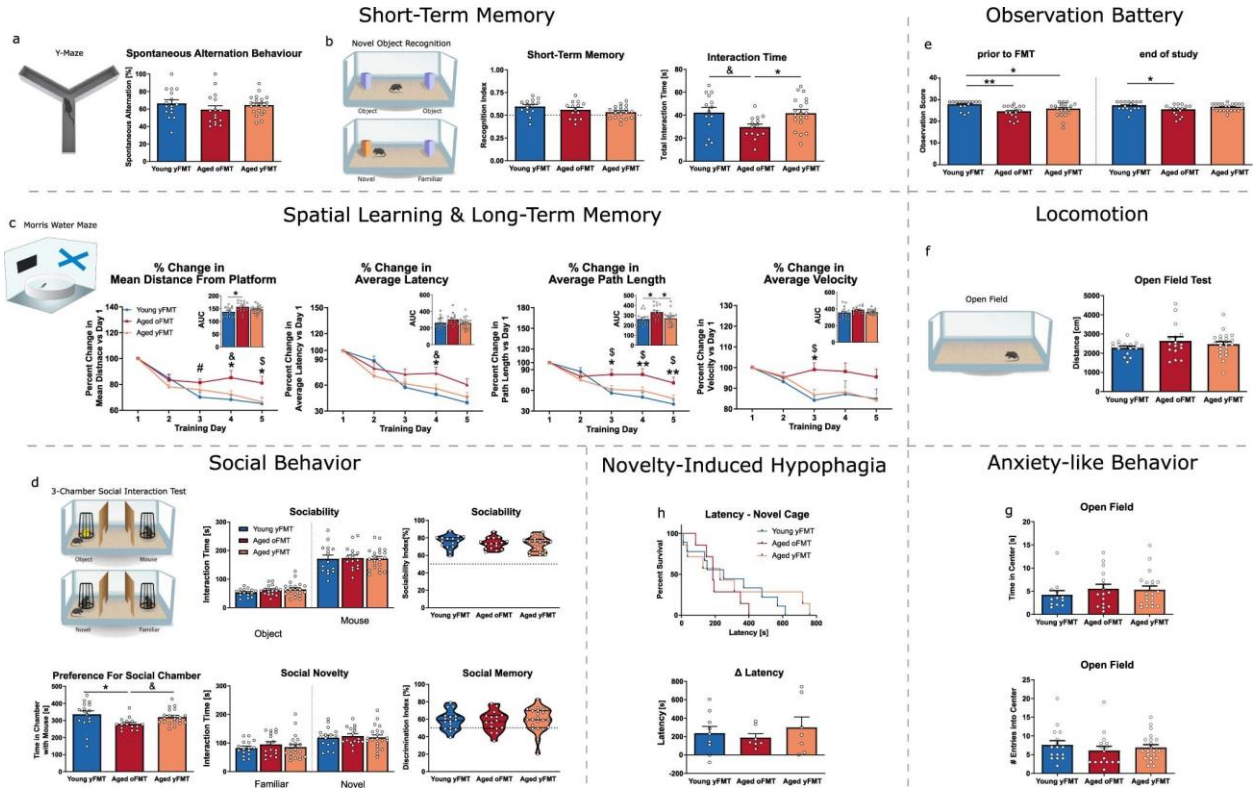
Extended data figure 2.3. Impact of aging on peripheral innate and adaptive immune system. Adaptive and innate immunity was assessed by Flow Cytometry in both the mesenteric lymph nodes (at the end of the study) and the systemic circulation (before mice were assessed for behavior). ***a****, Adaptive immunity in MLNs: A profound age effect was noted in the overall population of CD4$^+$ T-cells (H(3)=30.49, p < 0.001, Kruskal-Wallis post-hoc Dunn's) while CD8$^+$ T-cells were not affected by age nor FMT. Subsequently, we assessed subpopulations of both, CD4$^+$ and CD8$^+$ T-cells, which both, early activated (F(2,47)=31.79, p < 0.001) and memory CD4$^+$ T-cells (F(2,48)=45.47,*

$p < 0.001$), and memory $CD8^+$ T-cells ($F(2,44)=68.14$, $p < 0.001$) showed an age-associated increase which was unaffected by yFMT into aged mice. n = 15-16 (young yFMT), 16 (aged oFMT), 19-21 (aged yFMT). ***b****, Adaptive immunity in the circulation: A decrease of T-regulatory cells and their transcription factor, foxp3, was observed in both age groups ($F(2,50)=6.789$, $p = 0.003$ and $F(2,50)=12.90$, $p < 0.001$, respectively). In addition, $CD4^+$, $CD25^+$ T-cells were decreased in both age groups ($F(2,50)=6.554$, $p = 0.003$) while CD25 expression on $CD4^+$, $CD25^+$ T-cells were increased in aged yFMT compared to young yFMT ($H(3)=6.179$, $p = 0.046$, Kruskal-Wallis post-hoc Dunn's: $p = 0.041$). Similarly to MLNs, the overall population of $CD4^+$ T-cells was strongly decreased by age ($F(2,50)=89.4$, $p < 0.001$) while $CD8^+$ T-cells remained unchanged. Subsequent assessment of the respective subpopulations revealed a strong increase in both age groups in early activated ($H(3)=20.74$, $p < 0.001$, Kruskal-Wallis post-hoc Dunn's) and memory $CD4^+$ T-cells ($H(3)=23.05$, $p < 0.001$, Kruskal-Wallis post-hoc Dunn's), and memory $CD8^+$ T-cells ($F(2,49)=51.81$, $p < 0.001$) while early activated $CD8^+$ T-cells remained unchanged. Further, we assessed the expression of the adhesion marker, CD62L, on both overall $CD4^+$ and $CD8^+$ T-cells. A decrease of CD62L expression in both age groups were found in $CD4^+$ T-cells ($F(2,50)=4.875$, $p = 0.012$). In contrast, aged mice exhibited an increase in CD62L expression in $CD8^+$ T-cells ($F(2,50)=4.684$, $p = 0.014$) from which Aged yFMT was significantly different to Young yFMT ($p = 0.013$) while aged oFMT showed a trend ($p = 0.068$). n = 14-16 (young yFMT), 15-16 (aged oFMT), 20-21 (aged yFMT).* ***(c)*** *Innate immunity in circulation: assessment of three subpopulations of monocytes; $Ly6C^{hi}$, $Ly6C^{int}$ and $Ly6C^{low}$ monocytes revealed a strong age impact on the overall population and subsequent protein expression levels of activation and trafficking markers. An age-related increase was found in the overall population of $Ly6C^{hi}$ ($F(2,46)=5.489$, $p = 0.007$), $Ly6C^{int}$ ($F(2,45)=3.316$, $p = 0.045$) and $Ly6C^{low}$ monocytes ($H(3)=14.21$, $p < 0.001$, Kruskal-Wallis post-hoc Dunn's). Similarly, all three populations exhibited an age-related increased expression in the trafficking receptor CX3CR1 ($Ly6C^{hi}$: $F(2,44)=4.22$, $p = 0.021$, $Ly6C^{int}$: $F(2,44)=14.07$, $p < 0.001$, and $Ly6C^{low}$ monocytes: $F(2,46)=5.832$, $p = 0.006$) which remained unaffected by yFMT into aged mice. Moreover, aging triggered an increase in the activation marker CD11b on both $Ly6C^{int}$ and $Ly6C^{hi}$ monocytes $F(2,45)=29.51$, $p < 0.001$, post-hoc: $p < 0.001$, and $F(2,46)=3.872$, $p = 0.028$, post-hoc: $p = 0.032$, respectively), and while these aging-related effects persisted in the $Ly6C^{int}$ monocytes of aged yFMT mice ($p < 0.001$), there was no significant difference apparent in the CD11b marker on $Ly6C^{hi}$ monocytes in aged yFMT compared to young yFMT mice. Further, both aged groups showed a decreased expression of CD62L on $Ly6C^{int}$ monocytes ($F(2,43)=16.43$, $p < 0.001$) while both, $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes remained unaffected. In addition, Aged oFMT showed an increase in Ly6C expression on $Ly6C^{int}$ monocytes (($F(2,46)=6.819$, $p = 0.003$; post-hoc: $p = 0.002$) which tended to be rescued in aged yFMT ($p = 0.071$). Assessment of $CD11b^+$, $CD11c^+$ dendritic cells revealed an age-related increase in both age groups regarding the overall population of $CD11c^+$ dendritic cells in the circulation ($F(2,46)=8.221$, $p < 0.001$), the trafficking receptor CX3CR1 ($F(2,46)=6.467$, $p = 0.003$), and the activation marker CD11b ($F(2,46)=6.867$, $p = 0.003$). Notably, no differences were found in $Ly6G^+$ neutrophils while $CD49^+$ NK cells showed a strong increase in both aged groups ($H(3)=15.86$, $p < 0.001$, Kruskal-Wallis post-hoc Dunn's). n = 12-15 (young yFMT), 15-16 (aged oFMT), 19-21 (aged yFMT). Mean ± s.e.m. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, & $p < 0.1$. (a-c) If not otherwise indicated: data was analyzed by one-way ANOVA post-hoc Holm–Sidak. All statistical tests were conducted two-sided. Each datapoint represents one individual mouse.*



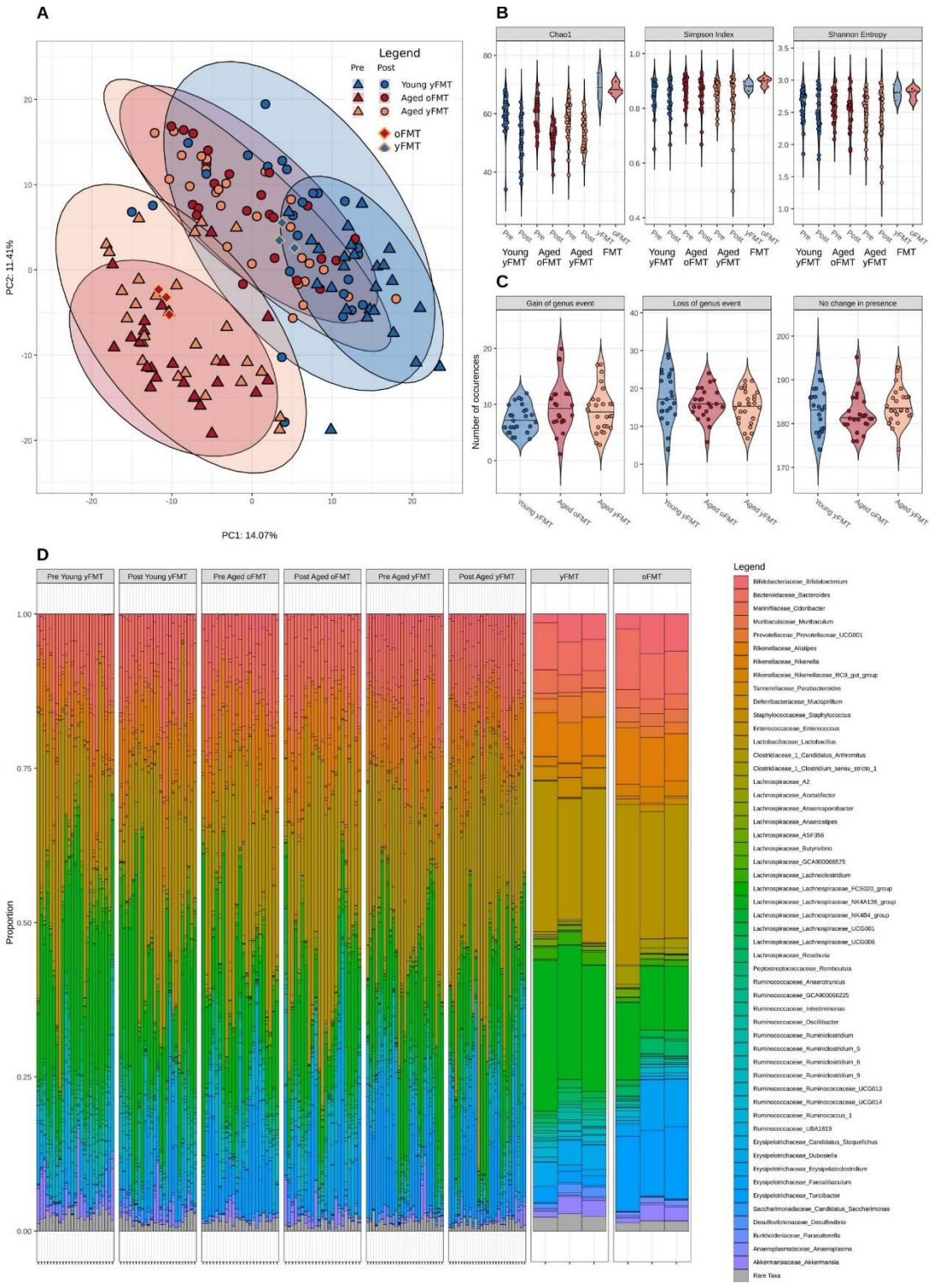
Extended data figure 2.4. Gating strategy. For MLNs, PBMCs were selected based on SSC-H and FSC-H using the FACSCalibur. F4/80^{+} Macrophages were further selected using CD11b^{+} and F4/80^{+}. T-cells were gated through CD4^{+} and CD8^{+}. To assess sub-populations of T-cells, each CD4^{+} and CD8^{+} T-cells were investigated with the markers for early activation (CD69) and memory cells (CD44^{hi}). In addition, we assessed monocytes / neutrophils by

gating on CD11b$^+$ and Ly6C$^+$, followed by differentiation with Ly6G for monocytes (CD11b$^+$, Ly6C$^+$, Ly6G$^-$) and neutrophils (CD11b$^+$, Ly6C$^+$, Ly6G$^+$). Monocytes were further determined for the proportion of Ly6C^{hi} monocytes which express Ly6C on a high level (CD11b$^+$, Ly6G$^-$, Ly6C^{hi}). Dendritic cells were investigated through gating for CD11c$^+$ and MHCII$^+$, and subsequent CD103$^+$ marker expression. For the circulation assessment, we used a more comprehensive panel and utilized the FACSCelesta, which enabled initial gating for singlets, followed by selection of the PBMCs and live cells. We gated for CD4$^+$ and CD8$^+$ T-cells followed by subsequent assessment of each population for markers for early activation (CD69) and memory cells (CD44^{hi}). We also investigated T-regulatory cells (CD4$^+$, CD25$^+$, Foxp3$^+$) through gating for CD4$^+$, CD25$^+$ T-cell subpopulation followed by Foxp3$^+$. In addition, we gated for CD49$^+$ Natural killer (NK) cells through the CD4$^-$, CD8$^-$ population. Neutrophils were selected based on SSC^{hi}, Ly6G$^+$ and CD11b$^+$. Monocytes were selected through SSC^{hi} and Ly6G$^-$. Based on CD11b$^+$ expression and their respective Ly6C expression, we differentiated the monocyte population into Ly6C^{int} monocytes and Ly6C^{hi} monocytes. Dendritic cells were assessed through Ly6C^{low} and CD11c$^+$. Ly6C^{low} monocytes were selected through Ly6C^{low} followed by CX3CR1$^+$.



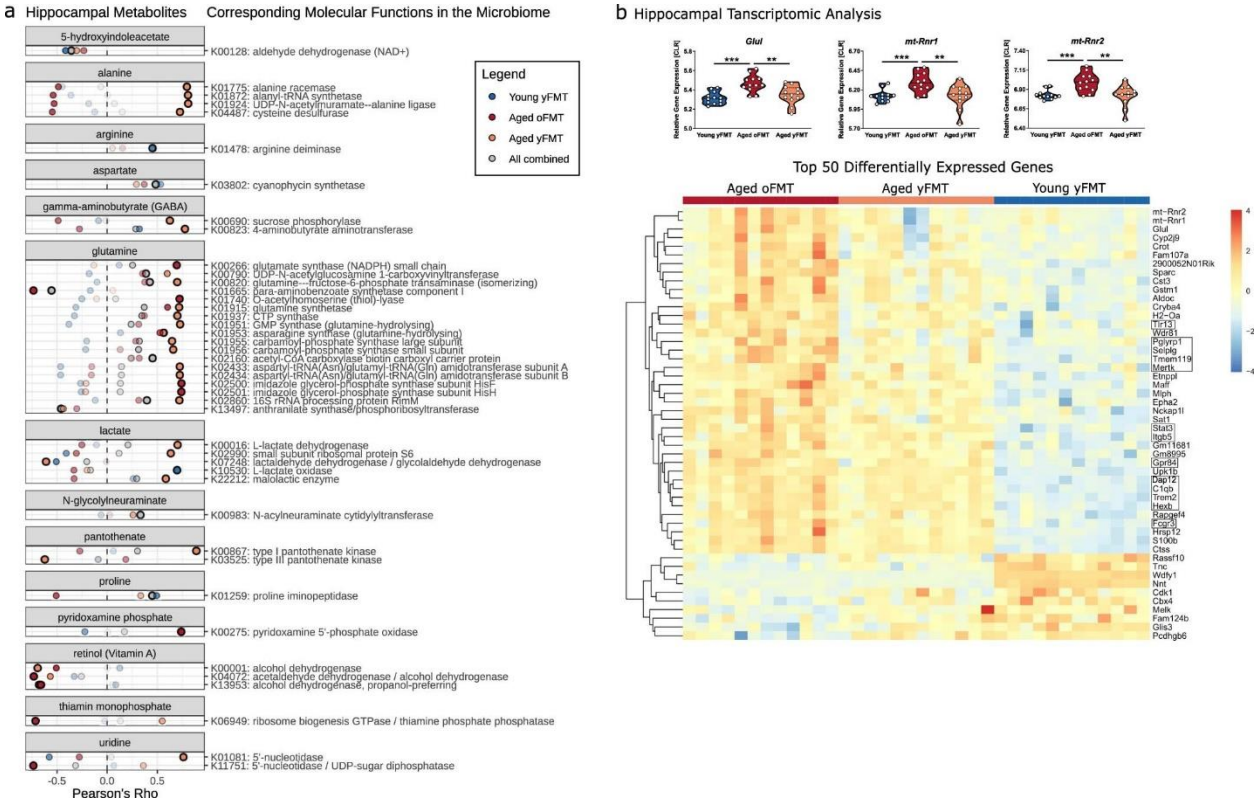
Extended data figure 2.5. Additional behavioural readouts. a, Short-term memory assessed by spontaneous alternation behavior (Y-maze). No significant differences were observed between groups. n = 16 (young yFMT), 15 (aged oFMT), 21 (aged yFMT). b, Short-term memory and exploration in the Novel Object Recognition (NOR) test. While long-term memory in the NOR was not significantly different across groups, there were significant differences in exploration of the objects (F(2,44)=3.388, p = 0.028), with aged mice receiving yFMT (aged yFMT) showing an increased exploration activity compared to aged oFMT (p = 0.043). n = 14 (young yFMT), 12 (aged oFMT), 19 (aged yFMT). c, Learning in Morris Water Maze: to better visualize the change in learning, readouts were further assessed by determining the % change from training day one (statistical analysis via RM One-Way ANOVA post-hoc Holm-Sidak followed by assessing differences per training day via one-way ANOVA; and AUC). Overall, there was a trend towards significant differences in learning in % change in mean distance from platform, path length, and average velocity, though there were no significance differences between groups following post-hoc corrections. Further assessment at each training day revealed significant age effects when comparing aged oFMT to young yFMT mice. Interestingly, aged yFMT mice showed no statistical difference compared to young yFMT mice, but significantly improved compared to aged oFMT mice in several parameters. First, aged oFMT mice displayed a reduced improvement in % change in mean distance from platform on Training Days 4 and 5 (F(2, 50)=4.349, p = 0.018, post-hoc p = 0.021; F(2,

*50)=4.031, p = 0.024, post-hoc p = 0.046) as well as a trend on Day 3 (F(2, 50)=2.395, p = 0.102, post-hoc p = 0.097). Aged yFMT mice showed significant improvement compared to aged oFMT mice on Training Day 5 (p = 0.046) and trended towards significant difference on Day 4 (p = 0.056). This recovery in the effect of aging by yFMT was also evident in AUC, where aged oFMT mice had significantly higher AUC than young yFMT mice (F(2, 50)=3.842, p = 0.028, post-hoc p = 0.047) which was ameliorated by yFMT into aged mice. While there was no overall difference in % change in latency across training days, a significant age impairment is present on Training Day 4 (F(2, 50)=3.814, p = 0.029, post-hoc p = 0.031) where aged yFMT mice also trended towards significantly improving compared to aged oFMT (p = 0.089). Significant effects of age were apparent in % change in average path length on Training Days 3, 4, and 5 (Day 3: F(2, 50)=4.481, p = 0.016, post-hoc p = 0.022; Day 4: F(2, 50)=6.165, p = 0.004, post-hoc p = 0.004; and Day 5: F(2, 50)=5.580, p = 0.007, post-hoc p = 0.007). Furthermore, aged yFMT significantly improved compared to aged oFMT on these three training days (p = 0.039; p = 0.027; p = 0.030, respectively). This clear age effect was further evident when looking at AUC, where aged oFMT mice showed significantly increased AUC compared to young yFMT and aged yFMT mice (F(2, 50)=5.277, p = 0.008, post-hoc p = 0.016 and p = 0.016, respectively). Aged oFMT mice had significantly different % change in average velocity compared to both young yFMT and aged yFMT mice on Training Day 3 (F(2, 50)=4.351, p = 0.018, post-hoc p = 0.026 and p = 0.04, respectively), though this significance was not apparent in AUC. n = 16 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). d, Social behavior and memory: Treatment affected social behavior with respect to the time the test mouse interacted with a con-specific mouse (over an object) in the second phase of the three-chamber social interaction test (F(2,49)=4.168, p = 0.021) while interaction with a novel mouse (over the now familiar mouse) in the third phase of the three-chamber social interaction test remained unaffected by the treatment. Post-hoc analysis indicated decreased interaction with a con-specific mouse in aged oFMT compared to young yFMT (p = 0.022), which trended towards being rescued by yFMT into aged mice (p = 0.077). n = 15 (young yFMT), 16 (aged oFMT), 21 (aged yFMT). e, Observation Battery: Bar plots show observation battery score prior to FMT compared to end of study. Both aged groups showed a decreased score prior to FMT compared to young yFMT (H(3)=13.33, p = 0.001; post-hoc: aged oFMT p = 0.001, and aged yFMT p = 0.038, respectively). In contrast, aged yFMT did not show a significant difference (p = 0.46) at the end of the study while aged oFMT retained a decreased score compared to young yFMT (H(3)=6.146, p = 0.046; Kruskal-Wallis post-hoc Dunn's: p = 0.04). n = 16 (young yFMT), 16 (aged oFMT), 21 (Aged yFMT). f, Locomotion: Locomotion was unaltered by age (Aged oFMT) or FMT from young to aged mice (Aged yFMT). g, Anxiety-like behavior: neither age nor FMT affected time in center and number of entries into the center zone in the open field. n = 12-16 (young yFMT), 16 (aged oFMT), 20-21 (aged yFMT). h, No significant differences were observed in the Novelty-Induced Hypophagia behavioral test. n = 9 (young yFMT), 9 (aged oFMT), 7 (aged yFMT). Mean ± s.e.m. For MWM Training Day figures, young yFMT vs aged oFMT: #p < 0.10, *p < 0.05, **p < 0.01; aged yFMT vs aged oFMT: &p < 0.10, $p < 0.05; For all other figures: & p < 0.10, *p < 0.05, **p < 0.01. (a-i) If not otherwise indicated: data was analyzed by one-way ANOVA post-hoc Holm-Sidak. All statistical tests were conducted two-sided. Each datapoint represents one independent mouse.*

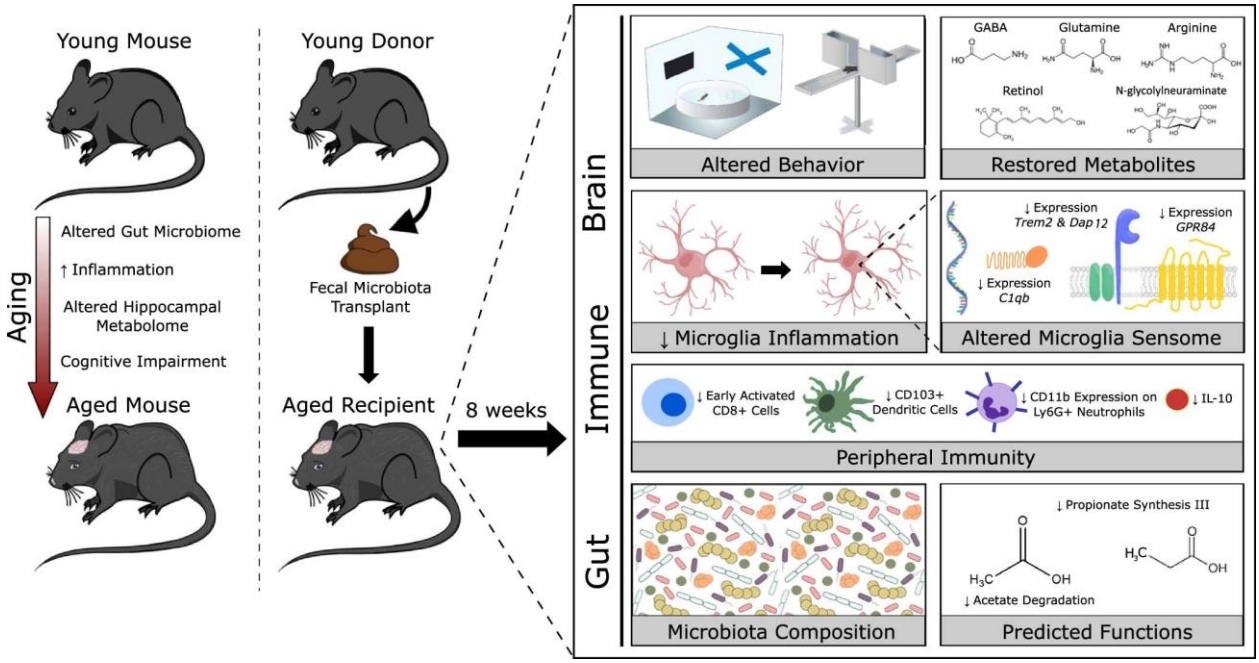


Extended data figure 2.6. FMT samples have a high diversity and cluster with the faecal microbiome of their donors. a, Principal component analysis showing the compositions of the faecal microbiome in young and aged mice as measured in Aitchison distance, as well as the donor samples in triplicates. Ellipses represent the 95% confidence intervals per group. b, Violin plots showing that pooled FMT samples have a higher alpha diversity compared to those of the murine microbiome. Black horizontal lines in violin plots represent the medians. c, Violin plots showing the number of genera gained, lost and unchanged per mouse after FMT. No differences in the amounts of genera gained,

lost or unchanged were found. d, Stacked bar plots showing the composition of the microbiome per treatment group per time point, including both types of FMT donor material. Genera that were never detected above 1% reads abundance were aggregated and labeled as 'Rare Taxa' for this figure. No statistical testing was done for the FMT donor samples due to the low sample number (n = 3 per group). n = 25 (young yFMT), 23 (aged oFMT), 27 (aged yFMT) independent mice.



*Extended data figure 2.7. yFMT influences hippocampal transcriptomics and yFMT-induced microbiome alterations functionally correlate with hippocampal metabolomics. a, Pearson correlation coefficients for the hippocampal metabolites that were found to be altered in aging and restored towards young levels after yFMT. The vertical dashed lines depict a Pearson correlation coefficient of 0. Colors depict the treatment groups, whereas grey points represent the correlation coefficients after pooling all three groups. Opaque points with thick black borders display significant correlations after Benjamini-Hochberg correction at q < 0.1. Non-opaque points display the correlation coefficient for the other treatment groups. Full statistical information is available in Supplementary Table 2.4. b, Transcriptomics analysis revealed three hippocampal genes altered in aging and reversed by yFMT. Relative gene expression is depicted as violin plots. All three genes, Glul and mt-Rnr1 and mt-Rnr2 were significantly altered by aging (aged oFMT vs. young yFMT: p < 0.001, p = 0.001, p < 0.001, and q = 0.004, q = 0.016, and q = 0.007, respectively), and restored by yFMT (aged yFMT vs. aged oFMT: p = 0.003, p = 0.006, p = 0.003, and q = 0.125, q = 0.159, and q = 0.125, respectively). Mann-Whitney U-test followed by Storey's q-value post-hoc tests: **p < 0.01, ***p < 0.001. n = 12 per group. Heatmap showing top 50 most differentially expressed genes between groups by FDR. Microglia sensome-related genes are indicated with a box.*



Extended data figure 2.8. Graphical summary. Microbiota from young mice rejuvenated aspects of aging-related alterations in the gut microbiome, peripheral and brain immunity, and restored metabolites and behaviour.

2.6.3 Supplementary tables

Due to the large size of some tables, not all tables are displayed here. All supplementary tables can be found with the published paper at https://www.nature.com/articles/s43587-021-00093-9#Sec36 (Boehme *et al.* 2021).

Supplementary Table 2.1. Differences in the genera per group following FMT. BH indicates Benjamini-Hochberg posthoc correction. Due to size of table, please see published version at https://www.nature.com/articles/s43587-021-00093-9#Sec36 (Boehme et al. 2021).

Supplementary Table 2.2. Differences in gut-brain modules per group following FMT. BH indicates Benjamini-Hochberg posthoc correction.

GBM ID	GBM name	Pre Young yFMT vs Post Young yFMT BH.adjusted.p.value	Pre Young yFMT vs Post Young yFMT Effect Size	Pre Aged yFMT vs Post Aged yFMT BH.adjusted.p.value	Pre Aged yFMT vs Post Aged yFMT Effect Size	Pre Aged oFMT vs Post Aged oFMT BH.adjusted.p.value	Pre Aged oFMT vs Post Aged oFMT Effect Size
MGB005	Tryptophan synthesis	0.3496	0.3453	0.7893	-0.0898	0.8867	-0.0590
MGB006	Glutamate synthesis I	0.4871	0.2170	0.3338	-0.2993	0.7374	-0.1727
MGB007	Glutamate synthesis II	0.1651	0.4332	0.5259	-0.2278	0.6872	-0.2034
MGB009	Histamine synthesis	0.1884	0.4134	0.0255	0.3927	0.0629	0.5433
MGB015	p-Cresol synthesis	0.8731	0.0643	0.1710	-0.3968	0.1570	0.4188
MGB016	p-Cresol degradation	0.6273	0.0755	NA	NA	NA	NA
MGB019	GABA degradation	0.2166	0.3646	0.0227	0.4182	0.0621	0.5391
MGB021	GABA synthesis II	0.6224	0.0750	0.6921	0.0210	0.7127	-0.0189
MGB022	GABA synthesis III	0.5382	-0.2317	0.0493	-0.3844	0.8295	0.0857
MGB023	Dopamine degradation	0.1082	-0.4583	0.7747	-0.0643	0.8750	-0.0834
MGB024	DOPAC synthesis	0.1146	-0.4165	0.8416	0.0257	0.8081	-0.1296
MGB025	Nitric oxide synthesis I (NO synthase)	0.6047	-0.0498	0.5521	0.1600	0.6320	0.1489
MGB027	Nitric oxide degradation I (NO dioxygenase)	0.0043	-0.6176	0.6629	0.1481	0.3589	0.2848
MGB028	Nitric oxide degradation II (NO reductase)	0.3303	0.2540	0.0009	-0.7478	0.0146	-0.8630
MGB029	ClpB (ATP-dependent chaperone protein)	0.4069	0.2938	0.6931	-0.1433	0.8818	-0.0420
MGB031	17-beta-Estradiol degradation	0.1948	0.4119	0.6812	0.1278	0.1163	0.5353
MGB032	Quinolinic acid synthesis	0.8662	0.0009	0.2513	-0.2868	0.1269	0.4396
MGB033	Quinolinic acid degradation	0.1159	0.4770	0.6470	0.1487	0.5553	0.2442
MGB034	Isovaleric acid synthesis I (KADH pathway)	0.5504	0.1754	0.8951	0.0199	0.8914	-0.0892
MGB035	Isovaleric acid synthesis II (KADC pathway)	0.4500	0.1377	0.7861	0.0171	0.8315	-0.0175
MGB036	S-Adenosylmethionine (SAM) synthesis	0.0877	0.5278	0.8035	0.0435	0.4125	0.3463
MGB037	Inositol synthesis	0.0120	0.7557	0.7328	-0.0984	0.8218	0.0657
MGB038	Inositol degradation	0.1060	-0.4511	0.7696	-0.0777	0.8797	-0.0688
MGB039	g-Hydroxybutyric acid (GHB) degradation	0.6723	0.0345	0.7013	0.0262	0.7184	0.0410
MGB040	Menaquinone synthesis (vitamin K2) I	0.2908	0.3512	0.8771	0.0055	0.1130	0.4423
MGB041	Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)	0.7930	-0.0916	0.4022	-0.1362	0.6795	0.0707
MGB043	Acetate synthesis I	0.0799	0.5356	0.7430	0.1057	0.6991	0.2239
MGB045	Acetate synthesis III	0.6238	0.0810	0.6228	0.1004	0.7031	0.0838
MGB047	Acetate degradation	0.8798	0.0319	0.0155	-0.6363	0.6095	-0.2144
MGB048	Propionate synthesis I	0.6459	0.0886	0.6655	0.0950	0.6142	-0.1192
MGB049	Tryptophan degradation	0.5539	-0.1355	0.0354	-0.4235	0.8778	0.0151
MGB051	Glutamate degradation II	0.6338	0.0583	NA	NA	NA	NA
MGB052	Butyrate synthesis I	0.7202	0.0113	0.6699	-0.1536	0.0165	-0.4562
MGB053	Butyrate synthesis II	0.5838	-0.1098	0.7219	0.0879	0.8406	0.0360
MGB054	Propionate synthesis II	0.5435	0.2021	0.4551	0.1355	0.0612	-0.3866
MGB055	Propionate synthesis III	0.7451	-0.1287	0.0110	-0.5393	0.6878	-0.1357
MGB056	Propionate degradation I	0.6191	0.0425	0.6857	0.0244	0.7201	-0.0274

Supplementary Table 2.3. Differences in hippocampal metabolomics between groups following FMT, including metabolite type and reference key for volcano plots in Figure 2.3. Due to size of table, please see published version at https://www.nature.com/articles/s43587-021-00093-9#Sec36 (Boehme et al. 2021).

Supplementary Table 2.4. Coorelations between hippocampal metabolomics and KO pathways in the gut microbiome. Due to size of table, please see published version at https://www.nature.com/articles/s43587-021-00093-9#Sec36 (Boehme et al. 2021).

Supplementary Table 2.5. Differences in hippocampal transcriptomics between groups following FMT.

name	gene_name	Aged oFMT vs Aged yFMT	Aged oFMT vs Aged yFMT	Young yFMT vs Aged yFMT	Young yFMT vs Aged yFMT	Young yFMT vs Aged oFMT	Young yFMT vs Aged oFMT	Aged oFMT vs All yFMT p.value	Aged oFMT vs All yFMT	Aged oFMT vs Aged yFMT	Young yFMT vs Aged yFMT	Young yFMT vs Aged oFMT	Aged oFMT vs All yFMT q.value	Aged oFMT vs Aged yFMTpq	Young yFMT vs Aged yFMTpq	Young yFMT vs Aged oFMTpq	Aged oFMT vs All yFMTpq
ENSMUSG00000000290	Itgb2	0.5137	0.3889	0.1005	-0.6480	0.0121	-1.0700	0.0669	0.7052	0.7829	0.1713	0.0397	0.2463	ns	ns	pq	ns
ENSMUSG00000000682	Cd52	0.1277	-0.4373	0.0000	-3.7152	0.0000	-3.0646	0.1660	0.7565	0.4699	0.0000	0.0001	0.4128	ns	pq	pq	ns
ENSMUSG00000000869	Il4	0.8428	-0.0917	0.4428	0.3585	0.1782	0.4088	0.3613	-0.2654	0.9016	0.5658	0.3093	0.6043	ns	ns	ns	ns
ENSMUSG00000001123	Lgals9	0.7553	0.2603	0.0205	0.8896	0.3186	0.5320	0.7036	-0.1158	0.8686	0.0428	0.4580	0.8747	ns	pq	ns	ns
ENSMUSG00000003420	Fcgrt	0.9323	0.0251	0.0000	2.9462	0.0000	2.6365	0.0134	-0.8564	0.9530	0.0000	0.0000	0.1123	ns	pq	pq	pq
ENSMUSG00000004040	Stat3	0.2657	0.5919	0.0083	1.2460	0.0387	0.8789	0.6079	-0.0633	0.5819	0.0231	0.0990	0.8224	ns	pq	pq	ns
ENSMUSG00000004609	Cd33	0.5512	0.1903	0.9774	-0.0793	0.3186	-0.3144	0.3439	0.2506	0.7829	0.9881	0.4580	0.6043	ns	ns	ns	ns
ENSMUSG00000004730	Adgre1	0.1978	0.4402	0.3186	-0.4431	0.0387	-0.9384	0.0486	0.6765	0.5515	0.4248	0.0990	0.2033	ns	ns	pq	p
ENSMUSG00000007613	Tgfbr1	0.6297	0.1597	0.0045	1.4298	0.0014	1.5294	0.1461	-0.4771	0.7829	0.0166	0.0063	0.3735	ns	pq	pq	ns
ENSMUSG00000009185	Ccl8	0.4095	-0.5019	0.0145	-1.0414	0.1432	-0.4139	0.7283	-0.0100	0.6850	0.0342	0.2744	0.8816	ns	pq	ns	ns
ENSMUSG00000010142	Tnfrsf13b	0.5512	-0.1983	0.8874	-0.0114	0.4776	0.1801	0.4358	-0.2020	0.7829	0.9173	0.5858	0.6796	ns	ns	ns	ns
ENSMUSG00000014361	Mertk	0.0597	0.8006	0.0242	0.9211	0.8428	0.2728	0.3439	0.2273	0.3430	0.0494	0.9016	0.6043	ns	pq	ns	ns
ENSMUSG00000015852	Fcrls	0.0519	0.8117	0.0000	3.4560	0.0000	3.2433	0.2115	-0.5607	0.3408	0.0000	0.0000	0.4746	ns	pq	pq	ns
ENSMUSG00000015947	Fcgr1	0.0887	0.7089	0.0780	0.6164	0.8874	-0.0299	0.3791	0.3678	0.3549	0.1380	0.9173	0.6229	ns	ns	ns	ns
ENSMUSG00000018774	Cd68	0.1782	0.5565	0.1432	-0.6856	0.0205	-1.0746	0.0317	0.8112	0.5288	0.2352	0.0571	0.1942	ns	ns	pq	pq
ENSMUSG00000018899	Irf1	0.3186	-0.5739	0.0083	1.2007	0.0001	1.9096	0.0063	-1.0269	0.6236	0.0231	0.0007	0.0831	ns	pq	pq	pq
ENSMUSG00000018920	Cxcl16	0.6297	-0.1875	0.2189	-0.5505	0.1782	-0.4486	0.6313	0.1125	0.7829	0.3302	0.3093	0.8297	ns	ns	ns	ns
ENSMUSG00000020009	Ifngr1	0.1277	0.6263	0.0000	2.1421	0.0005	1.7830	0.3271	-0.4100	0.4699	0.0004	0.0024	0.6043	ns	pq	pq	ns
ENSMUSG00000020787	P2rx1	0.4428	0.4642	0.0000	2.9317	0.0000	2.2738	0.0904	-0.5669	0.7275	0.0000	0.0003	0.2969	ns	pq	pq	ns
ENSMUSG00000021423	Ly86	0.3186	0.5081	0.1600	0.5780	0.8428	0.0690	0.6550	0.2326	0.6236	0.2538	0.9016	0.8370	ns	ns	ns	ns
ENSMUSG00000021624	Cd180	0.6297	-0.3862	0.2657	-0.5487	0.7553	-0.2734	0.9341	-0.0365	0.7829	0.3819	0.8686	0.9549	ns	ns	ns	ns
ENSMUSG00000021665	Hexb	0.1600	0.6101	0.0173	1.0128	0.1782	0.5232	0.9868	0.0638	0.4906	0.0370	0.3093	0.9868	ns	pq	ns	ns
ENSMUSG00000021876	Rnase4	0.0684	0.5901	0.0145	1.2838	0.0449	0.8710	0.9341	-0.0596	0.3494	0.0342	0.1116	0.9549	ns	pq	pq	ns
ENSMUSG00000022496	Tnfrsf17	0.3474	-0.6663	0.6297	-0.0016	0.2189	0.6119	0.1994	-0.7389	0.6556	0.7334	0.3533	0.4704	ns	ns	ns	ns
ENSMUSG00000022817	Itgb5	0.0332	0.9038	0.0083	1.1929	0.1782	0.4693	0.6791	0.1768	0.3058	0.0231	0.3093	0.8559	p	pq	ns	ns
ENSMUSG00000022901	Cd86	0.5899	-0.2144	0.2415	-0.5163	0.3777	-0.3636	0.8557	0.0634	0.7829	0.3584	0.5036	0.9218	ns	ns	ns	ns
ENSMUSG00000023992	Trem2	0.0332	0.9449	0.7125	-0.1376	0.0205	-1.0779	0.0088	0.9818	0.3058	0.8093	0.0571	0.1014	p	ns	pq	pq
ENSMUSG00000024401	Tnf	0.2657	0.5316	0.1600	-0.1666	0.0205	-0.9145	0.0447	0.6650	0.5819	0.2538	0.0571	0.1960	ns	ns	pq	pq
ENSMUSG00000024610	Cd74	0.5137	-0.3015	0.7987	0.1113	0.2189	0.4709	0.2651	-0.3935	0.7829	0.8447	0.3533	0.5544	ns	ns	ns	ns
ENSMUSG00000024621	Csf1r	0.0449	0.7078	0.0000	2.3658	0.0000	2.1336	0.3271	-0.3705	0.3408	0.0002	0.0003	0.6043	p	pq	pq	ns
ENSMUSG00000024810	Il33	0.7125	0.1650	0.5137	-0.2730	0.1978	-0.5275	0.3271	0.3254	0.8298	0.6219	0.3370	0.6043	ns	ns	ns	ns
ENSMUSG00000025491	Ifitm1	0.6297	-0.2198	0.0145	1.0662	0.0023	1.4251	0.0447	-0.7142	0.7829	0.0342	0.0093	0.1960	ns	pq	pq	pq
ENSMUSG00000025492	Ifitm3	0.4095	-0.2645	0.3186	-0.5972	0.9323	-0.1269	0.5848	-0.0865	0.6850	0.4248	0.9530	0.8152	ns	ns	ns	ns
ENSMUSG00000025804	Ccr1	0.5899	-0.0916	0.7553	0.0700	0.7987	0.1569	0.6313	-0.1335	0.7829	0.8272	0.8854	0.8297	ns	ns	ns	ns
ENSMUSG00000026180	Cxcr2	0.3777	-0.3923	0.5137	0.0166	0.2913	0.4836	0.2509	-0.4565	0.6556	0.6219	0.4394	0.5369	ns	ns	ns	ns
ENSMUSG00000026390	Marco	0.2913	0.2138	0.1782	0.3199	0.8874	0.1429	0.6079	0.0585	0.6092	0.2732	0.9173	0.8224	ns	ns	ns	ns
ENSMUSG00000026656	Fcgr2b	0.0887	0.6620	0.6707	-0.1913	0.0519	-0.8459	0.0317	0.7426	0.3549	0.7713	0.1223	0.1942	ns	ns	ns	pq
ENSMUSG00000026712	Mrc1	0.3186	-0.3980	0.1277	0.6716	0.0045	1.2297	0.0264	-0.7563	0.6236	0.2136	0.0166	0.1871	ns	ns	pq	pq
ENSMUSG00000027322	Siglec1	0.5899	-0.0479	0.7553	-0.0595	0.4776	-0.0044	0.4557	-0.0237	0.7829	0.8272	0.5858	0.6873	ns	ns	ns	ns
ENSMUSG00000027398	Il1b	0.2415	0.6264	0.5137	-0.3751	0.0887	-0.8294	0.0904	0.6882	0.5697	0.6219	0.1814	0.2969	ns	ns	ns	ns
ENSMUSG00000027399	Il1a	0.6297	0.3302	0.7987	0.0909	0.7987	-0.2160	0.6550	0.2694	0.7829	0.8447	0.8854	0.8370	ns	ns	ns	ns
ENSMUSG00000027848	Olfml3	0.1432	0.6443	0.0000	2.7989	0.0000	2.3127	0.1878	-0.5147	0.4878	0.0000	0.0002	0.4546	ns	pq	pq	ns
ENSMUSG00000027947	Il6ra	0.3777	0.3310	0.0000	2.2331	0.0000	2.0511	0.0971	-0.5704	0.6556	0.0004	0.0002	0.3081	ns	pq	pq	ns
ENSMUSG00000027995	Tlr2	0.3777	0.3380	0.0009	-1.4034	0.0005	-1.6871	0.0134	0.8939	0.6556	0.0042	0.0024	0.1123	ns	pq	pq	pq
ENSMUSG00000029417	Cxcl9	0.5899	-0.2623	0.0173	-1.0608	0.2415	-0.5545	0.7283	0.1560	0.7829	0.0370	0.3766	0.8816	ns	pq	ns	ns
ENSMUSG00000029468	P2rx7	0.0887	0.7558	0.0045	1.3204	0.2415	0.6033	0.7786	0.0628	0.3549	0.0166	0.3766	0.9184	ns	pq	ns	ns
ENSMUSG00000029470	P2rx4	0.5137	0.1988	0.0780	0.7756	0.3186	0.5164	0.8557	-0.1442	0.7829	0.1380	0.4580	0.9218	ns	ns	ns	ns
ENSMUSG00000030413	Pglyrp1	0.0145	1.2338	0.0083	1.2619	0.8428	-0.0026	0.2115	0.5651	0.2648	0.0231	0.9016	0.4746	p	pq	ns	ns
ENSMUSG00000030474	Siglece	0.5512	-0.1726	0.3777	0.3271	0.3474	0.4579	0.3613	-0.3303	0.7829	0.4894	0.4842	0.6043	ns	ns	ns	ns
ENSMUSG00000030577	Cd22	0.3777	0.2537	0.0000	-1.7860	0.0000	-1.9341	0.0024	0.8872	0.6556	0.0000	0.0000	0.0715	ns	pq	pq	pq
ENSMUSG00000030579	Dap12	0.0780	0.9914	0.4776	-0.3432	0.0036	-1.4309	0.0063	1.1740	0.3549	0.6019	0.0139	0.0831	ns	ns	pq	pq
ENSMUSG00000030786	Itgam	0.2415	0.4960	0.0887	0.8535	0.3777	0.4370	0.8817	-0.0012	0.5697	0.1540	0.5036	0.9218	ns	ns	ns	ns
ENSMUSG00000031392	Irak1	0.6707	0.1774	0.0000	2.4098	0.0002	2.0156	0.0840	-0.6935	0.8119	0.0001	0.0012	0.2969	ns	pq	pq	ns
ENSMUSG00000031639	Tlr3	0.4776	0.4238	0.0173	1.0670	0.0332	0.8205	0.4358	-0.1123	0.7708	0.0370	0.0899	0.6796	ns	pq	pq	ns
ENSMUSG00000032089	Il10ra	0.0887	0.6339	0.0145	0.9241	0.2657	0.2478	0.7533	0.1889	0.3549	0.0342	0.4074	0.9001	ns	pq	ns	ns
ENSMUSG00000032440	Tgfbr2	0.7987	0.1585	0.0000	2.4085	0.0000	2.0735	0.0447	-0.7427	0.8854	0.0001	0.0003	0.1960	ns	pq	pq	pq
ENSMUSG00000032508	Myd88	0.1432	0.6706	0.0001	1.9114	0.0121	1.0544	0.5848	-0.1608	0.4878	0.0009	0.0397	0.8152	ns	pq	pq	ns
ENSMUSG00000033777	Tlr13	0.0011	1.4473	0.5512	0.1805	0.0684	-0.7579	0.0031	0.9220	0.1025	0.6501	0.1534	0.0715	pq	ns	ns	pq
ENSMUSG00000034855	Cxcl10	0.2415	-0.6507	0.0002	-1.9062	0.0121	-1.1088	0.4761	0.2476	0.5697	0.0012	0.0397	0.7065	ns	pq	pq	ns
ENSMUSG00000035385	Ccl2	0.9323	-0.0470	0.0007	-1.4563	0.0000	-1.6044	0.0264	0.6471	0.9530	0.0034	0.0004	0.1871	ns	pq	pq	pq
ENSMUSG00000036353	P2ry12	0.2415	0.5230	0.0001	1.6615	0.0018	1.2886	0.3108	-0.2578	0.5697	0.0009	0.0077	0.6043	ns	pq	pq	ns
ENSMUSG00000036362	P2ry13	0.2913	0.4223	0.0387	1.0006	0.1782	0.6495	0.8817	-0.0857	0.6092	0.0758	0.3093	0.9218	ns	pq	ns	ns
ENSMUSG00000036905	C1qb	0.0173	0.9627	1.0000	0.0003	0.0519	-0.8680	0.0109	0.9513	0.2648	1.0000	0.1223	0.1116	p	ns	ns	pq
ENSMUSG00000039005	Tlr4	0.0056	1.2181	0.0684	0.7939	0.4428	-0.4802	0.0411	0.8096	0.2557	0.1258	0.5658	0.1960	p	ns	ns	pq
ENSMUSG00000040552	C3ar1	0.1600	0.1965	0.0004	-1.4758	0.0000	-2.4350	0.0014	0.8658	0.4906	0.0020	0.0002	0.0656	ns	pq	pq	pq
ENSMUSG00000040747	Cd53	0.1978	0.6741	0.0083	1.2599	0.0780	0.7202	0.8041	0.0161	0.5515	0.0231	0.1709	0.9218	ns	pq	ns	ns
ENSMUSG00000044583	Tlr7	0.1600	0.6897	0.0068	1.2166	0.3474	0.4746	0.8041	0.1152	0.4906	0.0224	0.4842	0.9218	ns	pq	ns	ns
ENSMUSG00000044827	Tlr1	0.2415	0.3873	0.7553	-0.1906	0.1432	-0.5303	0.1197	0.4943	0.5697	0.8272	0.2744	0.3240	ns	ns	ns	ns
ENSMUSG00000045382	Cxcr4	0.6707	-0.2316	0.0000	2.4562	0.0000	3.1326	0.0063	-1.0378	0.8119	0.0001	0.0000	0.0831	ns	pq	pq	pq
ENSMUSG00000048163	Selplg	0.0449	0.8988	0.0173	1.2759	0.7125	0.3597	0.3613	0.2866	0.3408	0.0370	0.8298	0.6043	p	pq	ns	ns
ENSMUSG00000048779	P2ry6	0.8874	-0.0635	0.0068	1.0780	0.0003	1.7324	0.0377	-0.6130	0.9384	0.0224	0.0015	0.1960	ns	pq	pq	pq
ENSMUSG00000049103	Ccr2	0.3777	-0.3107	0.1782	0.5041	0.0684	0.7312	0.1118	-0.5505	0.6556	0.2732	0.1534	0.3240	ns	ns	ns	ns
ENSMUSG00000051212	Gpr183	0.0519	0.8774	0.0003	1.8123	0.4428	0.4042	0.5183	0.2029	0.3408	0.0016	0.5658	0.7568	ns	pq	ns	ns
ENSMUSG00000051439	Cd14	0.2189	0.5643	0.3186	0.3009	0.8874	-0.0894	0.5400	0.2675	0.5697	0.4248	0.9173	0.7763	ns	ns	ns	ns
ENSMUSG00000051498	Tlr6	0.7125	0.2906	0.8428	-0.0518	0.3777	-0.2495	0.4557	0.2522	0.8298	0.8811	0.5036	0.6873	ns	ns	ns	ns
ENSMUSG00000051504	Siglech	0.9774	0.0527	0.0519	0.9263	0.0887	0.9137	0.3108	-0.4044	0.9774	0.0974	0.1814	0.6043	ns	ns	ns	ns
ENSMUSG00000052336	Cx3cr1	0.2657	0.4401	0.0145	1.2106	0.1277	0.7977	0.8298	-0.1150	0.5819	0.0342	0.2554	0.9218	ns	pq	ns	ns
ENSMUSG00000054675	Tmem119	0.0332	0.9935	0.0045	1.2666	0.4776	0.2850	0.4358	0.3506	0.3058	0.0166	0.5858	0.6796	p	pq	ns	ns
ENSMUSG00000055170	Ifng	0.8428	-0.1043	0.9774	-0.3234	0.9774	-0.2833	0.8817	0.0514	0.9016	0.9881	0.9774	0.9218	ns	ns	ns	ns
ENSMUSG00000055447	Cd47	0.7987	0.0539	0.0014	1.6151	0.0002	1.7802	0.0669	-0.6327	0.8854	0.0066	0.0012	0.2463	ns	pq	pq	ns
ENSMUSG00000056054	S100a8	0.5512	-0.3448	0.3186	-0.3383	0.5137	0.0734	0.9868	-0.2240	0.7829	0.4248	0.6219	0.9868	ns	ns	ns	ns
ENSMUSG00000056071	S100a9	0.0242	-1.0505	0.3474	-0.3861	0.6707	0.3801	0.1197	-0.6260	0.3058	0.4565	0.7910	0.3240	p	ns	ns	ns
ENSMUSG00000058427	Cxcl2	0.7125	-0.2175	0.5512	-0.3147	0.9774	-0.1005	0.8557	-0.0737	0.8298	0.6501	0.9774	0.9218	ns	ns	ns	ns
ENSMUSG00000058715	Fcer1g	0.0684	0.6874	0.2657	0.2609	0.4095	-0.3520	0.1197	0.5079	0.3494	0.3819	0.5382	0.3240	ns	ns	ns	ns
ENSMUSG00000059089	Fcgr4	0.6297	0.1982	0.0056	-1.3230	0.0014	-1.4926	0.0377	0.7634	0.7829	0.0197	0.0063	0.1960	ns	pq	pq	pq
ENSMUSG00000059108	Ifitm6	0.9774	-0.2313	0.0387	0.9160	0.0887	0.7645	0.3439	-0.5618	0.9774	0.0758	0.1814	0.6043	ns	pq	ns	ns
ENSMUSG00000059498	Fcgr3	0.0597	0.7524	0.2913	0.4159	0.2189	-0.4589	0.0669	0.6052	0.3430	0.4124	0.3533	0.2463	ns	ns	ns	ns
ENSMUSG00000059994	Fcrl1	0.9323	0.1495	0.7987	-0.2329	0.6707	-0.3434	0.8557	0.2550	0.9530	0.8447	0.7910	0.9218	ns	ns	ns	ns
ENSMUSG00000060591	Ifitm2	0.7987	-0.0344	0.0519	1.1055	0.0145	1.0450	0.1197	-0.5029	0.8854	0.0974	0.0460	0.3240	ns	ns	pq	ns
ENSMUSG00000062991	Nrg1	0.8428	-0.1279	0.0145	1.0513	0.0205	1.0728	0.1461	-0.5962	0.9016	0.0342	0.0571	0.3735	ns	pq	pq	ns
ENSMUSG00000063234	Gpr84	0.0145	0.9589	0.0029	-1.2464	0.0000	-2.4625	0.0001	1.4417	0.2648	0.0128	0.0001	0.0052	p	pq	pq	pq
ENSMUSG00000079227	Ccr5	0.0173	0.9919	0.0045	1.2183	0.7987	0.2176	0.2373	0.3628	0.2648	0.0166	0.8854	0.5198	p	pq	ns	ns

Supplementary Table 2.6. The known ability to cross the blood brain barrier for hippocampal metabolites that were altered in aging and reversed following yFMT.

Metabolite	yes/no/likely/unclear	Reference [PMID]
(R)-alpha-methylhistamine	yes	8083809
2-Deoxy-adenosine	likely	26443809
2-Deoxy-uridine	likely	2855575
5-hydroxy-indolacetate	no	
5-phosphoribosyl diphosphate	unclear	
Amino acids such as alanine, arginine, glutamine, phenylalanine, serine, proline	yes	8923485
Alpha-tocopherol	yes	
Anserine	yes	18543335, 16125861
Arachidonoylcarnitine	unclear	
Aspartate	yes	10736373
Carnosine	yes	18543335
Cytidine	yes	16769123
GABA	yes	11598501, 33041752
Gamma-glutamyl-leucine	yes	27740595
Glutamate	yes	8762179, 10736373
Glutamine	yes	8923485
Glutaryl-carnitine	unclear	
Heme	unclear	
Lactate	yes	27598136, 2050746
Leucyl-glycine	likely	
N-delta-acetyl ornithine	yes	8923485
N-glycolylneuraminate	yes	26098915
N-oleoyl-serine	likely	
N-stearoyl-serine	likely	
Panthotenate	yes	3734807
Pyridoxamine-Phosphate (=pyridoxal-5-phosphate, PLP)	yes	32759679
Pyroglutamine	unclear	
Retinol	yes	18296731
Suberate (Bissulfosuccinimidyl suberate)	unclear	
Thiamin Monophosphate	yes	3335853
Uridine	yes	16769123

Supplementary Table 2.7. Observation battery scoring rubric.

Assessment	Score
Presence of whiskers	
none	0
a few	1
most, but not a full set (less than half is gone)	2
a full set	3
Appearance of fur	
ungroomed and disheveled	0
somewhat disheveled	1
well-groomed (normal)	2
Patches of fur missing on body	
none	0
some	1
extensive (lower density)	2
Patches of fur missing on face	
none	0
some	1
extensive (lower density)	2
Palpebral Closure	
eyes wide open	0
eyes half closed	1
eyes closed	2
Respiration	
gasping, irregular	0
slow, shallow	1
normal	2
hyperventilation	3
Gait	
normal	0
fluid but abnormal/delayed	1
limited movement only (no exploration)	2
incapacity	3
Reaching reflex (cotton swab was hold in front of the mouse)	
none	0
upon nose contact	1
upon vibrissae contact	2
before vibrissae contact	3
early vigorous extension (1cm before)	4
Pinna reflex	
none	0
active retraction, moderately brisk flick	1
hyperactive repetitive flick	2
Eye reflex	
present	0
absent	1
Whisker reflex	
present	0
absent	1
Tremor	
none	0
mild	1
marked	2
Toe Pinch	
none	0
slight withdrawal	1
moderate withdrawal, not brisk	2
brisk, rapid withdrawal	3
very brisk, repeated extension and flexion	4
Trunk curl	
number of sec required to right	1 to 10
Righting reflex	
absent	0
present	1
Muscle strength	
number of sec resisting on cage lid	1 to 60

Supplementary Table 2.8 Flow cytometry antibodies, suppliers, dilutions, and tissue use.

Target antigen	Conjugation	Concentration	Vendor	Catalog number	Clone	Antibody Registry ID	Tissue
CD4	FITC	1:100	Thermo Fisher Scientific	11-0042-85	RM4-5	RRID:AB_464897	MLN
CD11b	FITC	1:25	Miltenyi	130-113-243	REA592	RRID:AB_2726049	MLN
CD49b	FITC	1:10	Miltenyi	130-102-258	DX5	RRID:AB_2660456	blood
CD103	FITC	1:10	Miltenyi	130-102-479	20000000	RRID:AB_2654397	MLN
CD11b	VFITC	1:10	Miltenyi	130-109-290	REA592	RRID:AB_2654643	blood
CD11c	PE	1:25	Miltenyi	130-110-701	REA754	RRID:AB_2654708	MLN / blood
CD69	PE	1:10	Miltenyi	130-103-946	H1.2F3	RRID:AB_2659083	MLN / blood
Ly-6C	PE	1:10	Miltenyi	130-102-391	1G7.G10	RRID:AB_2660027	MLN
CD62L	PE-Cy7	1:25	BioLegend	104418	MEL-14	RRID:AB_313103	blood
CD8a	PerCP-Vio700	1:10	Miltenyi	130-102-239	53-6.7	RRID:AB_2659898	MLN / blood
Ly-6G	PerCP-Vio700	1:10	Miltenyi	130-107-917	REA526	RRID:AB_2652831	MLN / blood
CX3CR1	PerCP-Cy5.5	1:150	BioLegend	149010	SA011F11	RRID:AB_2564494	MLN
CD44	APC	1:10	Miltenyi	130-110-084	REA664	RRID:AB_2658155	MLN
CD192 (CCR2)	APC	1:10	Miltenyi	130-108-723	REA538	RRID:AB_2655857	blood
F4/80	APC	1:10	Miltenyi	130-102-379	REA126	RRID:AB_2651705	MLN
FoxP3	APC	1:10	Thermo Fisher Scientific	17-5773-82	FJK-16s	RRID:AB_469457	blood
MHC-II	APC	1:10	Miltenyi	130-102-139	M5/114.15.2	RRID:AB_2660058	MLN
CD25	BV421	1:25	BioLegend	102034	PC61	RRID:AB_10895908	blood
MHC-II	BV421	1:100	BioLegend	107632	M5/114.15.2	RRID:AB_2650896	blood
CD4	BV605	1:100	BioLegend	100548	RM4-5	RRID:AB_2563054	blood
Ly-6C	BV605	1:25	BioLegend	128036	HK1.4	RRID:AB_2562353	blood
CX3CR1	BV785	1:25	BioLegend	149029	SA011F11	RRID:AB_2565938	blood
CD44	BV786	1:25	BD Biosciences	563736	IM7	RRID:AB_2738395	blood

Supplementary Table 2.9. Centre log transformed hippocampal metabolomics data per individual. Due to size of table, please see published version at https://www.nature.com/articles/s43587-021-00093-9#Sec36 (Boehme et al. 2021).

Chapter 3

Microbiota consortium derived from cognitively-impaired elderly patients does not impair cognition when transplanted into young rats

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In Preparation

3.1 Abstract

The process of aging instigates changes throughout the body, including distinct alterations in the gut microbiome and declining cognition. Recent evidence has shown that compositional changes in the gut microbiota correlate with cognitive health in elderly humans. Furthermore, rejuvenation of the gut microbiota via faecal microbiota transfer from young mice into aged mice was sufficient to improve memory outcomes in aged mice, sparking the question as to whether specific strains of bacteria regulate healthy or impaired cognition during aging. Here, we selected a consortium of bioactive bacteria derived from elderly patients with or without mild cognitive impairment and transferred these consortia into a rat model to investigate if these consortia would impact cognitive and gastrointestinal health in a younger host. Gastrointestinal measures included colonic monoamine levels, motility, and faecal water content. Cognitive performance was assessed via the novel object recognition test and Morris water maze, as well as antidepressant-like behaviour in the forced swim test, and additional microbiota-sensitive behaviours that may impact cognition were assessed including anxiety-like behaviour in the elevated plus maze and open field test, and grip strength as a measure of frailty. Treatment with bacteria derived from elderly individuals with mild cognitive impairment reduced colonic noradrenaline concentrations, but did not alter other metrics of gastrointestinal health, including colonic serotonin levels, faecal water content, or gastrointestinal transit time. Furthermore, the selected bacterial consortium identified in elderly humans with impaired cognition was not sufficient to alter the cognitive performance of the recipient rat. This research suggests that there are further additional factors that must be considered when attempting to recapitulate the clinical link between the gut microbiota and cognitive decline in aging.

3.2 Introduction

By the year 2050, the global population aged 65 and older is expected to double compared to just 35 years prior (He *et al.* 2016). This demographic transition, coupled with increased life expectancy, is anticipated to be accompanied with a greater burden of aging-related diseases, including age-related cognitive decline; healthcare costs; and emotional and physical load on family members and care givers (Jaul & Barron 2017). Therefore, it is increasingly imperative to decipher the differences between healthy biological aging and pathological changes underpinning aging-associated neurodegenerative diseases, including dementia. Understanding these differences may unravel insights into novel diagnostics and therapeutic treatments.

Aging is a gradual process of whole-body deterioration in multiple homeostatic functions, characterized by cellular senescence, stem cell exhaustion, immune dysregulation, genomic instability, neurotransmitter imbalance, and oxidative stress, resulting in increased prevalence of diseases (Klempin & Kempermann 2007; Lopez-Otin *et al.* 2013). In the brain, aging provokes a reduction in neuroplasticity and adult hippocampal neurogenesis, which may contribute to age-related cognitive decline (Moreno-Jimenez *et al.* 2019). These deficits in hippocampal neurogenesis and increased neuronal loss appears accelerated in individuals with cognition-related neurodegenerative diseases (West *et al.* 1994; Moreno-Jimenez *et al.* 2019), and has been associated with the progression of Alzheimer's Disease (AD) (Moreno-Jimenez *et al.* 2019). AD, the most common aging-related form of dementia, triggers progressive hippocampal deterioration leading to cognitive decline, especially in spatial perception and working memory (Reul *et al.* 2017). It is hypothesized that structural and pathological changes occur in the brain several years prior to the clinical onset of AD, allowing the progression to be divided into three progressive states: (1) pre-symptomatic (2) mild cognitive impairment (MCI), and (3) overt dementia (Trushina *et al.* 2013). The window of opportunity to successfully intervene appears limited to the earlier stages of disease pathology, suggesting that identification of predictive biomarkers for dementia, as well as potential therapeutics, may be most impactful during the MCI phase.

Gastrointestinal functions are also disrupted during aging, presenting in weakened gut barrier function, altered intestinal neurotransmitter levels, and distinct changes in the gut microbiota (Claesson *et al.* 2011; Claesson *et al.* 2012a; Konturek *et al.* 2015). The trillions of microorganisms residing in the gastrointestinal tract are increasingly recognized as important contributors to

maintaining host health throughout the lifespan (Cryan *et al.* 2019b). While the composition of the gut microbiota, if left undisturbed, is generally stable during adulthood, community stability gradually declines with old age, accompanied by a loss in microbial diversity and heightened variability in microbial composition (Biagi *et al.* 2010; Claesson *et al.* 2011; Biagi *et al.* 2017; Ghosh *et al.* 2020a; Wilmanski *et al.* 2021). Genera including *Bacteroides, Alistipes,* and *Parabacteroides* have been observed to be increased in people aged 65+ compared to younger, healthy adults (Claesson *et al.* 2011). Furthermore, a recent multi-cohort study involving clinical data, revealed that healthier elderly individuals showed an increase in gut microbial uniqueness over time, whereas retention of relative *Bacteroides* abundance was associated with decreased host survival over a 4-year follow-up window (Wilmanski *et al.* 2021). This research highlights that the microbiota may act as a marker for lifespan in elderly humans with specific bacterial genera potentially playing a role in these effects.

Accumulating evidence is revealing associations between the gut microbiota and diseases associated with aging, including MCI and AD (Alkasir *et al.* 2017; Vogt *et al.* 2017; Saji *et al.* 2019; Guo 2021). A Japanese cohort examining elderly patients with mild cognitive impairment but no dementia, versus healthy elderly found higher prevalence of *Bacteroides* in patients with cognitive decline (Saji *et al.* 2019). Additional research into gut microbiota of patients with MCI, AD, or healthy aging has revealed multiple bacterial genera are linked to AD, namely an increase in *Escherichia,* when compared to healthy controls (Li *et al.* 2019a). While correlative evidence clearly indicates that the gut microbiota is altered in elderly patients with cognitive decline, microbiota-targeted research suggests the gut microbiota play an active role in contributing host cognition in aging.

Clinical and preclinical microbiota-targeted interventions, including probiotics (Kobayashi *et al.* 2019a; Kobayashi *et al.* 2019b; Ueda *et al.* 2021), antibiotics (Guilherme *et al.* 2021), and faecal microbiota transplant (FMT) (Hazan 2020; Boehme *et al.* 2021; Park *et al.* 2021), have revealed that the gut microbiota plays an active role in host cognition during aging. Fascinatingly, an 82-year-old man with AD who was administered FMT to treat *Clostridioides difficile* infection showed improved cognitive score in the Mini-Mental State Examination (MMSE) from MCI levels to healthy cognition at his 2-month follow-up visit (Hazan 2020). Similarly, a 90-year-old woman suffering from AD treated with FMT for *Clostridioides difficile* displayed a marked improvement

in several cognitive function tests within 3 months (Park *et al.* 2021). This clinical FMT literature, albeit limited by sample size, supports the translatability of previous pre-clinical findings wherein FMT from young C57BL/6 mice rejuvenated aspects of memory when transplanted into aged C57BL/6 mice (Boehme *et al.* 2021). Furthermore, FMT from wild-type mice into male APP/PS1 transgenic mice, a genetic mouse model of AD, led to reductions in memory impairment, Aβ accumulation, synaptic dysfunction, and neuroinflammation (Sun *et al.* 2019), while FMT from an additional genetic AD mouse model known as 5xFAD mice into naïve C57BL/6 mice lead to decreased hippocampal neurogenesis and cognitive impairment (Kim *et al.* 2021). Numerous studies have shown that microbiota derived from aged organisms have been shown to induced aging-associated phenotypes when transplanted into a young recipient (Kundu *et al.* 2019; D'Amato *et al.* 2020). Furthermore, longevity has been associated the microbiota status of the host (Biagi *et al.* 2010; Smith *et al.* 2017; Barcena *et al.* 2019). While the mechanism(s) of action underlying microbiota-gut-brain communication in aging-related cognitive decline remain elusive, recent literature has showed that the microbially-derived metabolite acetate decreases cortical microglia phagocytosis and aids in the disease progression in 5xFAD mice, while germ-free 5xFAD mice have decelerated disease progression compared with their colonized counterparts (Erny *et al.* 2021). Overall, the gut microbiota are critical actors in maintaining host cognition, including in aging-associated cognitive disfunction.

Aging is commonly accompanied by an increase in medical drug usage which could impact the gut microbiota composition (Jackson *et al.* 2018; Vich Vila *et al.* 2020). It may therefore be difficult to decipher whether differences in the gut microbiota in AD and MCI are invoked by medications or disease pathology. Furthermore, the aged gut is marked with profound changes to physiology and function, including gastric motility (Firth & Prather 2002; Madsen & Graff 2004), which is accompanied by changes in monoamine levels (Hansen 2003; Mandl & Kiss 2007; Lopez-Otin *et al.* 2013; Seifi & Swinny 2019). Interestingly, the most common medications used to treat AD are cholinomimetics, and therefore may increase intestinal motility (Holzer *et al.* 1980) thereby potentially altering the gut microbiota (Martin *et al.* 2018). Inactive bowel movement and constipation are associated with an increased risk of developing MCI (Chen *et al.* 2020; Huang *et al.* 2020). Considering that the gut microbiota is known to influence gastrointestinal transit time (Muller *et al.* 2020), perhaps by altering the level of motility-mediating neurotransmitters in the gut, such as the inhibitory neurotransmitter noradrenaline and the motility stimulator serotonin

(Strandwitz 2018), it is plausible that alterations in gut microbiota in MCI might also contribute to the inactive bowel movement and constipation that have been associated with an increased risk of developing MCI, although this link has not yet been established.

Elucidating the role(s) that specific microbes play in the onset and progression of aging-associated cognitive impairment is critical to better understand microbiota-driven disease pathology and generate novel treatment options. Furthermore, intervention during MCI, and prior to the onset of AD appears to be the ideal timeframe for intervention to improve cognitive outcomes. Nonetheless, there is no published research which interrogates which bacteria may causatively instigate cognitive decline.

Herein, using data from the ELDERMET study (Claesson *et al.* 2011) (one of the most comprehensively studied aging cohort including microbiota to date (Wilmanski *et al.* 2021)), bacteria with known neuroactive potential which are more abundant in elderly individuals with diagnosed MCI or healthy cognitive function were identified. Using this as a guide, consortia of these microbes were constructed to represent the dominant and potentially neuroactive microbes of elderly with healthy cognition (HeCon) or elderly with mildly impaired cognition (MiCon). To determine whether the consortium of bacteria associated with impaired cognition could itself induce cognitive impairment, among other markers of aging, these consortia were transplanted into healthy young adult rats while control rats received sterile culture medium rather than a microbial consortium. Several weeks post-initial consortium transplant, cognitive performance, locomotor activity, anxiety-like and depressive-like behaviours, grip strength as a metric of frailty, gastrointestinal motility, and colonic neurotransmitter concentrations were then measured.

3.3 Methods

3.3.1 Microbiota consortia development

Microbiota consortia were developed to consist of either 10 or 11 culturable bacterial species from the Microbiome Culture Collection (Perez *et al.* 2021). Selection criteria initially included bacterial species that were highly prevalent in elderly individuals with either mild cognitive impairment or healthy cognition, and reduced in the opposing group, and correlated most strongly with cognitive scores in these patients. Shotgun sequenced microbiota data was used from the ELDERMET study, which has previously been published elsewhere (Claesson *et al.* 2011; Ghosh *et al.* 2020a). Within the ELDERMET study, 189 Irish elderly subjects aged >64 years were recruited (104 females, 85 males, mean ± standard deviation age 77 ± 8, ranging from 64 to 102 years of age). Subjects' lifestyles ranged from community-dwelling, attending outpatient day hospitals, in short-term rehabilitation care (<6 weeks), or in long-term care facilities. Exclusion criteria included advanced organ disease, alcohol abuse, and participation in a drug intervention. The abundances of the various faecal microbial species were correlated with the different physical and cognitive functional measures including Functional Independence Measure (FIM), Barthel Score, and Mini Mental State Examination (MMSE) scores. MMSE scores averaged at 26.1 ± 5.3 (mean ± standard deviation), with a range of 2 to 30, indicating a wide range of cognitive abilities were captured by this dataset. Bacterial species significantly associated with either two of these measures with the same directionality (either positive or negative) were identified. With this as a selection criterion, a further screening was performed *in silico*, selecting only for microbes with the known capacity to affect gut brain modules that have been associated with the cognitive parameters of the ELDERMET study (Valles-Colomer *et al.* 2019). The final consortiums of MiCon and HeCon bacteria were determined by assessing which of these microbes have previously shown reproducible associations with cognitive functional measures (either positive or negative). Therefore, the MiCon consortia was established to contain bacteria that have been associated with impaired cognition in the elderly and have cognition-relevant bioactive potential, while the HeCon consortia contained bacteria associated with healthy cognition in elderly that have cognition-relevant bioactive potential. The final bacterial consortiums for MiCon and HeCon are described in Table 1.

All strains of bacteria were cultured inside an anaerobic cabinet. Bacteria from pure glycerol stocks were streaked onto YCFA agar plates and allowed to grow for 24 h. Subsequently, a single colony from each plate was inoculated in liquid YCFA medium, prepared according to previously published literature (Duncan *et al.* 2002), and cultured for an additional 48 h. Optical density of each culture was measured via a spectrophotometer as an indicator for bacterial concentration. To determine the appropriate concentration ratio of each bacterium in the HeCon and MiCon consortia, the maximum relative abundance of each bacterium in the microbiome of healthy or cognitively impaired patients was calculated, and bacteria were combined based on their clinical maximum relative abundance and observed optical density. Using serial dilutions, the average colony-forming unit (CFU) was determined to be approximately 7.6×10^{-7} CFU/ml for the MiCon consortia and approximately 2.8×10^{-7} CFU/ml for the HeCon consortia. Consortia were aliquoted and stored at -80 °C until use.

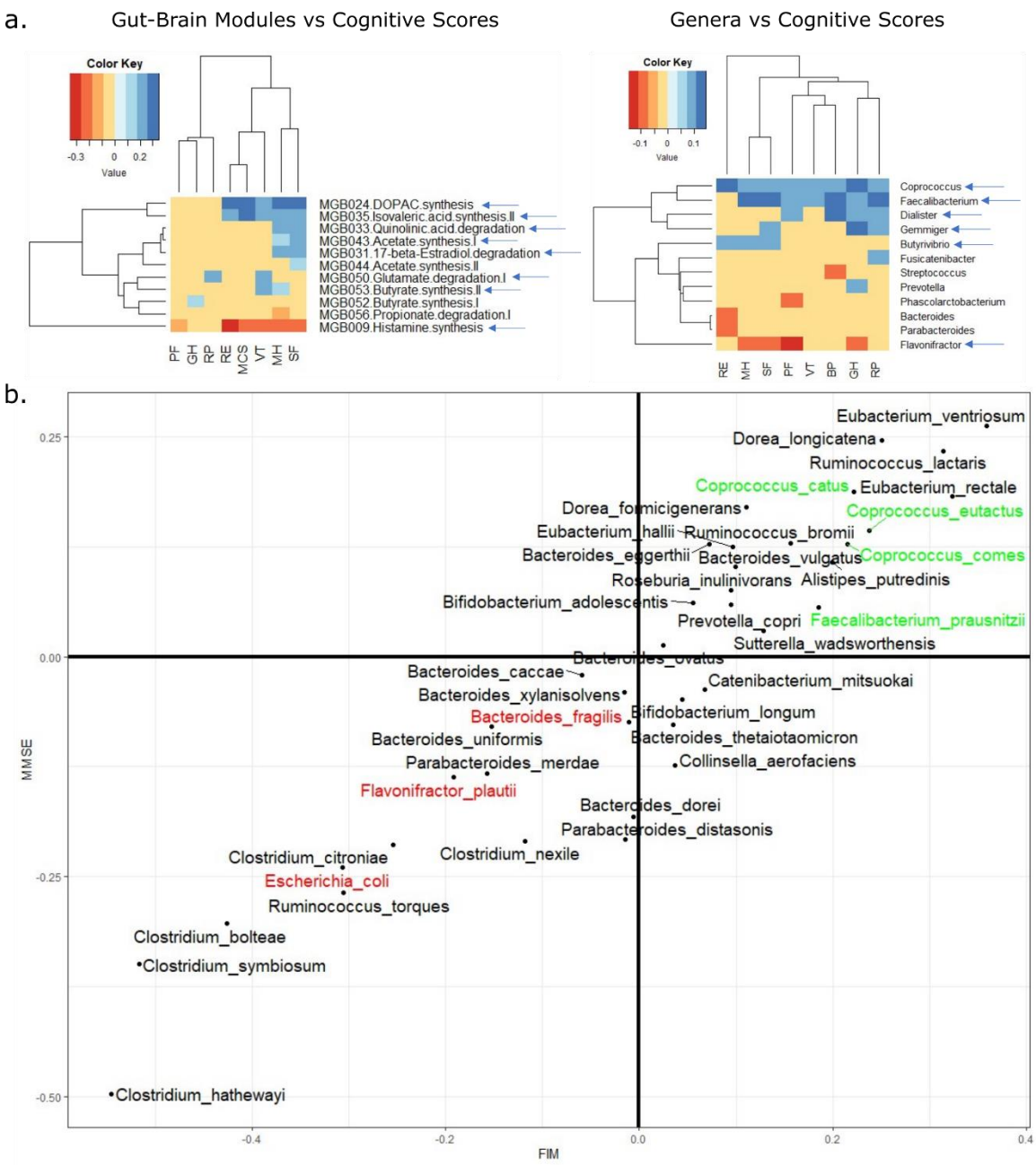


Figure 3.1. Development of the MiCon and HeCon microbial consortia. (A) Main gut-brain modules and bacterial genera driving the association between RAND mental well-being scores and the gut microbiome. Colour Key indicates the extent of the association, where blue represents a positive association and red represents a negative association. Abbreviations: PF, physical functioning; GH, general health perception; RP, role limitations caused by physical health; RE, role limitations caused by emotional health problems; MCS, mental component summary; VT, vitality; MH, emotional well-being; SF, social functioning. (B) Associations between microbes that contain multiple RAND-associated gut-brain modules observed to significantly correlate to mental well-being or bacteria that were significantly correlated with mental well-being with Functional Independence Measure (FIM) and Mini-Mental State Exam (MMSE) scores in the ELDERMET study (Ghosh et al. 2020a). Species in the bottom left quadrant showed strongest associations with worsened cognitive score and were candidates for MiCon. Species in the top right quadrant showed strongest positive correlations with healthy cognition and were potential candidates for inclusion in HeCon. Species in red showed additional independent negative associations with improved cognition in the Valles-

Colomer study (Valles-Colomer et al. 2019). Species in green showed additional independent positive associations with improved cognition in the Valles-Colomer study.

Table 3.1 displays the species of bacteria comprising each consortium, their maximum estimated abundance observed clinically in patients with or without mild cognitive decline, and their relative abundance within the cultured consortium. Both MiCon and HeCon were primarily comprised of bacteria classified under the phylum *Firmicutes*. MiCon additionally contained phyla *Bacteroidetes* and *Proteobacteria*, while HeCon additionally contained one species from the phylum *Actinobacteria*. Roughly half of MiCon was comprised of *Escherichia coli* and *Parabacteroides merdae*.

Table 3.1. Species consensus, phylogeny of bacteria, estimated abundance in each consortium. Maximum estimated abundance is in terms of the abundance observed in human faecal samples and used to generate the relative abundance of each species within the consortium.

MiCon Consortia Composition			
Phylum	**Species ID (consensus)**	**Maximum estimated abundance**	**Relative Abundance in Sample**
Firmicutes	*Clostridium hathewayi*	1.51	1.68
Firmicutes	*Clostridium symbiosum*	0.32	0.36
Firmicutes	*Clostridium bolteae*	2.91	3.25
Proteobacteria	*Escherichia coli*	26.87	29.97
Firmicutes	*Clostridium citroniae*	0.06	0.07
Firmicutes	*Clostridium nexile*	4.6	5.13
Firmicutes	*Ruminococcus torques*	7.59	8.47
Firmicutes	*Flavonifractor plautii*	0.18	0.20
Bacteroidetes	*Parabacteroides distasonis*	12.98	14.48
Bacteroidetes	*Bacteroides dorei*	13.4	14.95
Bacteroidetes	*Parabacteroides merdae*	19.24	21.46
HeCon Consortia Composition			
Phylum	**Species ID (consensus)**	**Maximum estimated abundance**	**Relative Abundance in Sample**
Firmicutes	*Eubacterium ventriosum*	1.02	1.26
Firmicutes	*Dorea longicatena*	5.5	6.80
Firmicutes	*Eubacterium rectale*	13.09	16.20
Firmicutes	*Coprococcus catus*	0.7	0.87
Firmicutes	*Coprococcus comes*	3.15	3.90
Firmicutes	*Coprococcus eutactus*	5.73	7.09
Firmicutes	*Ruminococcus bromii*	22.4753	27.81
Firmicutes	*Faecalibacterium prausnitzii*	10.64	13.16
Firmicutes	*Roseburia inulinivorans*	5.87	7.26
Actinobacteria	*Bifidobacterium adolescentis*	12.65	15.65

3.3.2 Animals

All experimental procedures involving animals were approved by the Animal Experimentation Ethics Committee of University College Cork AEEC (Application ID #2019-005) and conducted under HPRA project authorization AE19130/P107 in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposes. Male Sprague Dawley rats weighing 150-174g (approximately 6 weeks old) were ordered from Envigo UK and housed in the Biological Services Unit facility, University College Cork. Rats were pair-housed upon arrival in cages in a temperature and humidity-controlled room on a 12 h light, 12 h dark cycle, with light shifts occurring at roughly 7:00 and 19:00. Food and water were available *ad libitum*. Rats were handled for at least 5 minutes daily for two weeks leading up to the start of the study.

3.3.3 Experimental Design

Cages of rats were randomly assigned into three treatment groups: Control (n = 10), Healthy cognition-derived consortia (HeCon, n = 9) recipients, and Mild cognitive impairment-derived consortia (MiCon, n = 10). All rats underwent one week of antibiotic treatment, followed by 72 hr washout, then inoculation with microbial consortia, and colonization for three weeks prior to the initiation of the behavioural test battery to assess metrics associated with aging-related cognitive decline, antidepressant-like and anxiety-like behaviours, gut health, and frailty, prior to being euthanized. Rats were weighed weekly during the study. A timeline of the study is shown in Figure 3.1a.

3.3.4 Antibiotic treatment and consortia administration

Rats were treated with an antibiotic cocktail administered via drinking water for one week to remove existing gut microbiota. The antibiotic cocktail was composed of multiple antibiotics to target both gram negative and gram-positive bacteria and consisted of ampicillin (1 g/L), vancomycin (500 mg/L), ciprofloxacin HCl (200 mg/L) and imipenem (250 mg/L). Antibiotic cocktail was made fresh every other day.

Following a 72-hr washout post-antibiotic administration, rats were orally gavaged once daily for three days with 300 µL of either ~7.6 x 10^{-7} CFU/ml MiCon or ~2.8 x 10^{-7} CFU/ml HeCon bacteria consortia. Rats assigned to the Control group received an oral gavage of 300 µL sterile growth

medium. Following the initial three-day inoculation, rats were subsequently gavaged with the same dosage twice per week to promote engraftment of the microbial consortia.

Consortia and media solutions were brought to room-temperature prior to gavage administration. For oral gavage, rats were restrained in a vertical position with the loose skin pulled back to minimize the movement of the head and gently extend the head backwards to straighten the oesophagus. The gavage feeding needle (18-gauge, bulb-tipped) was inserted behind the incisors and directed towards the back of the throat, down the oesophagus and towards the stomach. Treatment was administered, and the gavage needle removed. Animals were monitored following each gavage to ensure no distress.

3.3.5 Faecal collection

Faeces were collected from the rats during daily handling sessions, according to the timeline in Figure 3.1a. For collection, clean gloves and forceps were sterilized with 70% ethanol. The anus of the rat was massaged to promote defecation. One to two faecal boluses were collected into sterile screwcap tubes and immediately snap frozen on dry ice and the rat returned to its cage.

3.3.6 Novel object recognition test

The novel object recognition test (NOR) was used to assess recognition memory and utilizes the inherent tendency of a rat to explore a novel object longer than a familiar object. The test occurred over three days. On the first day, rats were habituated to the open field arena (40 x 60 cm) for three 10-minute exploration trials, the first of which was used to assess locomotor activity. On the second day, two identical objects were placed on adjacent corners approximately 5 cm from the walls of the arena, and rats were allowed to explore for 10 min. Rats were immediately returned to their home cages following completion of testing. After 24 h, one of the familiar objects was replaced with a novel object with a distinctly different shape and colour, and rats were allowed to explore the arena for a further 10 min. Objects were counterbalanced, whereby animals were randomly assigned Object A or Object B as the familiar object to account for any potential preference for one type of object, and the location of the novel or familiar object alternated every trial to minimize the impact of external cues on the time rats spent with either object. Animals were allowed to habituate to the room for at least one hour prior to each trial, and objects and

testing arenas were cleaned using 70% ethanol between trials. All trials were recorded and the first 5 min of the final testing day was scored using BORIS software v. 7.9.7. Object exploration was defined by direct nose interaction and/or investigatory behaviour with the object, but not when the rat was sitting atop the object or circling it. Locomotor activity in the open arena was assessed via Ethovision v. 15 (Noldus). Recognition index was defined as Recognition Index = time interacting with novel object / total object interaction time.

3.3.7 Gastrointestinal motility

Constipation has been previously associated with aging-associated cognitive decline (Leta *et al.* 2021), and is considered a risk factors for MCI (Chen *et al.* 2020; Huang *et al.* 2020). Therefore, intestinal motility was assessed using intragastric administration of a non-absorbable carmine red dye. Time until the excretion of the first red faecal bolus was recorded as the time of whole gut transit. Animals were single-hosed the night prior to the assay to allow for habituation to a novel cage with fresh bedding. The following morning, each animal was administered with 200 µL of carmine red dye (6% carmine red in 0.5% methylcellulose) via oral gavage and the time of the gavage was recorded. For oral gavage technique, please see 3.3.4. Following each gavage, cages were inspected every 15 minutes for a coloured bolus. Time of first coloured bolus was recorded, and total intestinal transit time was determined. The test was concluded after 600 min due to the 12 h light cycle of the animal facility, to not disturb the circadian cycle of the rats.

3.3.8 Faecal water content

Faecal water content was assessed on the same day as the intestinal motility test to allow for the relation of differences in intestinal motility to differences in faecal water content. Faecal boluses were collected fresh, excreted during gavage for the intestinal motility test, prior to any coloured bolus being produced. Boluses were immediately placed into pre-weighed 1.5 mL screwcap tubes. The weight of the tube plus faecal bolus was recorded, and tubes were placed uncapped in an incubator set at 60 °C. After 48 hr, the weight of the tube plus dried faecal bolus was recorded. Weights of the fresh and dried faeces were determined by subtracting the weight of the tube from the weight of the tube plus fresh or dry faeces. Faecal water content was calculated with the following equation: $\text{Faecal water content} = \frac{[(\text{Weight of fresh faeces}) - (\text{Weight of dry faeces})]}{\text{Weight of fresh faeces}}$.

3.3.9 Elevated plus maze

Middle aged rodents demonstrate increased anxiety-like behaviour (Ennaceur *et al.* 2008), which may have an impact on cognitive readouts (Eysenck 1985; Wetherell *et al.* 2002). Therefore, the elevated plus maze was used to assess anxiety-like behaviour and was conducted under red light (lux 5.0 – 6.0). Rats were allowed to explore a plus-shaped maze consisting of two opposing, identical open arms (50 cm long x 10 cm wide) and two opposing, identical closed arms (50 cm long x 10 cm wide x 40 cm-high walls). The central square region of the maze, where the arms joined, was 10 cm x 10 cm. An individual rat was placed in the centre of the maze facing an open arm and allowed to explore for 5 min before being returned to its home cage. Between each trial, the arena was cleaned with 70% ethanol. Animals were recorded on a ceiling-mounted camera and scored later using BORIS software v. 7.9.7. Outputs included time spent the open or closed arms, entries into arms (defined by having all four paws in the arm), and ethological metrics including head dips, leans, and rears were measured.

3.3.10 Grip strength test

Frailty in elderly populations has been associated with alterations in the gut microbiota (Jackson *et al.* 2016), and MCI (Boyle *et al.* 2010), and if this phenotype transferred to rodent microbiota recipients, it may lead to physical deficits that could impact on behavioural testing. Therefore, the grip strength test was conducted to measure frailty. This test utilizes a device to measure the force exerted by the pulling motion of a rat on a trapeze bar. An individual rat was removed from its home cage and placed over the trapeze bar until it grasped the bar with both forepaws. Keeping its torso horizontal, the tail of the rat was gently pulled backwards, and maximal grip strength (peak force) was measured using the electronic grip strength meter (Ugo Basil, Italy). The test was performed in triplicate, and trials wherein the rat failed to properly grip the trapeze were eliminated. Average peak force was normalized to the individual's body weight. All equipment was cleaned with 70% ethanol in between trials.

3.3.11 Morris water maze

The Morris water maze (MWM) was used to assess hippocampal-dependent spatial long-term memory and learning (Vorhees & Williams 2006; Nunez 2008), and is known to be sensitive to both aging and aging-associated microbiota perturbations in rodents (Boehme *et al.* 2021). In this

test, rodents rely on spatial clues marked on the walls of the testing room to navigate within an open swimming arena to locate a clear, hidden platform located 1-1.5 cm below the surface of the water. The circular pool (fiberglass, 190 cm internal diameter x 90 cm height), is filled with water (25 ± 1 °C) to a depth of 60 cm. Spatial learning is assessed over a period of 4 training days, with 4 consecutive 2-min trials occurring on each training day, and each trial starting from a different position within the arena. Rats were allowed to swim freely, and latency to locate the hidden platform was scored manually. Failure to locate the hidden platform within 2 minutes resulted in the rat being gently guided to the hidden platform. Rats were allowed to remain on the platform for 10 seconds prior to initiating the next trial. Upon completion of the final trial, rats were immediately dried with a clean towel and returned to their home cage.

Long-term spatial memory was assessed on the fifth day in a single probe trial. The hidden platform was removed from the arena, rats were placed in a novel starting location, and the latency of each rat to enter the target quadrant where the platform was previously located was assessed. Rats were allowed to explore the arena for 2 min, then removed, thoroughly dried with a clean towel, and returned to their home cage. Latency to the target quadrant and time within the target quadrant were scored using Ethovision v. 15.

3.3.12 Forced swim test

The forced swim test (FST) is a common test used to assess antidepressant-like activity (Cryan & Mombereau 2004) and was herein used to determine whether either microbial consortium transplant impacted antidepressant-like behaviour. This test spans over two days, including a 15-minute pre-swim test on Day 1, and involves the rat being placed into a transparent plexiglass cylinder filled with water (25 ± 1 °C). On Day 2, the swim test was conducted; rats were placed into the water-filled cylinder for 5 minutes, then dried with a clean towel and returned to their home cage. Side-view video recordings of the test were captured and later scored with BORIS v. 7.9.7 to assess the frequency of swimming, climbing, and immobility behaviours every five seconds of the test. Additional ethological metrics including the number of head shakes and dives were also recorded.

3.3.13 Tissue collection

Five days after completion of behaviour, rats were decapitated, and gut tissue dissected. Previous research has shown that animal models of microbiota disturbance, such as germ-free status or antibiotic treatment, can affect weight of the cecum (Savage & Dubos 1968; Spichak *et al.* 2018). Therefore, to determine whether treatment impacted metrics associated with gut health, the weight of the caecum and length of the small and large intestine were measured. Additionally, as the gut microbiota are known to influence the immune system and the hypothalamus-pituitary-adrenal (HPA) stress response axis of their host (Cryan *et al.* 2019b), the spleen and adrenals were dissected and weighed. Tissue was snap frozen on dry ice then stored at -80 °C for later use.

3.3.14 Determination of neurotransmitter levels in the gut

The gut microbiota can influence intestinal neurotransmitter levels by producing and metabolizing neurotransmitters, such as noradrenaline, which may allow bacteria to influence intestinal motility (Strandwitz 2018). Monoamine neurotransmitter levels were quantified in colonic tissue using high-performance liquid chromatography (HPLC), similar to as previously described (van de Wouw *et al.* 2020b). Mobile phase was comprised of HPLC-grade 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate monohydrate, 5.6 mM octane-1-sulphonic acid (Sigma Aldrich), 0.01 mM EDTA disodium salt (Alkem/Reagecon), and 11% (v/v) methanol (Alkem/Reagecon). Mobile phase pH was adjusted to 2.8 using 1 M sodium hydroxide (Alkem/Reagecon). The homogenization buffer contained 20 ng/20 µL of internal standard N-Methyl 5-HT (Sigma Aldrich) diluted in mobile phase. Colonic tissues were weighed prior to being sonicated three times in 500 µL ice-cold homogenization buffer for 5 sec each. During intervals, samples were kept cool on wet ice. Homogenized samples were then centrifuged at 14,000 RPM at 4 °C for 20 minutes, and 30 µL of the supernatant was diluted in 270 µL of mobile phase to create a 1:10 dilution. An injection volume of 20 µL was used for each sample on the HPLC system (Shimadzu, Japan). External standards of each monoamine, including Noradrenaline, 5-HIAA, 5-HT (serotonin) and the internal standard N-Methyl 5-HT (Sigma Aldrich), were prepared at 2 ng/20 µL and injected at regular intervals in between sample runs. The instrument included a SCL 10-Avp system controller, LC10AS pump, SIL-10A autoinjector, CTO-10A oven, EC3000 detector cell, and Class VP-5 software. Samples flowed at 0.9 mL/min using a Kinetex 2.6 u C18 100 A × 4.6 mm, with a pressure threshold of 30 to 400 bar, column oven temperature of 30 °C, and detector settings of +

0.8 V. The identity of each monoamine was confirmed by comparison to external standards. The ratio of peak height:internal standard was determined compared with the external standards ratio, and are displayed in ng/µg of tissue.

3.3.15 Statistical analysis

All data was assessed for normality using the Shapiro-Wilk test. Outliers were determined using the ROUT test (Q = 0.05) in neurotransmitter data, resulting in one outlier being removed in the MiCon noradrenaline dataset. In the case of normally distributed data, the effect of consortia treatment was determined by one-way ANOVA and Tukey post-hoc test. When data was non-parametric, a Kruskal-Wallis test was conducted followed by Mann-Whitney U tests. Time in centre of the open field, Novel Object Recognition Index and time spent with each object, dips in the elevated plus maze, and climbing events in the forced swim test were non-parametric and were therefore assessed via Kruskal-Wallis test followed by a Mann-Whitney U test. All other data were parametric and were assessed via one-way ANOVA and where appropriate followed by Tukey post-hoc test. Furthermore, in the carmine red gastrointestinal motility test, whereby majority of rats failed to produce a red bolus prior to the overhead lights turning off and thus conclusion of the test, a chi-square test of independence was used to assess whether treatment group affected whether a rat produced a bolus prior to the conclusion of the test. Statistical analysis and graph generation were performed using SPSS software v. 24 (IBM Corp) and GraphPad Prism v. 8.3.0 respectively.

3.4 Results

3.4.1 Treatment with MiCon or HeCon did not affect rodent weight gain or organ weight

As both the gut microbiota and the process of aging are known to disturb metabolic functions related to body weight, the weight of each rat was measured weekly and at the time of culls (Figure 3.2b). There was no effect of treatment on body weight gain throughout the study ($F(2, 26) = 0.01788$, $p = 0.9823$) or body weight at the time of culls ($F(2, 26) = 0.06223$, $p = 0.9398$) Alterations in the gut microbiota, such as those induced by antibiotics, have previously been shown to increase caecum weight (Ge *et al.* 2017). Neither bacterial consortia impacted the caecum weight ($F(2, 26) = 0.1.175$, $p = 0.3247$) or intestinal length ($F(2, 26) = 0.2465$, $p = 0.7834$; Figure 3.2c).

Bacteria in the gut are known to influence immune cell activity through a variety of methods, including histone modification, inflammatory signalling, and altering the gut barrier function, allowing for leakage of pro-inflammatory bacterial compounds (Cryan *et al.* 2019b). The spleen acts as a critical organ in immune regulation, including immune cell homeostasis and turnover (Bronte & Pittet 2013). Interestingly, the weight of the spleen is reduced in germ-free, likely due to their immature immune status (Hrncir *et al.* 2008). Therefore, differences in the weight of the spleen may indicate an immune response to inflammation. There was no difference in the weight of the spleen at culls (Figure 3.2d).

Furthermore, research involving germ-free rodents and rodents with disrupted gut microbiota have shown that the gut microbiota plays a role in the hypothalamus-pituitary-adrenal (HPA) stress response axis (Cryan *et al.* 2019b). Increased HPA signalling leads to an increase in the size of the adrenal glands. Thus, the weight of the left and right adrenal gland was measured as a proxy for altered HPA axis signalling. No differences in the weight of the left or right adrenal were observed (Figure 3.2d).

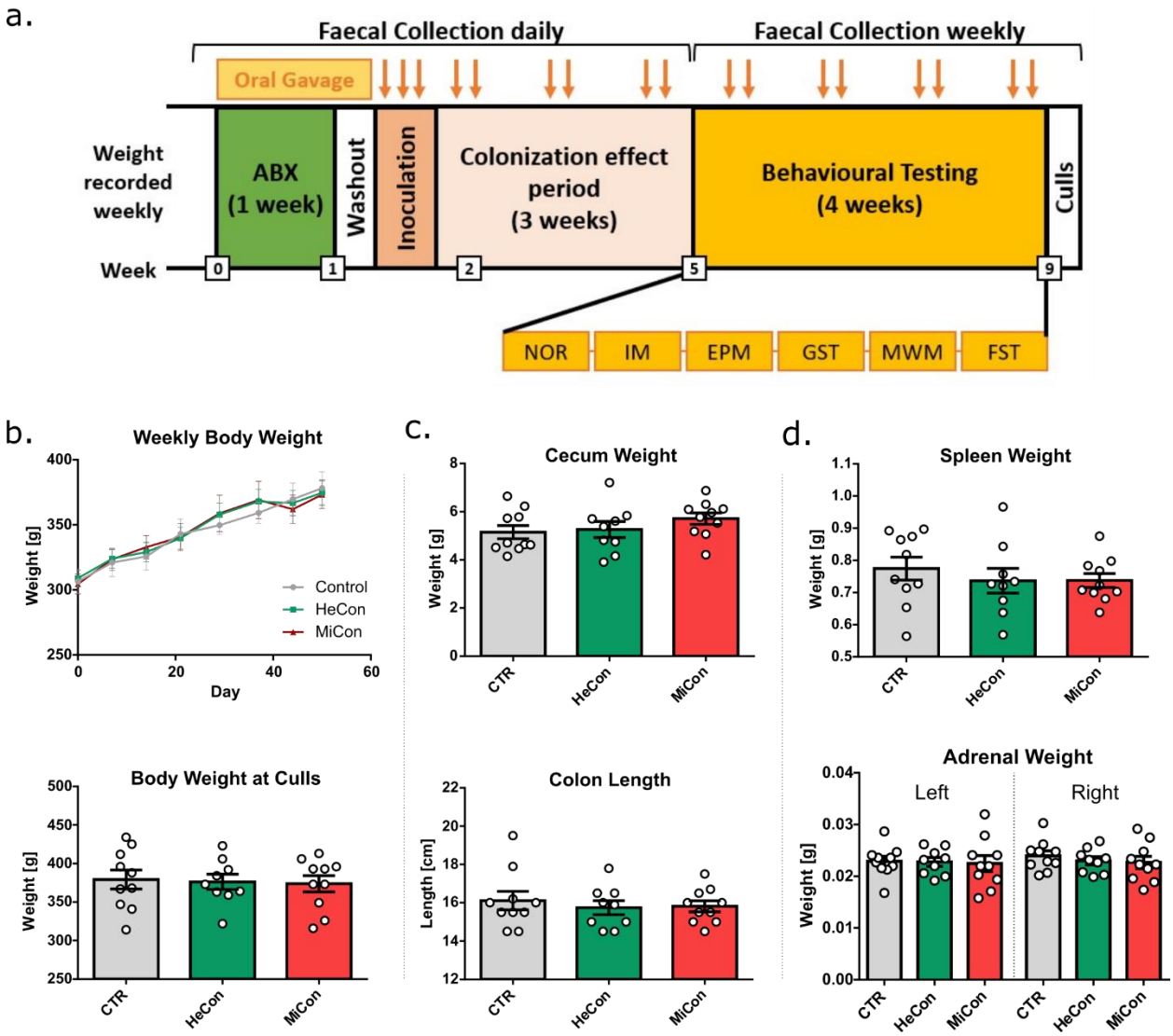


Figure 3.2. Administration of cognition-associated microbiota fails to modify physiological parameters in the young male rat. (a) Experimental timeline of the experiment, including behavioural battery; novel object recognition (NOR), intestinal motility (IM), elevated plus maze (EPM), grip strength test (GST), Morris water maze (MWM) and forced swim test (FST). (b) Average weekly body weight per group and body weight at the time of culls. (c) Intestinal measurements at culls. (d). Spleen and adrenal weights measured at time of culls.

3.4.2 Microbiota associated with cognitive decline reduced noradrenaline levels in the colon, but did not affect other metrics of gut physiology

Inactive bowel movement and constipation are risk factors for MCI (Chen *et al.* 2020; Huang *et al.* 2020), and has been linked to altered gut microbiota (Muller *et al.* 2020). Thus, whether either of the bacterial consortia induced differences in gastrointestinal transit time was assessed. Rats in the MiCon group trended towards having a slower gastrointestinal transit time than HeCon recipient rats (Figure 3.4b, $F(2,26) = 4.167$, $p = 0.0269$; MiCon vs HeCon $p = 0.0811$). However,

in the carmine red intestinal motility test, majority of rats failed to produce a faecal bolus prior to the animal facility lights switching to the dark cycle, which may skew the results of this test. Therefore, to determine whether rats had quicker gastrointestinal transit time based on treatment, a chi-square test of independence was used to determine whether or not one treatment group was more likely to produce a red bolus prior to the cut off time of the test. There was a trend that more rats in the HeCon group produced a faecal bolus than their counterparts, though the results were not statistically significant, X^2 (2, N = 39) = 5.221, p = 0.0735. There were no significant difference in faecal water content between groups (F(2, 20) = 0.3782, p = 0.6899; Figure 3.4a).

Colonic neurotransmitters, including serotonin (5-HT) and noradrenaline, are key regulators of gastrointestinal motility (Kendig & Grider 2015; Costa *et al.* 2021). Previous studies have shown that specific gut bacteria are able to regulate neurotransmitter levels in the gut, including serotonin and noradrenaline (Strandwitz 2018). To further explore the relationship that bacterial consortia treatment may have on gastrointestinal motility and general gut function, monoamine neurotransmitters in the distal colon of rats was assessed using HPLC *post-mortem*. Interestingly, colonic noradrenaline was significantly decreased in rats treated with MiCon (F(2, 25) = 3.939, p = 0.0072) compared to HeCon recipients (p = 0.0306) and control rats (p = 0.0088) (Figure 3.4c). The level of colonic 5-HT was not effected by treatment with microbial consortia (F(2, 26) = 2.482, p = 0.1031), although there was a trend towards MiCon rats having reduced colonic 5-HT compared to control rats (p = 0.0905). No differences were observed in the level of 5-hydroxyindoleacetic acid (5-HIAA), the primary metabolite of serotonin (F(2, 26) = 0.6672, p = 0.5217), or the ratio between 5-HIAA:5-HT (F(2, 26) = 0.1091, p = 0.8971), indicating that the serotonergic system is not affected by consortium treatment (Figure 3.4c).

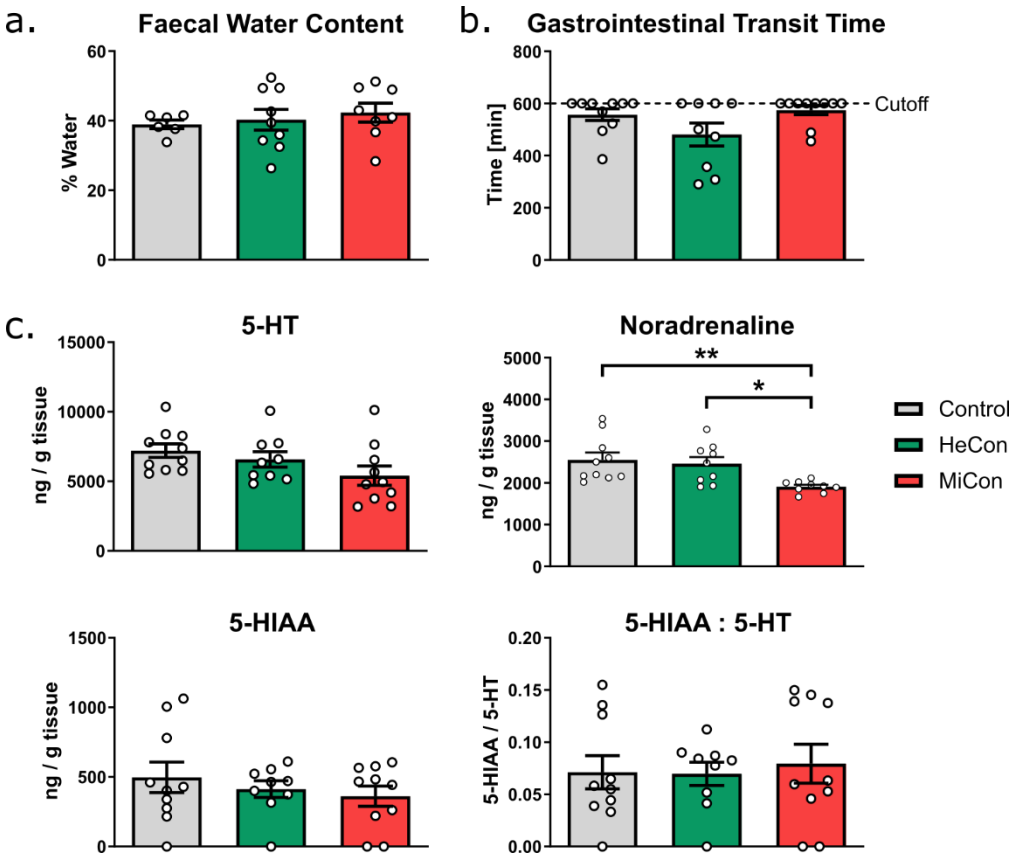


*Figure 3.4. Noradrenaline was reduced in the intestine of rats administered MiCon while other gastrointestinal measurements remained uneffected. (a) Faecal water content, (b) Gastrointestinal transit time, measured via carmine red dye, (c) Colonic neurotransmitter levels assessed by HPLC. Significant differences are displayed as *p < 0.05, **p < 0.01; One-way ANOVA post-hoc Tuckey.*

3.4.3 Microbial consortia administration from patients with mild cognitive impairments did not alter rat recipient behaviour

To determine whether administration of microbiota consortia altered host behaviour, a series of diverse tests were conducted to assess locomotor activity, anxiety-like and antidepressant-like behaviour, short-term memory, long-term spatial memory and learning, and grip strength as an indication for frailty.

None of the bacterial treatments had a significant effect on locomotor activity the total distance travelled in the open field ($F(2, 26) = 0.09344$, $p = 0.9111$) or mean velocity ($F(2, 26) = 0.09565$, $p = 0.9091$) indicating no differences in locomotor activity between groups (Figure 3.3a). There was no difference in the time spent in the centre of the open field arena ($F(2, 26) = 1.181$, $p = 0.3228$), thus suggesting no alterations in exploratory and anxiety-like behaviour.

Aging is also associated with an increased in anxiety-like behaviour in rodents (Ennaceur *et al.* 2008; Scott *et al.* 2017). Furthermore, behaviour in the elevated plus maze has previously been altered by microbiota perturbation in aged rodents (Boehme *et al.* 2021). Thus, the impact of microbial consortia transplant on anxiety was measured in the elevated plus maze wherein rats are considered to have increased anxiety-like behaviour when they spend less time in the open arms. Neither microbial treatment altered time in the closed and open arms of the elevated plus maze (Figure 3.3b, closed arms ($F(2, 25) = 1.097$, $p = 0.3494$), open arms ($F(2, 25) = .6992$, $p = 0.5065$). There was no difference in the percentage of entries into the open arm observed between groups ($F(2, 26) = 0.06200$, $p = 0.9400$). Additional ethological measures, such as head dips, leans, and rears, can indicate differences in exploratory behaviours. There were no difference between groups in any of these behaviours (Figure 3.3b;).

MCI in humans is associated with a reduction in learning ability and long-term and short-term memory. Thus, spatial learning ability and long-term spatial memory was assessed using the Morris water maze (Figure 3.3c). Over the period of four training days, the latency for a rat to find the platform significantly reduced regardless of treatment group (two-way ANOVA effect of time x consortia treatment ($F(6, 78) = 0.7818$, p = 0.5868; effect of time F (2.458, 63.92) = 52.31, $p <$ 0.0001). However, there was no effect of either microbial consortia treatment (consortia treatment $F(2, 26) = 0.4707$, $p = 0.6298$; AUC ($F(2, 25) = 1.613$, $p = 0.2194$). Long-term spatial memory, assessed by the latency to enter the target quadrant during the probe trial and time in the target quadrant were not affected by any treatment (latency: $F(2, 25) = 1.804$, $p = 0.1854$; time in target quadrant: $F(2, 25) = 0.3821$, $p = 0.6862$).

Short-term recognition memory was assessed using the novel object recognition test (Figure 3.3d). In this test, there was no effect of treatment on the recognition index ($H(2) = 2.864$, $p = 0.2389$), or time spent with either novel or familiar object (novel object: $F(2, 22) = 0.8207$, $p = 0.4531$; familiar object ($H(2) = 2.384$, $p = 0.3036$). Therefore, it appears that neither treatment with HeCon nor MiCon was able to induce changes in spatial learning, spatial long-term memory, or short-term recognition memory.

Given that the gut microbiota has been shown to play a causative role in depressive-like behaviour (Kelly *et al.* 2016), whether HeCon or MiCon treatment could induce changes in antidepressant-like behaviour in the forced swim test was also assessed. There were no differences between groups

in immobility ($F(2, 22) = 0.03852$, $p = 0.9623$), swimming ($F(2, 22) = 0.08984$, $p = 0.9144$), or climbing ($H(2) = 1.288$, $p = 0.5251$).

Finally, grip strength, a measure of frailty which has been associated with changes in the gut microbiome in aging (Haran & McCormick 2021) and MCI (Boyle *et al.* 2010) was assessed. There were no differences in grip strength between groups ($H(2) = 0.4314$, $p = 0.8060$).

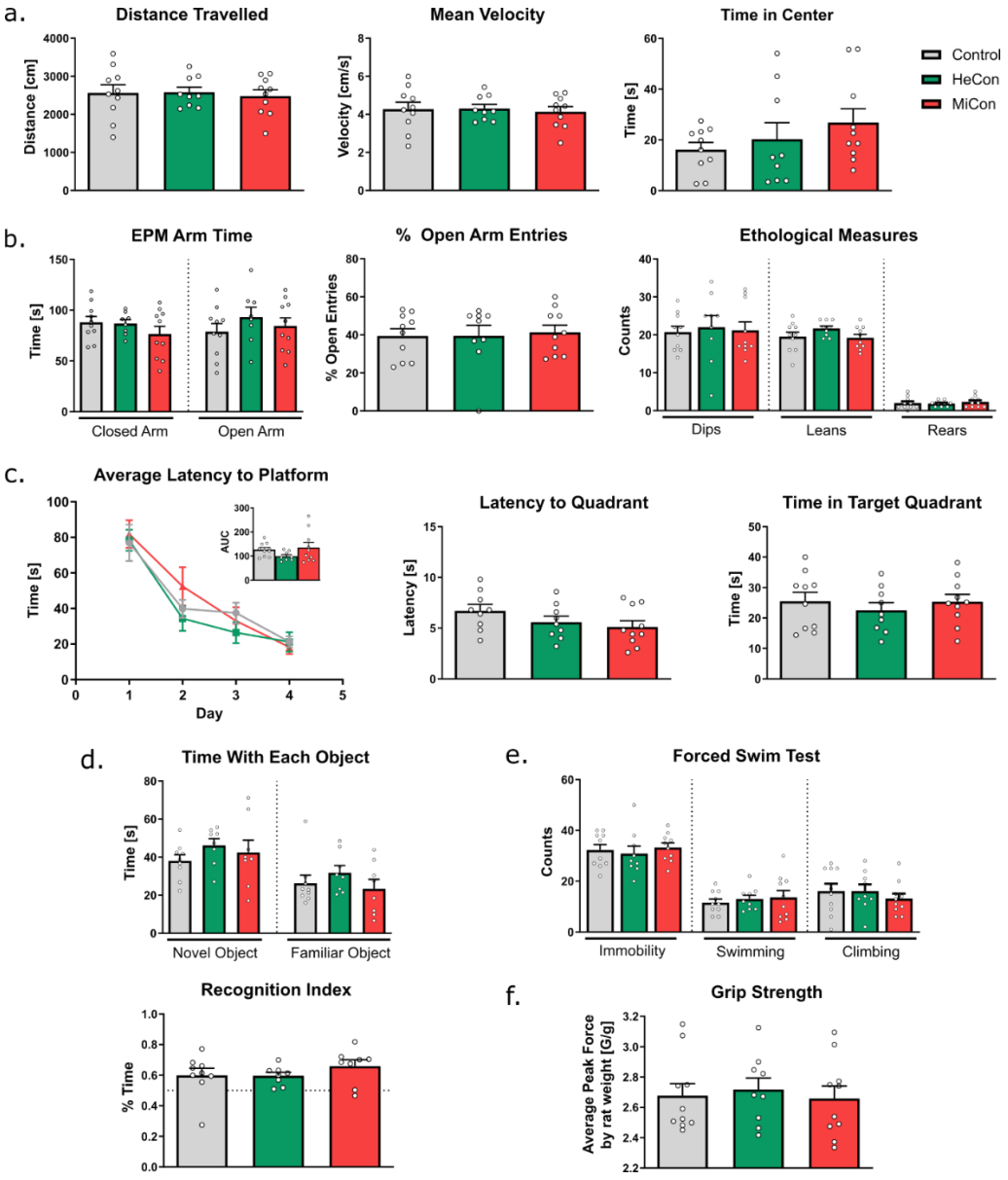


Figure 3.3 No changes were observed in behaviour following microbiota transplant. (a) Open field test; total distance travelled, mean velocity, and time spent in the centre of the arena. (b) Elevated plus maze; Time spent in the closed and open arms of the arena, percentage of entries that occurred into the open arm, and ethological measures including head dips, leaning onto the arena wall, and rears. (c) Morris water maze; average latency to locate the platform during each training day and inlaid area under the curve (AUC), latency to the quadrant target during the probe trial,

and time spent in the target quadrant during the probe trial. (d) Novel object recognition; time spent exploring either the novel or familiar object, and recognition index. (e) Forced swim test; occurrence of immobility, swimming, or climbing behaviours. (f) Average grip strength, normalized by the body weight of each rat.

Overall, these findings indicate that neither MiCon nor HeCon were able to induce changes in host cognition, locomotion, frailty, anxiety-like or antidepressant-like behaviours.

3.5 Discussion

This study investigated whether consortia of bacteria identified in elderly individuals with cognitive impairment or healthy cognitive functioning would induce similar cognitive states when transferred to young adult rats. First, bacteria were identified that have been known to harness the ability to interact with gut-brain signalling, and which were differently abundant in elderly populations with cognitive deficits or healthy cognitive function. Based on these findings, consortia of microbes with bioactive potential were developed to mimic components of the gut microbiomes of elderly with health or impaired cognition. Whether these bacterial consortia have any impact on behaviour, frailty, and gastrointestinal metrics was then investigated by transplanting these consortia into young adult rats.

The transplantation of the identified microbial consortia was not sufficient to elicit changes in behaviours related to short-term memory, long-term spatial memory and learning, anxiety-like behaviour, depressive-like behaviour, or grip strength. Furthermore, there was no difference in the faecal water content, intestinal transit time, caecum weight, intestinal length, spleen weight or adrenal weight. Upon examining neurotransmitter levels in the colon, noradrenaline appeared significantly reduced in MiCon-treated rats compared to HeCon-treated or Control rats. Furthermore, the level of 5-HT in MiCon-treated rats trended towards being reduced compared to control rats, although this was not statistically significant. There were no differences in the levels of serotonin metabolite 5-HIAA, or 5-HIAA:5-HT turnover ratio.

While this study is highly novel in its development of a bacterial consortium with predicted functionalities within the gut-brain axis, and transplantation of these consortia to assess host cognition, the lack of impact on cognitive function was unexpected because specific bacterial species were selected and administered based on their genetic capabilities and correlation to healthy or impaired cognition. For instance, studies examining elderly people with MCI have observed a decreased abundance of *F. prausnitzii* in the gut microbiome of these individuals, and better cognitive test scores in MCI patients positively correlated with a higher abundance of *F. prausnitzii* (Ueda *et al.* 2021). Meanwhile, male mice with Aβ-induced cognitive impairment that were treated with various strains of *F. prausnitzii* showed improved short-term and long-term memory (Claesson *et al.* 2011; Ueda *et al.* 2021). The HeCon consortia used here included *F. prausnitzii* but was unable to elicit similar effects on cognition.

Various species of bacteria can alter gastrointestinal neurotransmitter levels (Strandwitz 2018), and thereby may be able to induce alterations in the gut-brain axis. *Escherichia coli*, for instance, has been shown to take up exogenous noradrenaline in culture, increasing its rate of growth (Kinney *et al.* 2000). Nearly 30% of MiCon was comprised of *Escherichia coli*, which may potentially be the cause for the reduction in colonic noradrenaline observed in MiCon-treated rats. While reduction in colonic noradrenaline was not accompanied by alterations in gastrointestinal motility or behaviour, it is nevertheless interesting to observe this effect. Whether this reduction in colonic noradrenaline could translate to long-term consequences including healthspan is yet to be determined.

While there was clear support for the selected bacterial consortia to promote or hinder cognitive performance, this study has several limitations or confounding factors that should be considered when interpreting the data and that should be addressed in future research. For instance, male Sprague Dawley rats were the recipients for the microbiota consortium transplant. However, the bacterial consortia were identified and cultured from human faecal samples and therefore may not be adapted to thrive in a rat gastrointestinal environment. Therefore, these bacteria may not be performing the same functions as they would in a human host. Additionally, the engraftment of the bacteria was not assessed, and although consortia were gavaged twice weekly, a more frequent or longer treatment, may be needed to observe effects. Additionally, limitations existed on the concentration at which the microbiota could be grown in culture. A higher dosage of these bacteria might be required in order to observe effects. Furthermore, the bacterial candidates were identified from elderly patients, then transferred into young, healthy rats. Previously research in germ-free mice has shown that the age of the recipient is a critical factor determining how an individual responds to an FMT (Kundu *et al.* 2019), likely due to differences that occur during the aging process. Therefore, future research which seeks to investigate how microbes harbouring potential implications in aging-related diseases should consider applying these bacteria consortia interventions in middle-aged or aged rodent models. Additionally, this study did not seek to assess the impact of these consortium on health span or lifespan. It may be that MiCon-treated rats have shortened healthspan and display symptoms of cognitive decline sooner than HeCon-treated rats, although this is not possible to determine with the current study design. Furthermore, while the length of bacterial consortia treatment administered in this study is known to be sufficient to observe effects on cognition in other models of microbiota intervention (Sun *et al.* 2019; Boehme

et al. 2021), a longer intervention period may be needed in order to see effects of the consortia transplant. Additionally, as AD and MCI share genetic and hereditary underpinnings, it would also be of interest to explore the impact of these microbial consortia in genetic rodent models of AD to determine whether the consortia of microbes from MCI patients or healthy patients exacerbates or alleviates the effects of host genetics on the development of cognitive decline. This experiment did not investigate the complex interbacterial dynamics that occur within each consortium. It may be possible that the selected bacteria require the support from coexisting strains which were not included in the consortium (Oliveira *et al.* 2012). Additionally, it may be possible that the effect(s) of an individual bacterium is blunted or blocked by another bacterium in the consortium (Chen & Weimer 2001). Therefore, future research should consider investigating how individual strains of bacteria impact the host when administered individually, as well as determining the impact each bacteria play within the dynamics of the consortia.

Overall, this research suggests that not all bacteria which may have the capacity to alter the gut-brain axis have the ability to demonstrate this ability in a host. Furthermore, the relative abundance within a population of elderly people with or without cognitive impairment, may not be sufficient to determine whether it will affect the cognition of young, healthy rats. More work needs to be conducted to further determine how bacteria associated with aging-related cognitive impairment impact their host.

Chapter 4

Understanding the relationship between early life stress-induced gut microbiota changes and hippocampal neuroplasticity

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In Preparation

4.1 Abstract

Stress occurring early in life leaves long-term consequences on brain health, including impairments in neuroplasticity in the adult hippocampus, a region critical for functional cognitive and emotional behaviour. Moreover, the scars of enduring early life stress have also been identified in the gut microbiota, which is a crucial facilitator of nutritional availability and homeostasis within the host. The gut microbiota has recently been demonstrated to play an important role in regulating host cognition through its ability to influence hippocampal neuroplasticity. Perturbances to the gut microbiota, including those incurred by stress, may therefore underly the stress-induced impairments in hippocampal neuroplasticity, cognitive performance, and emotional difficulties later in life. Therefore, strategies intervening at the level of the gut microbiota may prove efficient for mitigating the consequences of early life stress. Recently, dietary supplementation with fish oil or polyphenols has been shown to alleviate some of the early life stress-induced consequences on adult rat behaviour and alter the gut microbiome, although the mechanism of action of these micronutrients have yet to be elucidated. Here, we attempt to further understand the impact of fish oil or polyphenol supplementation on host health in a rat model of early life stress by characterizing changes in diversity, composition, and predicted function of the gut microbiome using 16S rRNA sequencing and correlating these microbial factors to alterations in gene expression of neuroplasticity markers in the hippocampus. This research provides additional insights into the impacts of early life stress on the gut microbiome and host hippocampal neuroplasticity and reveals that dietary supplementation with fish oil and some polyphenols may exert their beneficial effects on hippocampal neuroplasticity following early life stress through gut microbiota-based mechanisms. Overall, by assessing correlations between alterations in the gut microbiome and neuroplasticity-related gene expression in the hippocampus, these findings reveal novel potential therapeutic targets that may mitigate the effects of early life stress on hippocampal plasticity, including bacterial genera whose potential ability to alleviate the effects of stress on hippocampal neuroplasticity should be further investigated.

4.2 Introduction

The neurobiological consequences of stress exposure during early life are well evidenced. These effects are particularly striking in the hippocampus, a brain region critical for regulating mood and emotions, as well as cognition. In humans, stress during early life, including insufficient maternal care, is associated with decreased hippocampal volume (Campbell *et al.* 2004; Videbech & Ravnkilde 2004; Frodl *et al.* 2010) and a higher risk of developing depression (Heim & Nemeroff 2001; Chapman *et al.* 2004; LeMoult *et al.* 2020) and social and developmental problems (Field 1998) later in life. To better understand the repercussions of early life stress on neurobiology and behaviour, the rodent model of early life maternal separation (MS) stress has become invaluable (O'Mahony *et al.* 2011; Nishi *et al.* 2014; Mansouri *et al.* 2019). Preclinical models have further revealed that early life stress leaves pervasive consequences on hippocampal synaptic plasticity and neurogenesis (Oomen *et al.* 2009; Oomen *et al.* 2010; Loi *et al.* 2014; Lajud & Torner 2015; Naninck *et al.* 2015; Rule *et al.* 2021), reduces hippocampal mossy fibre density (Huot *et al.* 2002), alters hippocampal gene expression including the expression of glutamate and GABA transporters (Martisova *et al.* 2012; Daskalakis *et al.* 2015), and perturbs the function and development of hippocampal microglia (Delpech *et al.* 2016) which are important supporters of hippocampal plasticity (Sierra *et al.* 2010). These consequences to the hippocampus likely contribute to the impairments in spatial learning and memory (Oomen *et al.* 2010; Naninck *et al.* 2015) and emotional behaviour (Bolton *et al.* 2017) observed in rodent early life stress models. Furthermore, the ability for antidepressants to exert their beneficial effects is believed to be dependent on their ability to restore hippocampal plasticity and neurogenesis (Santarelli *et al.* 2003; Mateus-Pinheiro *et al.* 2013; Hill *et al.* 2015), highlighting the importance of hippocampal neuroplasticity in regulating mood and behaviour.

Stress during early life also creates profound, long-lasting impacts on the trillions of microbes inhabiting the gastrointestinal tract, yielding altered microbial diversity and changing the abundance of key bacterial genera (Bailey & Coe 1999; O'Mahony *et al.* 2009; De Palma *et al.* 2015; Gur *et al.* 2015; Donoso *et al.* 2020). These microorganisms, known collectively as the gut microbiota, are crucial for supporting the health, including neurological health, of their host (Cryan *et al.* 2019b). For instance, the gut microbiota can manipulate host cognitive and depressive-like behaviour (Desbonnet *et al.* 2010; Savignac *et al.* 2015; Kelly *et al.* 2016; Abildgaard *et al.* 2017b;

Boehme *et al.* 2021; Knudsen *et al.* 2021; Tian *et al.* 2021), as well as influence the birth and development of new neurons in the adult hippocampus (Ogbonnaya *et al.* 2015) through immune (Mohle *et al.* 2016; Erny *et al.* 2021), metabolite-driven (Erny *et al.* 2021; Wei *et al.* 2021), and potentially vagal (O'Leary *et al.* 2018; Liu *et al.* 2021) pathways.

Diet plays a major role in shaping the gut microbiome on a daily basis (Johnson *et al.* 2019) and presents as a non-invasive strategy for gut microbiota-targeted interventions. The term *prebiotic* has been minted to highlight that a specific nutrient that is utilized by the gut microbiota and excerpts a benefit to host health (Gibson *et al.* 2017). Furthermore, traction has been growing to better understand the potential for diet to alleviate the effects of stress and stress-related pathologies (Kang *et al.* 2014; Burokas *et al.* 2017; Provensi *et al.* 2019). For instance, diets including various fruits and vegetables, which are high in prebiotic polyphenols (Vauzour 2012), have been clinically associated with improvements in depressive symptomology (Bayes *et al.* 2020), and yields antidepressant and anti-anxiolytic effects in various rodent models (Anjaneyulu *et al.* 2003; Kulkarni *et al.* 2008; Bouayed 2010), suggesting polyphenols might act through altering the gut microbiome to improve depressive-like behaviour. Meanwhile, a Mediterranean-style diet, which is high in fish oils, was sufficient to improve mental health scores in individuals with depression (Parletta *et al.* 2019), perhaps due to its ability to promote hippocampal neurogenesis (Beltz *et al.* 2007), implicating fish oil as another nutritional component whose efficacy towards improving stress-related symptomology may be rooted in its ability to restructure the gut microbiome. Nonetheless, the mechanisms of action for how polyphenols and fish oil exert their prebiotic effects remains unknown, though their ability to influence the gut microbiota may allow for their potential effects on hippocampal neuroplasticity-related behaviours.

Understanding the mechanism(s) of action by which polyphenols and fish oil can ameliorate the disruptions in cognitive and emotional behaviour following early life stress may provide new insights into potential novel therapeutics for early life stress-associated consequences, including increased risk of depression. Therefore, we investigated the impacts of fish oil and three polyphenol dietary supplementations (xanthohumol, phlorotannins, and quercetin) on the diversity and predicted functional capacity of the gut microbiome. Furthermore, changes in the gut microbiome and its predicted bioactive gut-brain pathways were correlated with alterations in the

expression of hippocampal genes related to neuroplasticity to further understand the role the gut microbiome plays in facilitating the effects of these dietary supplements.

4.3 Methods

4.3.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork AEEC #2015-022 and conducted under HPRA project authorization AE19130/P053 in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposes. Adult female Sprague Dawley rats (250–300 g) were mated in-house in the Biological Services Unit facility, University College Cork. Upon observance of a mucus plug, female rats were housed individually in plastic cages and maintained with minimal disturbance until birth (15 × 22 × 9 cm). All procedures were conducted in a temperature and humidity-controlled room on a 12:12-h light-dark cycle (lights on from 7.00 to 19.00 h) with food and water provided *ad libitum*.

Additional outcomes, including behavioural measures, were collected from the rodents involved in this study and these data were previously published (Donoso *et al.* 2020; Egerton *et al.* 2020).

4.3.2 Maternal separation stress procedure

Maternal separation stress was conducted as previously described (O'Mahony *et al.* 2009; Pusceddu *et al.* 2015; Donoso *et al.* 2020; Egerton *et al.* 2020) daily from postnatal day (P)2 through P12. Briefly, the litter of pups were separated from their mother and placed into plastic cages warmed to 30–33 °C in a separate room to avoid potential communication with the mother (Hofer *et al.* 1994). After three hours of separation, pups were returned to their mother and home cage. Separation occurred daily between 9 am and 12 pm. An additional group of litters that were not subjected to the maternal separation stress paradigm were left untouched. After P12, pups were left undisturbed apart from cage cleaning which occurred every two days. At weaning, male rats were housed in groups of 2-4.

4.3.3 Drugs

All diets were generated by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany). Fluoxetine was obtained from Sigma Aldrich (PHR1394, Sigma Aldrich Ireland Ltd., Wicklow, Ireland) and prepared in the standard chow at 0.015% fluoxetine. Fish oil was added to the standard chow in

place of soybean oil and comprised 7% of the total feed (ssniff Spezialdiäten. Ferdinand-Gabriel-Weg, Germany). A phlorotannin-rich extract derived from *Fucus vesiculosus* (Gite *et al.* 2019) was provided by National University of Ireland, Galway (Galway, Ireland). Quercetin was purchased from Sigma (Q4951). Xanthohumol was obtained from Hopsteiner, GmbH, Mainburg, Germany (A-4-2014).

4.3.4 Dietary supplementation and treatment groups

This study consists of seven groups with rats randomly assigned to their respective group. Groups included a non-maternally separated control group (NS-Control, n = 12) fed with a standard chow diet (E15712-04); a maternally separated control group fed the same standard chow diet (MS-Control, n = 12); a maternally separated group fed a diet containing 0.015% fluoxetine (MS-Fluoxetine, n = 12); a maternally separated group fed a diet supplemented with fish oil in place of soybean oil in the standard chow (MS-Fish Oil, n = 10); a maternally separated group fed a diet supplemented with Quercetin (MS-Quercetin); a maternally separated group fed a diet supplemented with phlorotannin (MS-Phlorotannin); and a maternally separated group fed a diet supplemented with xanthohumol (MS-Xanthohumol). All diets were generated by ssniff Spezialdiäten (Ferdinand-Gabriel-Weg, Germany) and were isoenergetic and contain equal amounts of macronutrients. The fatty acid, vitamin, and mineral composition of the fish oil diet is described elsewhere (Egerton *et al.* 2020).

Food was provided *ad libitum*, and administration of dietary intervention chow began at eight weeks of age until sixteen weeks of age. All dietary supplementation was administered with calculations based on previously published literature defining the average daily consumption of food and average Sprague Dawley weight between 9 to 16 weeks (Laaksonen *et al.* 2013). MS-Fluoxetine rats were estimated to receive a dose of 10 mg/kg/day fluoxetine, a standard dosage to observe preclinical behavioural effects of fluoxetine (Arndt *et al.* 2015). Fish oil, swapped for soybean oil in the standard chow, was estimated to provide 467 mg/kg/day of the ω-3 fatty acid docosahexaenoic acid (DHA). Quercetin supplementation was estimated to provide 20 mg/kg/day quercetin (Haleagrahara *et al.* 2009). Phlorotannin supplementation was estimated to provide 20 mg/kg/day phlorotannin (Ahn *et al.* 2017). Xanthohumol supplementation was estimated to provide 10 mg/kg/day xanthohumol (Ceremuga *et al.* 2013)

4.3.5 Hippocampal gene expression

Rats were decapitated and the brain was immediately removed from the skull. The whole hippocampus was dissected from both hemispheres and immediately snap frozen on dry ice, then stored at -80 °C until future use. Total RNA was extracted from the right hippocampus following the Invitrogen TRIzol™ Reagent RNA extraction method provided by the supplier (ThermoFisher Scientific), using 500 µL of TRIzol™ Reagent per sample, and a resuspension in 100 µL RNase-free water. Complimentary DNA was generated from 80 ng/ml Total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-Time PCR (Roche) using the SYBR Green SensiFAST™ SYBR No-ROX Kit (Bioline) was carried out to assess differences in gene expression levels. Relative mRNA expressions were calculated with the 2-ΔΔCt method (Livak & Schmittgen 2001), normalizing against the reference gene β-actin. Primer sequences are described in Table 5.1.

Table 5.1. Sequences of SYBR Green Rat PCR Primers. F indicates forward primer, R indicates reverse primer.

Target mRNA	**Froward Primer 5'-3'**	**Reverse Primer 5'-3'**
β-actin	AAG TCC CTC ACC CTC CCA AAA G	AAG CAA TGC TGT CAC CTT CCC
DCX	ATC TCT ACA CCC ACA AGC C	AAT GTC TAA GCA CCA ATA GCC
KI67	ATT CCC CAC AAC CCA AAA C	ATG CCT ACT TTC CCC TGA C
Bdnf	AAG TCT GCA TTA CAT TCC TCG A	GTT TTC TGA AAG AGG GAC AGT TTA
Bdnf IV	ACT GAA GGC GTG CGA GTA TT	TGG TGG CCG ATA TGT ACT CC
GRIN1	CAG CCT TTT CAG AGC ACA C	ATC TTC CTC CTC CTC CTC AC
GRIN2A	ATA GAC CCC CTG ACT GAG AC	CAC CTA TCA TTC CAT TCC ACA C
GABA(B1a)	ACA CAC ACA CAC ACA CAC C	CAC ACA CAC ACA CAC ACA G
Nr3c1	GCA GCA GTG AAA TGG GCA AAG	TCA GGA GCA AAG CAG SGC AG
Nr3c2	CCC TTC CAA CAA CAC CAA C	AAC TGC TGA AAG CCC CAT C

4.3.6 Gut microbiota 16S sequencing

During dissection, faecal pellets were removed from the distal lower intestine and immediately snap frozen on dry ice then stored at -80 ° C until later analysis. Faecal microbial DNA was extracted using the QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen) as per the manufacturer's directions. Quality and concentration of DNA was assessed with a NanoDrop® ND-1000 UV–vis Spectrophotometer (Thermo Fisher Scientific). Using the Illumina 16S metagenomic sequencing library protocol, the V3-V4 variable region of the bacterial 16S rRNA gene was amplified. Following index PCR and purification, samples were examined by PCR using

the KAPA HiFi HotStart PCR Kit (KAPA Biosystems) and cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter Life Sciences). Subsequently, indexing PCR was performed to attach Nextera XT barcodes and Illumina sequencing adapters to the 5′ overhangs and samples underwent additional AMPure XP clean-up. Samples were sequenced with standard Illumina sequencing protocols on the MiSeq™ System (Illumina®) with a 2 × 250bp cycle kit according to manufacturer's guidelines.

4.3.7 Gut microbiome analysis

Paired-end reads were pre-filtered using a quality score threshold of >30, then trimmed and filtered for chimeras and quality using the DADA2 library in R Studio (v.1.4.1717). The fastqc program was used to assess sample quality in Ubuntu v.20.04. Samples with fewer than 10,000 reads post-filtering were eliminated. Taxonomy was assigned using the SILVA SSURef database (release v.138) and DADA2 according to the DADA2 manual unless otherwise indicated. Amplicon sequence variants were aggregated at the genus level, and reads with an unknown genus were removed from downstream analysis, as this effected fewer than 10% of total samples. After filtering, 162 genera remained in all microbiome samples.

Microbiome bioinformatics was performed in R Studio (v.1.4.1717). Values were centre-log transformed using the clr_lite function (Lubbe *et al.* 2021) in the Tjazi library (https://github.com/thomazbastiaanssen/Tjazi) with a permutation number of 1,000, and principal-component analysis was performed. α-diversity was calculated using the iNEXT library and the Chao1, Shannon, and Simpson indices. β-diversity assessed via Aitchison distance was visualized on a principal-component analysis. Differences in β-diversity were assessed with a PERMANOVA followed by a pair-wise PERMANOVA Benjamini-Hochberg post-hoc correction to assess the effects of treatment on maternal separation stress-treated rats.

Gut microbiome functional capacity was inferred in terms of KEGG orthology (KO) using PICRUSt 2. Gut-Brain Modules (GBMs) were then calculated from KOs using the omixerRpm R library. Linear modelling was performed to determine the differential abundance of microbial genera and GBMs. To correct for multiple testing (FDR) in tests involving microbiota features, the Storey's Q-value was used (Storey & Tibshirani 2003) with 0.2 as the q-value threshold. Cohen's d was used to measure effect size.

4.3.8 Corelations between hippocampal gene expression and microbiome features

Corelations were performed in RStudio to assess the relationship between the expression of hippocampal neuroplasticity-related genes and the clr-transformed relative abundance of genera and GBMs. Linear relationships between the data were assessed using Pearson's correlation coefficient followed by a Benjamini–Hochberg correction for False Discovery Rate (FDR) with a threshold of 0.1.

4.3.9 Gene expression statistics and visualizations

Gene expression data was assessed for normality using the Shapiro-Wilk test. To determine whether early life maternal separation stress altered gene expression in the hippocampus, an unpaired t-test, or a Mann-Whitney test in the case of nonparametric data, was performed between NS-Control and MS-Control rats. This study was designed to assess the impact of each individual dietary supplementation on hippocampal gene expression in maternally stressed rats. Therefore, the effect of individual dietary supplementation on gene expression following early life maternal separation stress was then determined by t-test or a Mann-Whitney test in the case of nonparametric data. All data is presented as Mean ± SEM. Statistical significance was defined as $p \leq 0.05$. Statistical analysis and graph generation were performed using SPSS software v. 24 (IBM Corp), R Studio, and GraphPad Prism v. 8.3.0.

4.4 Results

4.4.1 Prebiotic consumption impacts gut microbiota diversity and composition in rats subjected to early life stress

Changes in the gut microbiota have been observed in patients with stress-related psychiatric disorders, including major depressive disorder, and preclinical models of stress (O'Mahony *et al.* 2009; Cryan & Dinan 2012; Kelly *et al.* 2016; Tillmann *et al.* 2019; Knudsen *et al.* 2021). Therefore, 16S sequencing was performed to determine whether a treatment period of eight weeks with the antidepressant fluoxetine, fish oil, or various polyphenolic compounds can reshape the MS-afflicted gut microbiome and reverse effects of early life maternal separation stress was examined using α-diversity, β-diversity, and compositional analysis.

α-diversity refers to the diversity within an individual sample. α-diversity can be quantified through a variety of methods which provide different information about the within-sample diversity through unique statistical calculations and assumptions. The Chao1 index, a measurement of species richness, and was reduced in rats subjected to early life stress (Fig 4.1a; NS-Control vs MS-control $t(20) = 2.4695$, $p = 0.02325$). Neither fluoxetine treatment nor supplementation with polyphenols or fish oil were able to rescue this stress-induced phenomenon ($F(5, 56) = 1.199$, $p = 0.3217$). Shannon Entropy is an estimation of sample richness and evenness. Shannon Entropy was unaffected by maternal separation stress (Fig 4.1a; NS-Control vs MS-control $t(20) = 1.2078$, $p = 0.2425$) and fluoxetine treatment ($F(5, 56) = 3.319$, $p = 0.01076$; MS-Control vs MS-Fluoxetine $p = 0.1319$). However, two polyphenols, phlorotannins and xanthohumol, reduced the Shannon Entropy index compared to MS alone (MS-Control vs MS-Phlorotannins $p = 0.0119$; MS-Control vs MS-Xanthohumol $p = 0.0500$). The final alpha diversity metric assessed was the Simpson Index, which considers both the number of species present and the relative abundance of each species but is less influenced by rare taxa compared to the Shannon Entropy measure. In line with the Shannon Entropy score, neither maternal separation stress nor fluoxetine treatment had an impact on the Simpson Index diversity score (Fig 4.1a; t-test NS-Control vs MS-control $t(20) = 1.361$, $p = 0.1902$; One-way PERMANOVA $F(5, 56) = 3.155$, $p = 0.01405$; MS-Control vs MS-Fluoxetine $p = 0.1041$). Furthermore, phlorotannin and xanthohumol supplementation reduced Simpson Index diversity score in rats subjected to early life stress (MS-Control vs MS-Phlorotannins $p = 0.0210$; MS-Control vs MS-Xanthohumol $p = 0.0104$). This data suggests that the gut microbiomes of rats

exposed to maternal separation stress had reduced quantities of OTUs although similar distribution, while both phlorotannin and xanthohumol supplementation skewed the evenness of microbial genera distribution.

β-diversity refers to the diversity in the gut microbiome observed between groups. Previous literature has shown that β-diversity is altered by maternal separation stress (Donoso *et al.* 2020). These effects were replicated herein; maternal separation stress significantly altered β-diversity in adult rats (Fig 4.1b; NS-Control vs MS-Control $F = 1.8034$, $R^2 = 0.08271$, $p = 0.0249$). Treatment of MS rats with dietary supplements also yielded strongly significant differences in β-diversity using a pairwise PERMANOVA approach ($F = 2.678$, $R^2 = 0.19579$, $p = 0.00001$).

Compositionally, early life maternal separation stress substantially altered the abundance of four bacterial genera, leading to significantly decreased abundance of *Rothia* and *Christensenellaceae* and increased abundance of *Lachnospiraceae NK3A20 group* and *Colidextribacter* following stress (Figure 4.1c, $q < 0.1$). Given that the gut microbiome demonstrates sensitivity to treatment with the antidepressant fluoxetine (Cussotto *et al.* 2019; Lyte *et al.* 2019; Egerton *et al.* 2020), it is unsurprising that fluoxetine supplementation altered the compositional structure of the gut microbiome of MS rats (Figure 4.1c), yielding increased abundance of *Butyricicoccus*, *Frisingicoccus,* and *Ruminococcaceae UBA1819* and decreased the abundance of *Pygmaiobacter* and *Parasutterella* ($q < 0.1$). Fluoxetine's ability to reshape the gut microbiome has been hypothesized to underly its beneficial neurobehavioural effects (Lyte *et al.* 2019). Therefore, the ability for fish oil or polyphenols to exert similar effects on the gut microbiome may be relevant for understanding their neuroactive potential. Fascinatingly, supplementation with fish oil, phlorotannins and quercetin induced a similar increase in the abundance of *Butyricicoccus* (Figure 4.1c). Supplementation of MS rats with fish oil yielded the most differences in the gut microbiota genera-level composition, increasing the abundance of 17 bacterial genera while decreasing the abundance of 14 genera, inducing similar effects as fluoxetine treatment on the abundance of *Pygmaiobacter* and *Parasutterella,* in addition to *Butyricicoccus,* and reducing *Lachnospiraceae NK3A20 group* abundance beyond the extent observed in stress-naïve rats ($q < 0.1$). Supplementation with phlorotannins resulted in five bacterial genera having increased abundance and four observed to decrease. Of these, *Butyricicoccus* was the only genera to be significantly higher in both the MS-Phlorotannins and MS-Fluoxetine groups relative to the MS-Control, though

MS-Phorotannin rats shared no significant microbial general differences in similarity with NS-Control rats, indicating that phlorotannins do not allow the gut microbiome to recover in the relative abundance of any bacterial genera following stress. Xanthohumol consumption increased only the genera *Lachnospiraceae UCG-010* while decreasing levels of four bacterial genera, including *Lachnospiraceae NK3A20 group* and *Pygmaiobacter* which were observed to be decreased to the same effect size in rats naïve to stress and MS-Fluoxetine rats respectively (Fig 4.1c). Meanwhile, quercetin displayed the weakest ability to influence gut microbiota composition in MS rats, although its ability to increase *Butyricicoccus* and decrease *Lachnospiraceae NK3A20 group* were similar to MS rats treated with fluoxetine or non-stressed stress, respectively ($q < 0.1$).

Overall, stress reshaped the gut microbiome at the α-diversity, β-diversity, and compositional levels, with some stress-induced characteristics in the gut microbiome reversed or altered following prebiotic or antidepressant treatment. Fish oil consumption following MS induced the most differences in the relative genera abundance and β-diversity, while both phlorotannins and xanthohumol reduced measures of α-diversity. At the genera level, maternal separation stress led to an insignificant increase in the abundance of some bacterial genera, including *Lachnospiraceae ruminantium* and *Ruminococcacaea pygmaiobacter*, which were lowered to some degree by fluoxetine, fish oil, and polyphenol treatments. Similarly, *Oscillospiraceae pseudoflavonifractor* was enriched, albeit mostly insignificantly, in the NS-Control and MS rats receiving fluoxetine, fish oil, and polyphenol treatments compared to MS-Control rats.

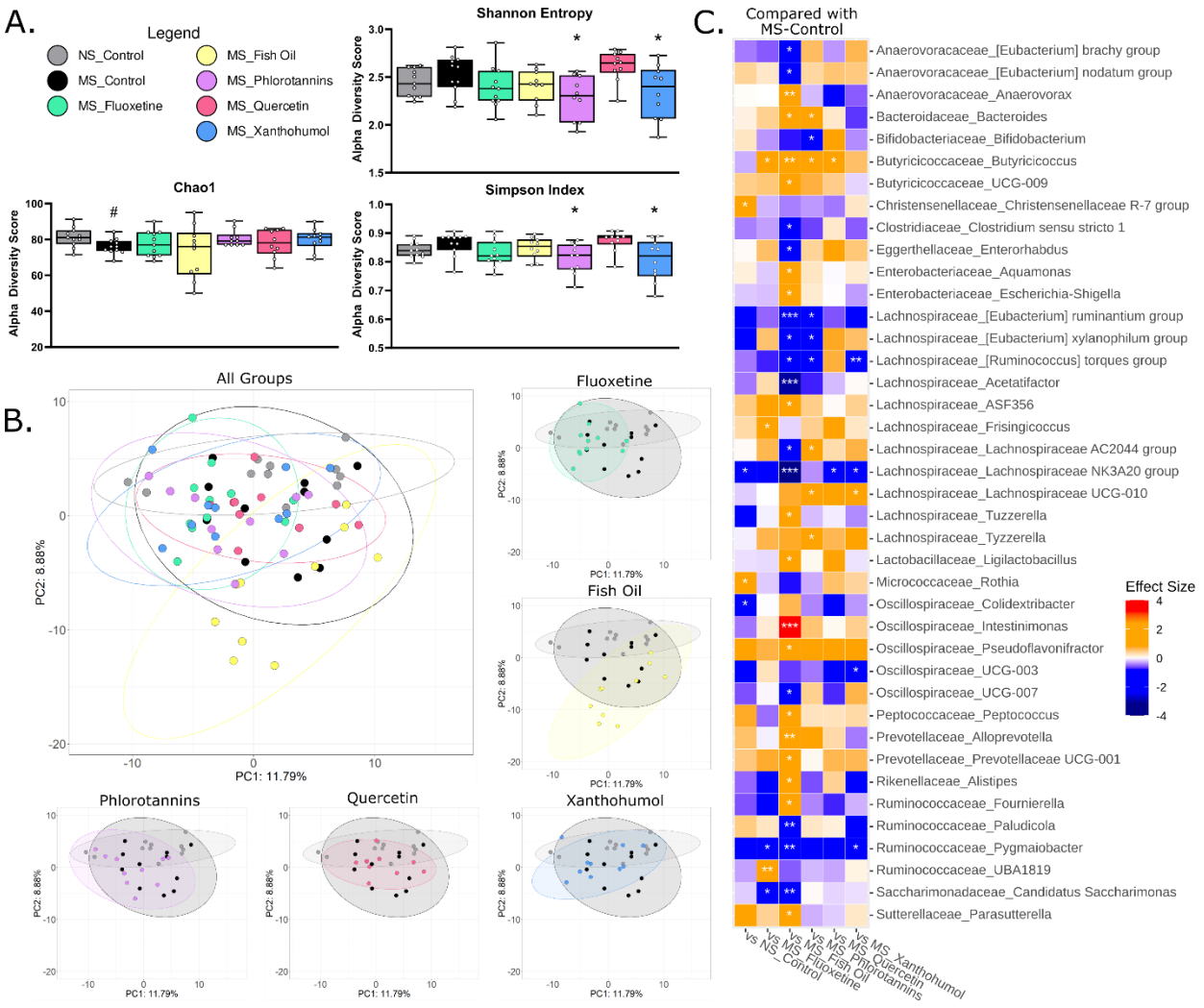


*Figure 4.1.Modulation of the effects of early life stress on the gut microbiome by fluoxetine, fish oil, and polephenol supplementation. (a) α-diversity of the gut microbiomes assessed via Chao1, Shannon Entropy, and Simpson Index, displayed as Mean ± SEM. Stress effect was determined by comparing NS-Control vs MS-Control using a t-test and is visualized as # $p < 0.05$. Effects of interventions on rats exposed to early life stress was determined via one-way ANOVA post-hoc LSD and significance is displayed as * $p \leq 0.05$ versus the MS-Control group (b) β-diversity of the gut microbiomes visualized using principal-coordinate analysis. Elipses indicate 95% confidence intervals. (c) Differential abundance of genera compared with the MS-Control group. Effect size was measured using Cohen's D and visualised with purple showing a higher abundance in the MS-Control group and orange indicating a lower abundance in the MS-Control group of each comparison. Only genera found to be significantly different in at least one comparison are shown. * $q < 0.1$; ** $q < 0.01$; *** $q < 0.001$. n = 10 – 11 rats per group. Results generated from this figure are a more advanced analysis on previously published work (Donoso et al. 2020; Egerton et al. 2020).*

4.4.2 Dietary supplementation with fish oils impacts the predicted function of the gut microbiota

Genetic-based potential functionality of a microbial community upon the gut-brain axis can be inferred from 16S microbial sequencing, although it is unknown whether the predicted function is indeed occurring at a given time (Valles-Colomer *et al.* 2019). Previously, specific Gut-Brain

Modules (GBMs) were found to be impacted by early life maternal separation stress in rats, including pathways for decreased Nitric oxide degradation I and nitric oxide synthesis II and increased pathways for tryptophan degradation and p-Cresol synthesis (Donoso *et al.* 2020), although the bioinformatics sequencing library used in this study has since undergone substantial improvements. Nonetheless, to our knowledge, the impacts of either fluoxetine or fish oil supplementation on the predicted function of the gut microbiome has never been examined in the context of maternal separation.

Analysis of predicted gut microbiota functions revealed immense differences in GBMs of adult rats triggered by early life maternal separation stress, including an increase in the abundance of 18 GBMs, including GBMs for acetate, butyrate and propionate synthesis and a reduction in the abundance of six GBMs, including nitric oxide synthesis and p-Cresol degradation, in rats exposed to MS (Figure 4.2). Fish oil supplementation decreased the abundance of 20 GBMs while increasing the abundance of 11 GBMs. Of note, 14 of the 31 GBMs altered by fish oil supplementation to MS rats yielded effect sizes in similar directions to those of non-stressed rats, indicating that fish oil may be able to restore some of the early life stress-induced alterations in GBMs. These GBMs included functions for decreased acetate synthesis I and III, butyrate synthesis II, DOPAC synthesis, increased p-Cresol degradation, among others. Interestingly, no polyphenol treatment elicited a significant effect on GBMs compared to MS-Control rats. Meanwhile, treatment with the antidepressant fluoxetine only significantly decreased Propionate Degradation I relative to MS-Control rats, an effect which was similar to that observed in non-stressed rats (Figure 4.2). The statistics of significant interactions is displayed in Supplementary Table 4.1.

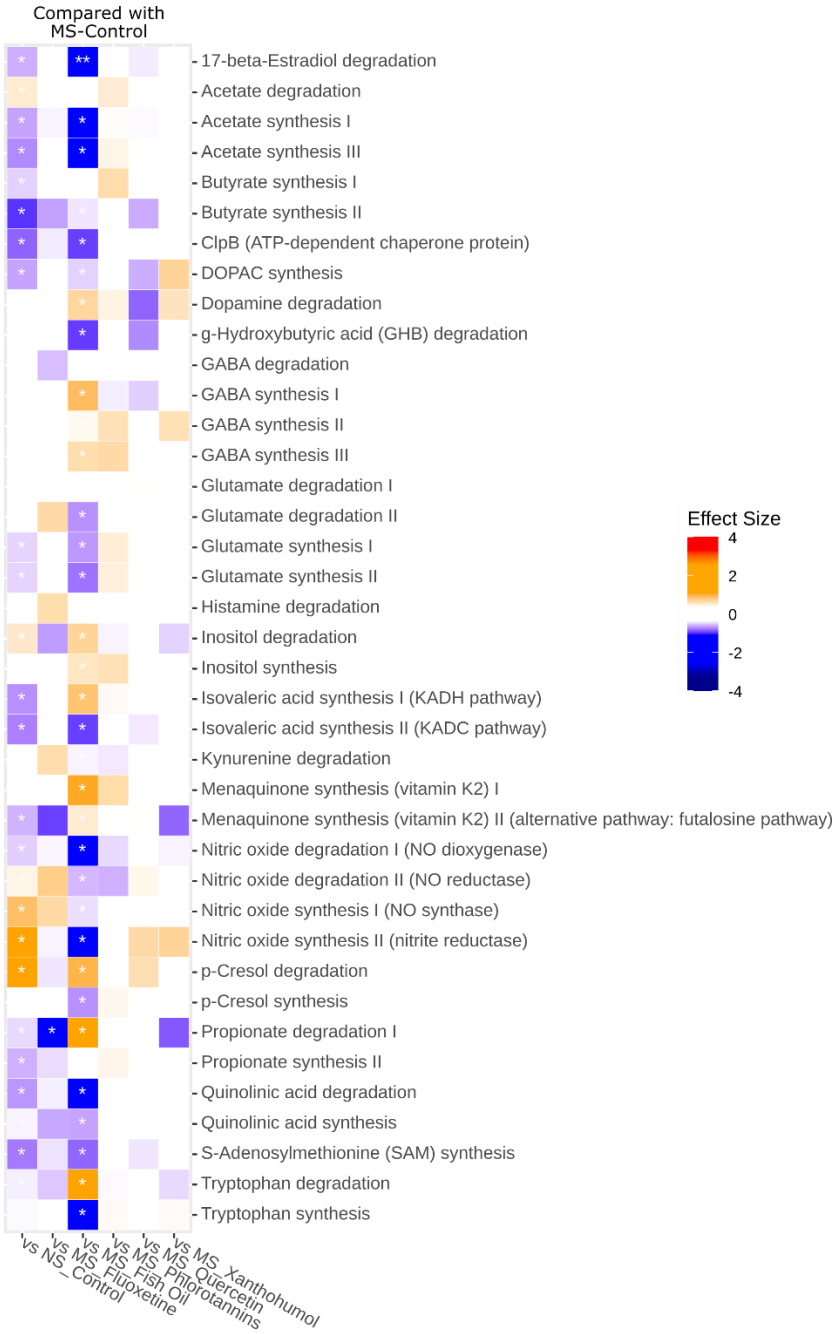


*Figure 4.2. Fish oil, but not polyphenol supplementation reshapes the predicted function of the stressed gut microbiome. Abbundance of gut-brain modules in each group compared with the MS-Control group. Effect size was measured using Cohen's D and visualised with purple indicating a higher GBM abundance in the MS-Control group and orange indicating a lower abundance in the MS-Control group of each comparison.. *q < 0.2; ** q < 0.02; *** q < 0.002;. n = 10 – 11 rats per group. Results generated from this figure are a more advanced analysis on previously published work (Donoso et al. 2020; Egerton et al. 2020).*

4.4.3 Some of the stress-induced alterations to hippocampal gene expression were reversed with various prebiotics

To further evaluate the impact maternal separation stress and dietary supplementation with polyphenols and fish oil on neuroplasticity, the hippocampal expression of several neuroplasticity genes, some of which have been shown to be sensitive to gut microbiota alterations, were measured via semiquantitative RT-PCR. Given that previous research has demonstrated that fish oil, fluoxetine, and polyphenols can impact the expression of or quantity of proteins related to neuroplasticity-related genetic markers, including those assessed herein (Vignes *et al.* 2006; Cutuli *et al.* 2014; Myhrstad *et al.* 2014; Fan *et al.* 2016; Ortiz-Lopez *et al.* 2016; Dang *et al.* 2018; Donoso *et al.* 2019), planned comparisons were made between each individual dietary treatment versus the MS-Control group.

Brain-derived neurotrophic factor (BDNF) is a key regulator of hippocampal progenitor cell proliferation, development, and survival (Vicario-Abejon *et al.* 1995; Choi *et al.* 2009) and its expression is sensitive to early life stress, although the directionality of this effect is not always consistent (Daskalakis *et al.* 2015). Furthermore, chronic treatment with the antidepressant fluoxetine has previously been shown in increase *BDNF* gene expression (Molteni *et al.* 2006). BDNF is also sensitive to the gut microbiota and BDNF protein and gene expression are reduced in the germ-free mouse hippocampus (Sudo *et al.* 2004; Diaz Heijtz *et al.* 2011; Clarke *et al.* 2013). Additionally, hippocampal BDNF exon IV is sensitive to early life stress as well as probiotic consumption (O'Sullivan *et al.* 2011). Although the gene expression of *BDNF IV* was not altered with stress or treatment (Figure 4.3), early life stress increased *BDNF* expression in the hippocampus (Fig 4.3; NS-Control vs MS-Control $t(23) = 2.423$, $p = 0.0241$). While fluoxetine was unable to significantly reduce the elevation in *BDNF* expression following early life stress ($t(22) = 1.640$, $p = 0.1151$), supplementation with fish oil ($t(22=0) = 2.585$, $p = 0.0177$) or quercetin ($t(20) = 2.767$, $p = 0.0119$), reduced this stress-induced rise in hippocampal *BDNF* expression.

Doublecortin (DCX) is expressed by immature newly born hippocampal neurons and the abundance of cells expressing DCX in the dentate gyrus is sensitive to both stress and temporal dynamics, whereby mice exposed to early life stress showed decreased *DCX* expression at 12, 16, and 21 days old, though this effect was not present from 28 to 50 days of age (Bath *et al.* 2016). Additionally, immunohistochemical experiments have found that germ-free mice have altered

hippocampal DCX cell density depending on their sex and age (Scott *et al.* 2020), displaying the sensitivity of hippocampal neurogenesis to the gut microbiota. Ki67 is an endogenous marker of cellular proliferation (Kee *et al.* 2002). Bacterial infection in the neonatal period led to an increase in hippocampal DCX and Ki67 in adulthood (Hennessey *et al.* 2021), demonstrating that perturbance to the early life gut microbiota can yield pervasive alterations in DCX and Ki67 gene expression. Herein, while there were no significant differences observed in *DCX* expression following early life stress, treatment of maternally separated rats with fish oil increased hippocampal *DCX* expression relative to the MS-Control rats ($t(20) = 2.244$, $p = 0.0363$). Furthermore, the expression of *Ki67* was significantly decreased in the hippocampus of adult rats exposed to early life maternal separation stress (Figure 4.3; NS-Control vs MS-Control $t(22) = 2.767$, $p = 0.0112$). Meanwhile, maternally separated rats treated with xanthohumol showed significantly increased hippocampal *Ki67* relative to MS-Control rats ($t(20) = 2.645$, $p = 0.0155$), and treatment with either fluoxetine ($t(21) = 1.914$, $p = 0.0693$) or quercetin ($t(20) = 1.913$, $p = 0.0702$) trended towards increasing hippocampal *Ki67* expression.

Furthermore, the expression of the $GABA_{B1}$ receptor expression in the hippocampus has exhibited sensitivity to prebiotic consumption (Burokas *et al.* 2017). The $GABA_{B1a}$ receptor subunit isoform in the hippocampus is considered to be a possible candidate for eliciting antidepressant response and stress resilience (O'Leary *et al.* 2014), potentially due to its relationship with $5\text{-}HT_{1A}$ receptor responses (Jacobson *et al.* 2017). While the expression of hippocampal $GABA_{B1A}$ was not affected by stress (Figure 4.3; NS-Control vs MS-Control $t(18) = 0.5798$, $p = 0.5692$), or treatment with fish oil or any polyphenols, treatment of MS rats with fluoxetine significantly reduced the level of $GABA_{B1A}$ expression in the hippocampus ($t(22) = 2.660$, $p = 0.0143$).

Both the Glutamate Ionotropic Receptor NMDA Type Subunit 1 (Grin1) and 2a (Grin2a) play important roles in neurodevelopment and synaptic plasticity (Platzer *et al.* 1993; Hasan *et al.* 2013; Franchini *et al.* 2020). Neither stress or treatment effected the hippocampal expression of *Grin2a*. Meanwhile, hippocampal *Grin1a* expression was significantly increased in rats exposed to early life stress (NS-Control vs MS-Control $t(22) = 3.005$, $p = 0.0065$), and this increase was not reversed by treatment with fluoxetine, fish oil, or any polyphenol supplementation.

The gene *nr3c1* encodes for a glucocorticoid receptor which can regulate neurogenesis by inhibiting the cell cycle (Shin *et al.* 2015). Indeed, hippocampal neurogenesis is reduced when this

glucocorticoid receptor is knocked down (Kronenberg *et al.* 2009). Additionally, increasing the mineralocorticoid receptor *Nr3c2* has been shown to alleviate the consequences of early life stress on hippocampal neurogenesis (Kanatsou *et al.* 2017). However, no differences were observed in hippocampal *Nr3c1* or *Nr3c2* mRNA expression following stress or dietary supplementation.

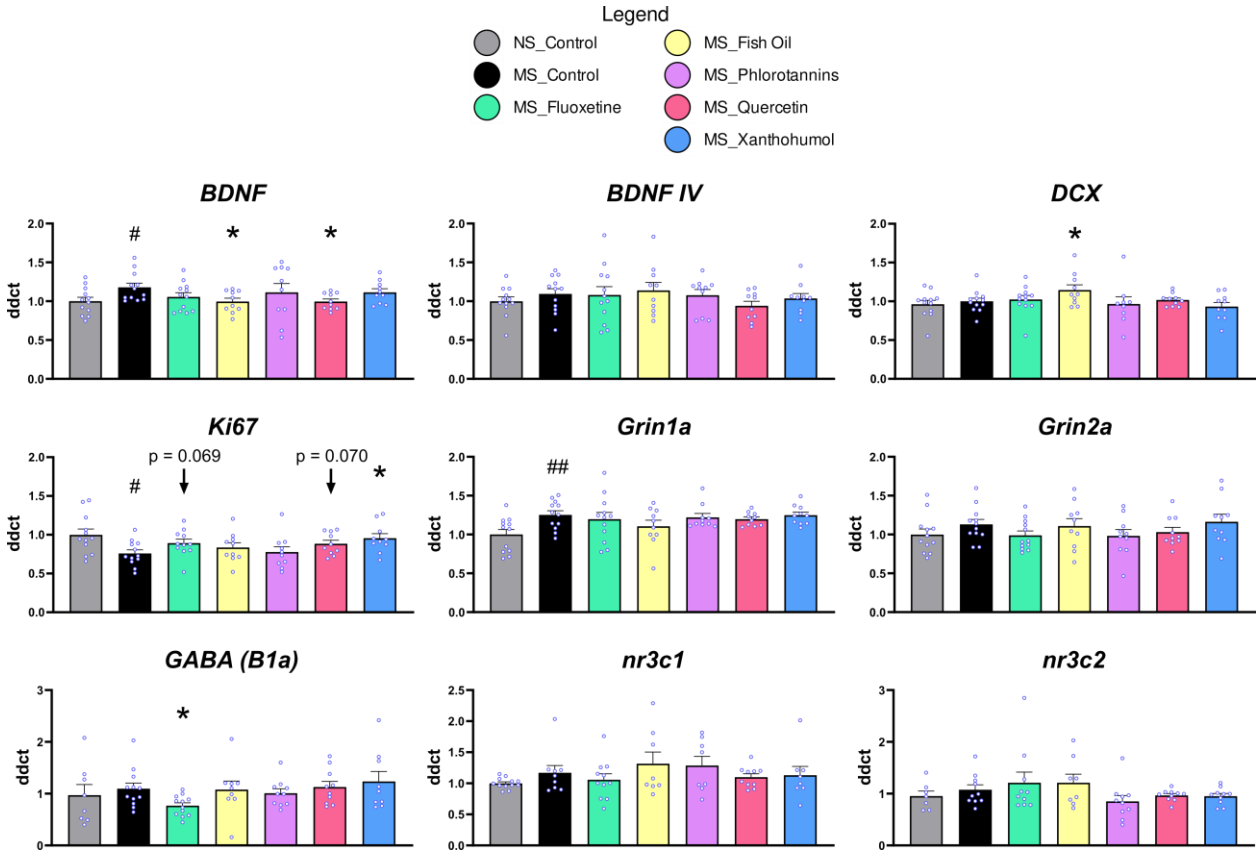


*Figure 4.3. Maternal separation stress and dietary supplementation with polyphenols alters hippocampal neuroplasticity-related gene expression. Following removal of datapoints for individuals whose triplicate RT-qPCR outcomes did not suit the inclusion criteria on a gene-by-gene basis, n = 7-12 per group, displayed as Mean ± SEM. Stress effect was determined by comparing NS-Control vs MS-Control using a t-test and is visualized as # p < 0.05. Effects of interventions on rats exposed to early life stress was determined via t-test between each intervention and the MS-contro group and significance is displayed as * p < 0.05.*

4.4.4 Alterations in the gut microbiome corelated with gene expression levels in the hippocampus

To further understand the relationship between the gut microbiome and neuroplasticity, the expression of neuroplasticity-related genes was correlated to the clr-transformed relative abundance of genera and GBMs (Fig 4.4 and 4.5). Interestingly, harbouring an increased relative abundance of *Bifidobacterium* or *Faecalibaculum* was associated with a decrease in the level of

BDNF ($r(73) = -0.384$, $q = 0.0871$ and $r(73) = -0.382$, $q = 0.0871$, respectively) and *Ki67* ($r(73) = -0.384$, $q = 0.0871$ and $r(73) = -0.382$, $q = 0.0871$, respectively) expression in the hippocampus which often indicates a reduction in pro-neurogenic signalling (Lee *et al.* 2002a). Furthermore, higher abundance of *Anaerovorax* was significantly related to increased hippocampal expression of immature neuronal marker *DCX* ($r(72) = 0.388$, $q = 0.0871$). Both $GABA_{B1A}$ and *Grin2a* significantly corelated with the abundance of *Roseburia*, albeit in opposing directions; $GABA_{B1A}$ correlates positively while *Grin2a* correlates negatively with *Roseburia* ($r(67) = 0.404$, $q = 0.0871$ and $r(71) = -0.437$, $q = 0.0643$, respectively). *Grin2a* also corelated significantly the genera *Bacteroides* ($r(71) = 0.483$, $q = 0.0183$). The hippocampal expression of *BDNF IV*, *Grin1a*, *Nr3c1*, and *Nr3c2* did not significantly corelate with any changes in microbial genera (Figure 4.4).

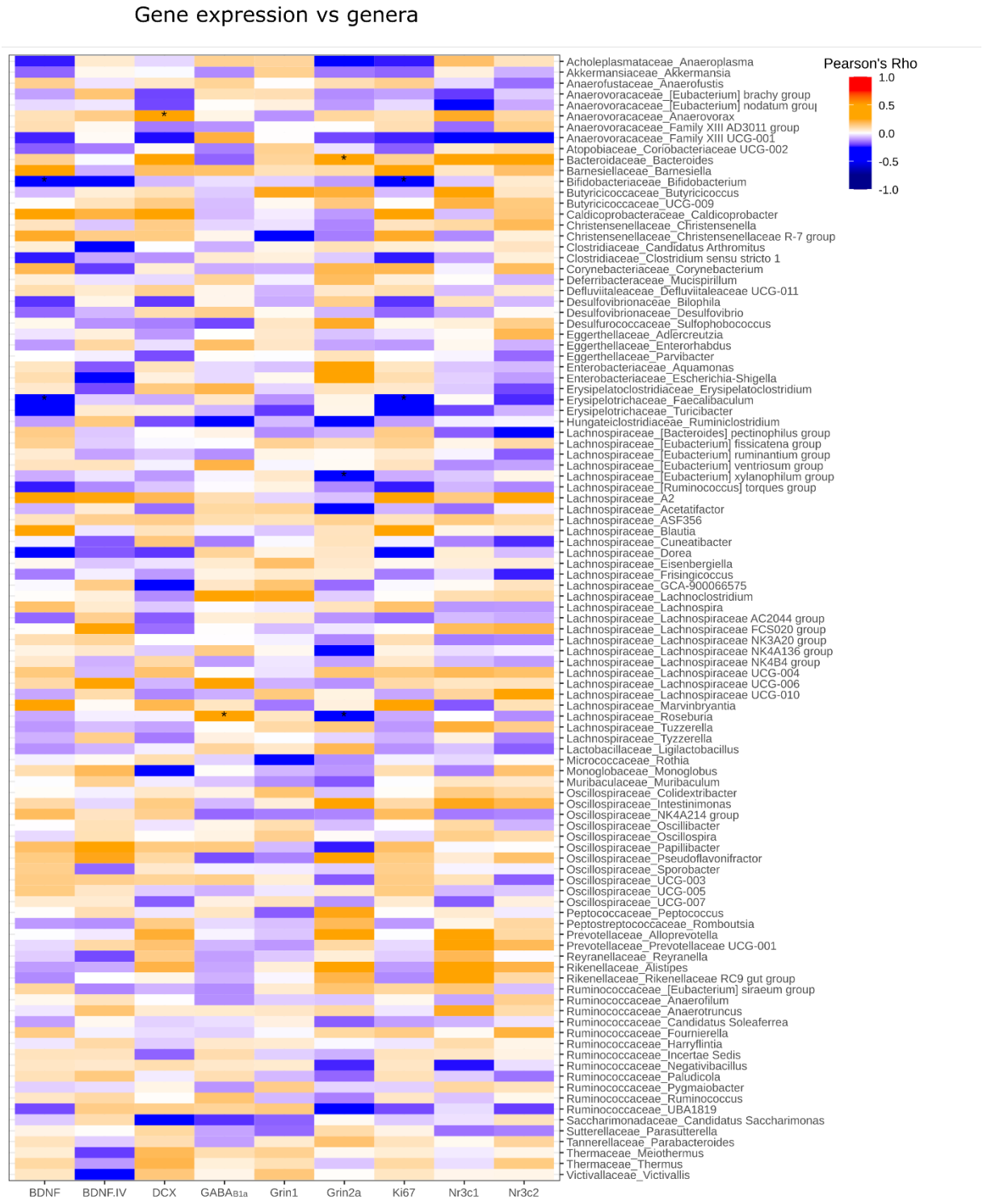


*Figure 4.4. Gene expression levels derived from RT-qPCR correlate to the abundance of specific bacterial genera in the gut. * $q < 0.05$, $n = 57$ to 73 rats per comparison.*

A similar technique was applied to assess whether corelations existed between hippocampal gene expression and predicted GBMs (Figure 4.5). However, only one correlation was strong enough

to be considered significant. Propionate Degradation I positively and significantly corelated to the expression of the hippocampal gene *Grin2a* $r(71) = 0.488$, $q = 0.0059$).

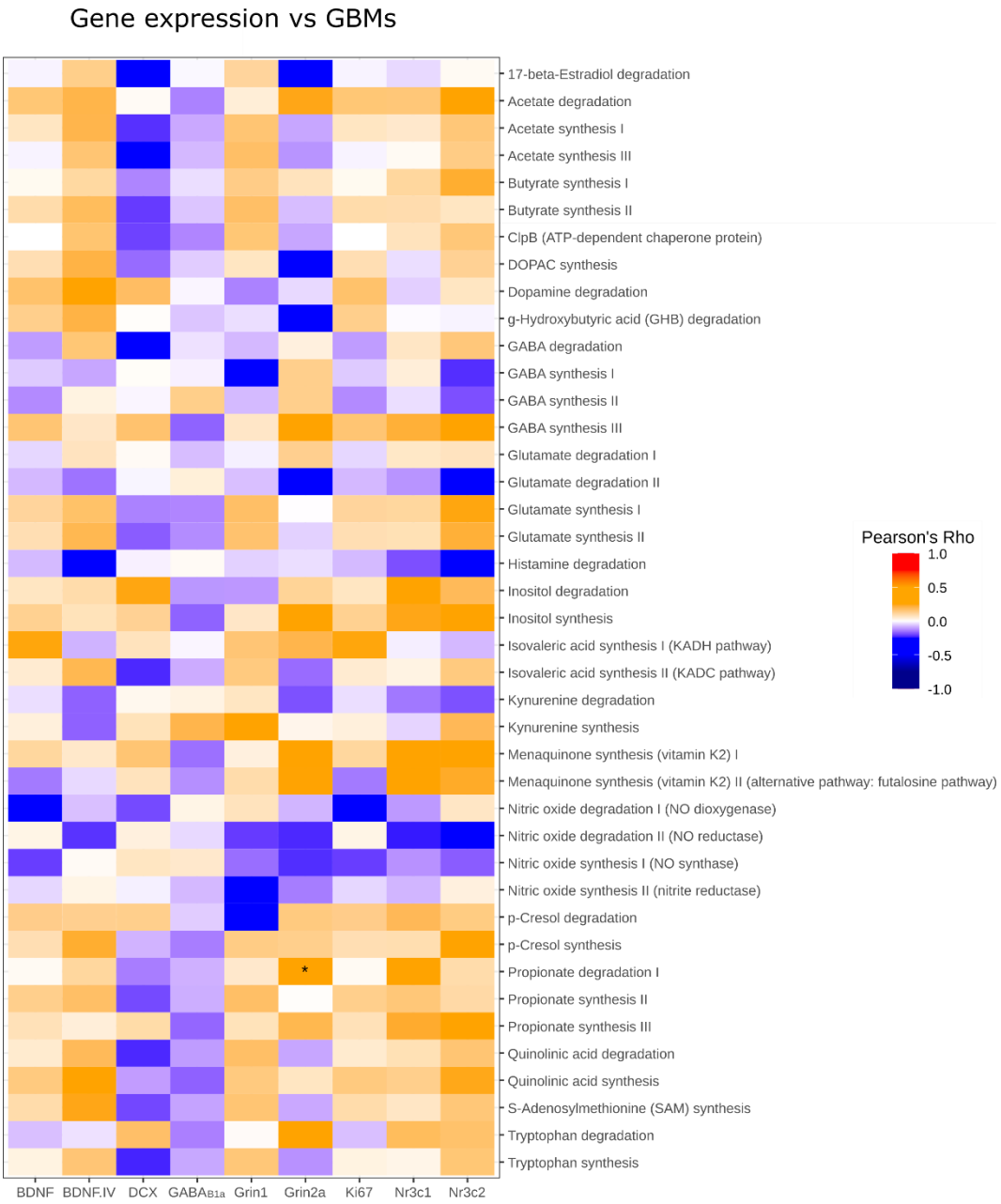


*Figure 4.5. Gene expression levels derived from RT-qPCR correlate to the abundance of specific bacterial genera in the gut. * $q < 0.05$, $n = 57$ to 73 rats per comparison.*

4.5 Discussion

The ability for the gut microbiome to contribute to or ameliorate stress-related phenotypes has been gaining increased attention (Stilling *et al.* 2015; Huo *et al.* 2017; Bastiaanssen *et al.* 2021; Berding *et al.* 2021a). Furthermore, increasing attention has been given towards better understand the potential for diet to alleviate the effects of stress (Kang *et al.* 2014; Burokas *et al.* 2017; Provensi *et al.* 2019). Indeed, previous research has found that dietary supplementation with fish oil and various polyphenols can restore behavioural alterations induced by early life stress, as well as alterations to the gut microbiome (Bayes *et al.* 2020; Donoso *et al.* 2020; Egerton *et al.* 2020), though the mechanisms underlying these effects have remained elusive. This research provides valuable insights into the relationships between early life stress, the gut microbiome, and neuroplasticity in the hippocampus, and reveals that supplementation with fish oil and some polyphenols might potentially exert their beneficial impacts on hippocampal neuroplasticity through remodelling the gut microbiome, though further research involving germ-free mice or faecal microbiota transplant would be required to fully elucidate this potential. Early life stress elicited alterations in the adult rat gut microbiome, including in the microbial diversity and relative abundance of specific genera of bacteria, and resulted in changes in the predicted functional ability of the gut microbiome in the context of gut-brain communication. Furthermore, stress during early life reduced the expression of the cell proliferation marker *Ki67* in the hippocampus of adult rats, while increasing the expression of *BDNF*, which were both corelated to the abundance of *Bifidobacterium* or *Faecalibaculum* in the gut microbiome, albeit in the same, negative direction. Supplementation with fish oil showed a remarkable ability to alter the gut microbiome and its predicted function, as well as reverse the early life stress-induced elevation in *BDNF* expression in the adult hippocampus.

Exposure to early life stress induced distinctions in the abundance of multiple predicted gut-brain modules between the NS-Control and MS-Control groups, including predicted increased synthesis of short-chain fatty acids acetate, butyrate, and propionate in rats subjected to early life stress and decreased acetate degradation in in rats subjected to early life stress (Figure 4.2). It is counterintuitive that we observed an increase in the predicted function of the gut microbiota to produce acetate while previously research demonstrated that acetate is reduced in the gut of adult rats exposed to early life maternal separation stress (Donoso *et al.* 2020). Perhaps the reduction in

local acetate levels facilitates the growth of bacteria which are capable of producing acetate to fill this gap, although further research would need to be conducted to investigate this phenomenon. Short chain fatty acids are a crucial component of the microbiota-gut-brain axis. *In vitro*, the administration of short-chain fatty acids at physiological levels to neural stem cells was sufficient to induce neurogenic differentiation (Ribeiro *et al.* 2020; Yang *et al.* 2020). Furthermore, gut-derived acetate has been shown to translocate to the brain, where it can influence the function of microglia (Erny *et al.* 2021) which are important regulators of hippocampal neurogenesis (Diaz-Aparicio *et al.* 2020). Early life separation stress has previously been shown to impair the development and function of hippocampal microglia (Delpech *et al.* 2016), so perhaps it might be that stress-induced changes in the gut microbiome's capacity to alter acetate levels may contribute to microglia impairments following stress, which in turn could impact neuroplasticity. However, the role of gut microbiota-derived acetate in facilitating hippocampal microglia disruption, neuroplasticity, and associated behaviour during stress has not yet been observed.

Fascinatingly, supplementation of fish oil to maternally separated rats reversed this predicted functional difference in acetate production in the gut microbiome. While previous research has not shown an effect of fish oil on acetate in the gut of adult rats exposed to stress (Egerton *et al.* 2020), this alteration in the predicted function of the gut microbiome may foreshadow other mechanisms by which fish oil exerts its beneficial effects on host hippocampal neuroplasticity. Moreover, acetate synthesis capability negatively corelated with the expression of hippocampal immature neuronal marker *DCX*, albeit this effect was not statistically significant. Fish oil supplementation to tars exposed to early life stress resulted in an increase in hippocampal *DCX* expression. Therefore, it would be interesting to further explore the links between stress-induced alterations in microbially produced acetate, hippocampal microglia functionality, and hippocampal neurogenesis-related behaviour in stressed animals.

Interestingly, the GBM Propionate Degradation I significantly and positively correlated with the expression of *Grin2a* in the hippocampus (Figure 4.4). *Grin2a* encodes for the neuronally expressed protein GluN2A, which is a component a subset of NMDA receptors which are critical for regulating structural and synaptic plasticity in the hippocampus (McEwen & Magarinos 2001; Bliss & Collingridge 2013) as well as microglia proliferation, including during stress (Nair & Bonneau 2006). Since microglia are crucial mediators of hippocampal neurogenesis and

neuroplasticity (Diaz-Aparicio *et al.* 2020), this research indicates that the gut microbiota may be able to influence hippocampal microglia, and potentially subsequently hippocampal plasticity, through propionate-related and NMDA-related mechanisms, although this link needs to be further researched.

Several bacteria significantly correlated with the expression of neuroplasticity-related genes in the hippocampus. For instance, *Bifidobacterium* and *Faecalibaculum* were associated with a decrease in the level of *BDNF* and *Ki67* expression in the hippocampus. BDNF is a neurotrophic factor that supports the production of new neurons in the brain (Lee *et al.* 2002a), and was found to be counterintuitively increased in MS-Control rats, perhaps indicating the host is responding to a deficit in newly born neurons and is attempting to counteract this. This suggests that *Bifidobacterium* and *Faecalibaculum* might be potential novel probiotic targets for alleviating the stress-induced impairments in hippocampal neuroplasticity, although hesitation is warranted given that under non-stressed conditions, increased expression of both BDNF and Ki67 have been associated with increased hippocampal neurogenesis (Waterhouse *et al.* 2012; Mathews *et al.* 2017). Indeed, a specific strain of *Bifidobacterium breve* has been shown to reduce depressive-like behaviour preclinically, as well as symptoms of major depressive disorder in diagnosed patients (Tian *et al.* 2019a; Tian *et al.* 2021). Furthermore, a higher abundance of *Anaerovorax* was significantly related to increased hippocampal expression of immature neuronal marker *DCX*. While no effects of stress were observed on *DCX* expression in the hippocampus, this suggests that *Anaerovorax* may be able to increase hippocampal neurogenesis following stress, although further research involving *Anaerovorax* supplementation would need to be conducted to determine this relationship herein.

There appears to be critical windows in early life during which the brain, gut microbiome, and microbiota-gut-brain axis exhibit increased sensitivity to external stimuli, including stress. For instance, studies have found that stress during the first two weeks of life in rodents yields pervasive effects on hippocampal neurogenesis, and depressive-like and anxiety-like behaviour in adulthood (Loi *et al.* 2014). However, recent research has shown that stress during the timeframe of puberty did not induce consequential outcomes on adult behaviour or hippocampal neurogenesis (Harris *et al.* 2022). Similarly, the microbiota-gut-brain axis exhibits time-sensitivity during early life, especially around the period of weaning (Al Nabhani *et al.* 2019). Delaying the weaning window

was sufficient to alter immunological features in rodents which lasted into adulthood (Al Nabhani *et al.* 2019). Therefore, it appears that there is a critical time period for which stress-induced alterations may lead to pervasive changes in the gut microbiome. Perhaps interventions targeting the gut microbiome during this critical window, and instead of during adulthood, may facilitate stronger effects on hippocampal neuroplasticity than those observed in this study (Bath *et al.* 2016).

Notably, these experiments were only conducted in male rats. Biological sex is a critical factor influencing the susceptibility of an individual to stress. Research has shown that women have increased prevalence of depression throughout their lifetime (Weissman *et al.* 1993; Kessler *et al.* 2005), which may be due to differences in vulnerability to early life stress-related insults, including in the hippocampus (Loi *et al.* 2014). For instance, in male rats, maternal separation stress during early life led to a short-term increase in prepubertal hippocampal neurogenesis which was followed by a reduction in hippocampal neurogenesis during adulthood (Loi *et al.* 2014). Meanwhile, female rats exposed to the same early life stress displayed disrupted prepubertal hippocampal neurogenesis, although this effect appeared to normalize in adulthood (Loi *et al.* 2014). These sex-specific differences in the temporal dynamics of hippocampal neurogenesis following early life stress may underly differences in the severity of depressive-like behaviours observed in mouse models of early life stress (Goodwill *et al.* 2019). Furthermore, biological sex has been implicated as a factor in the composition of the gut microbiome, especially in early life (Cong *et al.* 2016; Nagpal *et al.* 2017; Jaggar *et al.* 2020). Therefore, it is essential that future research investigate the impacts of dietary supplementation with fish oil and polyphenolic compounds on the stressed female microbiome and brain.

Overall, this research highlights the ability of dietary prebiotics to remodel the gut microbiome and its predicted functionality following early life stress. Additionally, by investigating changes in stress-sensitive neuroplasticity-related makers in the hippocampus and corelating these findings to alterations in the gut microbiome, this research provides novel insights into potential novel probiotics that may be able to rescue deficits in hippocampal plasticity. Future research should examine whether these dietary interventions induce differences in functional plasticity readouts within the hippocampus, including long-term potentiation, and examine hippocampal neurogenesis via immunohistochemical analysis, and assess additional genes related to

hippocampal neuroplasticity, such as the receptor for BDNF and other neurotrophic factors, such as VEGF to broaden the current findings that early life stress and specific dietary interventions can induce changes in key genes related to hippocampal neurogenesis. Moreover, clinical research should be conducted to investigate the translational relevance of these findings.

4.6 Supplemental material

Supplemental Table 4.1: *Statistical significance of treatment on GBMs. Q indicates Storey's Q value. Effect size is in terms of Cohen's D. The threshold for significance was set to q = 0.2. Bolded q value highlights a significant interaction.*

	MS_Control vs NS_Control		MS_Control vs MS_Fluoxetine		MS_Control vs MS_Fish Oil		MS_Control vs MS_Phlorotannins		MS_Control vs MS_Quercetin		MS_Control vs MS_Xanthohumol	
GBM	Q	Effect Size	Q	Effect Size	Q	Effect Size	Q	Effect Size	Q	Effect Size	Q	Effect Size
p-Cresol synthesis	0.25	-0.11	0.36	-0.29	**0.08**	-0.70	0.27	0.42	0.94	0.08	0.99	0.05
Inositol synthesis	0.26	0.08	0.36	-0.19	**0.11**	0.56	0.27	0.59	0.97	0.03	0.99	-0.02
p-Cresol degradation	**0.09**	1.13	0.32	-0.44	**0.05**	0.95	0.27	0.33	0.90	0.61	0.99	0.33
Inositol degradation	**0.14**	0.53	0.27	-0.67	**0.08**	0.71	0.27	-0.40	0.90	0.27	0.99	-0.50
g-Hydroxybutyric acid (GHB) degradation	0.26	0.09	0.36	0.20	**0.05**	-0.97	0.30	-0.21	0.90	-0.73	0.99	-0.29
Kynurenine degradation	0.20	-0.28	0.27	0.63	**0.16**	-0.40	0.27	-0.43	0.90	-0.21	0.99	-0.02
Quinolinic acid degradation	**0.13**	-0.69	0.32	-0.41	**0.03**	-1.11	0.27	0.33	0.90	-0.36	0.99	0.04
Propionate synthesis III	0.24	0.16	0.40	0.05	0.26	0.22	0.27	0.44	0.97	0.04	0.99	0.26
Isovaleric acid synthesis I (KADH pathway)	**0.13**	-0.71	0.36	0.32	**0.06**	0.83	0.27	0.39	0.93	0.14	0.99	0.00
Propionate degradation I	**0.14**	-0.47	**0.15**	-1.21	**0.03**	1.22	0.30	0.22	0.94	-0.09	0.99	-0.89
Isovaleric acid synthesis II (KADC pathway)	**0.13**	-0.76	0.36	-0.30	**0.04**	-0.96	0.30	0.25	0.90	-0.43	0.99	0.25
S-Adenosylmethionine (SAM) synthesis	**0.13**	-0.78	0.32	-0.45	**0.06**	-0.84	0.27	0.36	0.90	-0.44	0.99	0.00
Glutamate degradation II	0.22	-0.21	0.27	0.65	**0.08**	-0.70	0.28	-0.31	0.97	0.01	0.99	-0.16
Butyrate synthesis I	**0.14**	-0.50	0.36	-0.20	0.33	-0.08	0.27	0.64	0.90	-0.35	0.99	-0.16
17-beta-Estradiol degradation	**0.13**	-0.61	0.36	-0.16	**0.01**	-1.90	0.36	0.09	0.90	-0.42	0.99	0.30
Butyrate synthesis II	**0.10**	-1.00	0.27	-0.65	**0.15**	-0.44	0.36	0.09	0.90	-0.62	0.99	-0.26
Histamine degradation	0.26	-0.05	0.27	0.63	**0.18**	0.37	0.27	-0.35	0.97	-0.01	0.99	-0.02
Quinolinic acid synthesis	**0.17**	-0.40	0.27	-0.63	**0.09**	-0.65	0.27	0.34	0.93	-0.14	0.99	0.05
Propionate synthesis II	**0.13**	-0.61	0.32	-0.47	0.37	0.01	0.27	0.43	0.90	-0.33	0.99	0.01
Glutamate degradation I	0.20	-0.28	0.36	0.19	0.33	0.07	0.30	-0.22	0.90	0.37	0.99	-0.35
GABA degradation	**0.18**	-0.35	0.32	-0.56	0.27	0.20	0.39	-0.02	0.93	-0.15	0.99	-0.23
Nitric oxide synthesis II (nitrite reductase)	**0.07**	1.34	0.32	-0.40	**0.03**	-1.35	0.39	-0.05	0.90	0.65	0.99	0.69
Kynurenine synthesis	0.23	-0.18	0.40	0.07	0.24	0.25	0.36	-0.11	0.90	0.28	0.99	0.17
Tryptophan synthesis	**0.17**	-0.38	0.36	-0.14	**0.02**	-1.44	0.27	0.39	0.90	-0.25	0.99	0.39
Nitric oxide degradation I (NO dioxygenase)	**0.14**	-0.51	0.32	-0.39	**0.03**	-1.20	0.27	-0.48	0.90	0.21	0.99	-0.40
Tryptophan degradation	**0.17**	-0.41	0.32	-0.54	**0.03**	1.10	0.27	-0.37	0.90	0.24	0.99	-0.47
Glutamate synthesis I	**0.14**	-0.49	0.35	-0.35	**0.08**	-0.68	0.27	0.49	0.90	-0.32	0.99	0.07
Nitric oxide degradation II (NO reductase)	**0.16**	0.45	0.27	0.74	**0.10**	-0.58	0.27	-0.61	0.90	0.42	0.99	-0.33
Glutamate synthesis II	**0.14**	-0.50	0.36	-0.25	**0.06**	-0.80	0.27	0.47	0.90	-0.30	0.99	0.23
ClpB (ATP-dependent chaperone protein)	**0.13**	-0.85	0.32	-0.42	**0.04**	-0.96	0.36	0.08	0.90	-0.29	0.99	-0.04
GABA synthesis III	0.24	0.14	0.36	-0.15	**0.09**	0.62	0.27	0.66	0.94	0.12	0.99	-0.01
Dopamine degradation	**0.18**	0.32	0.36	0.28	**0.09**	0.68	0.27	0.45	0.90	-0.85	0.99	0.57
Acetate synthesis III	**0.13**	-0.73	0.36	-0.22	**0.03**	-1.20	0.27	0.44	0.90	-0.27	0.99	0.03
DOPAC synthesis	**0.13**	-0.65	0.40	-0.07	**0.14**	-0.50	0.30	0.21	0.90	-0.62	0.99	0.69
Acetate degradation	**0.14**	0.52	0.36	-0.19	0.27	-0.19	0.27	0.50	0.97	0.03	0.99	0.21
Nitric oxide synthesis I (NO synthase)	**0.13**	0.86	0.27	0.66	**0.14**	-0.47	0.29	-0.28	0.90	0.21	0.99	-0.07
Menaquinone synthesis (vitamin K2) I	0.24	0.16	0.38	-0.11	**0.03**	1.05	0.27	0.64	0.94	0.09	0.99	-0.04
Menaquinone synthesis (vitamin K2) II (alternative pathway: futalosine pathway)	**0.13**	-0.60	0.27	-0.96	**0.13**	0.51	0.29	-0.27	0.90	0.37	0.99	-0.85
GABA synthesis I	**0.18**	0.35	0.36	-0.15	**0.05**	0.88	0.27	-0.42	0.90	-0.51	0.99	-0.19
GABA synthesis II	0.22	0.22	0.36	-0.15	**0.16**	0.41	0.27	0.60	0.93	0.16	0.99	0.60
Acetate synthesis I	**0.13**	-0.65	0.32	-0.40	**0.03**	-1.12	0.27	0.38	0.90	-0.38	0.99	0.07

Chapter 5

Sex differences in the effects of caesarean section birth on neurogenesis in the juvenile hippocampus

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In Preparation

5.1 Abstract

Early life is a critical time period during which the brain undergoes immense structural and biological changes that prime the baby for future health. Specifically, the hippocampus, a key brain region supporting cognitive and emotional health, continues to rapidly develop during the postnatal weeks, evidenced by the birth, development, and migration of new neurons. In parallel, the gut microbiota is highly dynamic during early life and starts to stabilize around the period of weaning, which, in mice, is postnatal day 21. Research involving germ-free mice and microbiota manipulations has shown that the gut microbiota plays a crucial role in shaping host neurobiological processes, including hippocampal neurogenesis, although colonization of germ-free mice post-weaning did not alter these outcomes. Therefore, disruption to the gut microbiota during early life may perturb neurodevelopment of the host. Previous research has shown that babies born by caesarean section have distinctly altered gut microbiota, which persists into infancy. Furthermore, mice born by caesarean section display altered hippocampal physiology, although the effect of birth mode on hippocampal neurogenesis has not yet been investigated. Caesarean section birth typically requires the administration of antibiotics to the mother post-partem, which may yield currently undescribed impacts on offspring health and neurodevelopment when coupled with caesarean section birth. Herein, using mice we explored the consequences of birth mode, coupled with exposure to early life antibiotics, on hippocampal neurogenesis at postnatal day 21. We found that caesarean section birth led to sex-specific and region-specific differences in the number of doublecortin cells in the dentate gyrus. Female but not male offspring born by caesarean section had a significantly higher number of immature neurons in the dorsal dentate gyrus, although coupling caesarean section with early life maternal antibiotic consumption increased dorsal hippocampal neurogenesis independent of the sex of the offspring. Further studies are needed to understand the mechanisms underlying these sex differences.

5.2 Introduction

The perinatal period is a crucial window for neurodevelopment, during which the brain undergoes immense structural and biological changes that prime the baby for future health (Cowan et al. 2020). Within the rodent hippocampus, the dentate gyrus takes shape during late embryogenesis, though approximately 85% of dentate granule cells are produced postnatally (Bayer 1980; Rakic & Nowakowski 1981; Huang 2014). During the first weeks following birth, a substantial amount of neuronal birth, maturation, and migration continues to occur in the hippocampus (Altman & Bayer 1990a; Gould & Cameron 1996), a process known as hippocampal neurogenesis. In rodents, the generation of new granule cells peaks around the postnatal day (P) 10 (Bayer 1980; Altman & Bayer 1990a; Piatti *et al.* 2006) before tapering off around P20 (Angevine 1965) and neural stem cells are highly abundant until P14 (Malatesta *et al.* 2000), while synaptogenesis continues for several weeks after (Seress *et al.* 2001). Furthermore, there is a clear separation in the physiology and function of the adult hippocampus along its longitudinal axis (O'Leary & Cryan 2014; Levone *et al.* 2021a; Levone *et al.* 2021b). The dorsal hippocampus is widely understood to be primarily involved in spatial learning and memory (Moser *et al.* 1995; Ferbinteanu & McDonald 2001; Pothuizen *et al.* 2004), while the ventral hippocampus is implicated in modulating the stress response and anxiety-like behaviour (Kim & Fanselow 1992; O'Leary & Cryan 2014; Levone *et al.* 2021a; Levone *et al.* 2021b).

The hippocampus is one of two brain regions, along with the olfactory bulb, in which neurogenesis occurs not only during early postnatal development, but also continues through adolescence and during adulthood (Klempin & Kempermann 2007). In adulthood, ablation of hippocampal neurogenesis impacts spatial learning and memory, pattern separation, cognitive flexibility, stress response, and response to antidepressant drugs (Deng *et al.* 2009; Sahay *et al.* 2011; Snyder *et al.* 2011; Surget *et al.* 2011; Marin-Burgin & Schinder 2012). However, early life insults, such as maternal separation stress, have been shown to leave lasting impacts on hippocampal neurogenesis, in a sex-specific manner, whereby male rats exposed to early life stress displayed increased hippocampal neurogenesis until puberty which declines in adulthood, and female rats displayed a reduction in hippocampal neurogenesis that subsides in adulthood (Loi *et al.* 2014). Therefore, disturbances which disrupt hippocampal neurogenesis during early hippocampal development may in turn have functional consequences on an individual's anxiety, cognition, and

stress regulation in a sex-specific manner (Mirescu *et al.* 2004; Loi *et al.* 2014; Kozareva *et al.* 2019a).

In parallel to hippocampal development, the gut microbiome undergoes immense change during early life. Bacterial colonization of the gut is believed to begin at birth with bacteria classified under the families *Bifidobacteriaceae*, *Enterobacteriaceae*, and *Clostridiaceae* largely dominating the gut shortly after birth (Yatsunenko *et al.* 2012; Bokulich *et al.* 2016; Chu *et al.* 2017). At weaning, commonly defined as postnatal day 21 (P21) in mice, there is a rapid expansion in the gut microbiome, resulting in critical microbial priming of the host immune system (Al Nabhani *et al.* 2019), which may influence hippocampal neurogenesis later in life (Ziv *et al.* 2006). Literature examining the human developing gut microbiome has shown that quickly following the introduction of solid food (weaning), the diversity of species within the gut microbiome resembles that of adults, although it is still unique in the abundance of specific bacterial taxa (Koenig *et al.* 2011; Yatsunenko *et al.* 2012; Hollister *et al.* 2015). However, the developing gut microbiome is highly sensitive to perturbations. For instance, caesarean section (CS) birth dramatically alters the initial colonization of bacteria in the baby, which leads to the gut microbiome more closely resembling the mother's skin rather than the mother's vaginal microbiome, which is standard following vaginal birth (Dominguez-Bello *et al.* 2010). Furthermore, colonization of the early life gastrointestinal system by key beneficial microbes, including Bifidobacterium, is blocked in CS-born infants (Shao *et al.* 2019). The fingerprint of CS birth on the gut microbiome is apparent in humans until infancy (Bokulich *et al.* 2016; Shao *et al.* 2019), and differences in relative abundance of certain bacterial taxa have been observed in rodent models into adulthood, though diversity of the murine gut microbial community appears to normalize after weaning, which, in rodents, occurs around P21 (Hansen *et al.* 2014; Morais *et al.* 2020). Interestingly, shifting the weaning period to postnatal day 15 (P15) in mice, rather than the common weaning timepoint P21, lead to fewer cells proliferating in the dentate gyrus of male, but not female, mice at 3 weeks of age (Kikusui & Mori 2009). All of this evidence suggests that the weaning period is an important time of life, contributing to the development of the gut microbiota and hippocampal neurogenesis.

Accumulating research suggests that the gut microbiota plays a crucial role in shaping host neurobiological processes including adult hippocampal neurogenesis. In 2015, research involving germ-free male Swiss Webster mice, which live in sterile isolators and have never been in contact

with microbiota, showed that the absence of microbes increased the survival of newly born neurons in the adult hippocampus (Ogbonnaya *et al.* 2015). Neurogenesis in the dorsal hippocampus was particularly responsive to the absence of microbiota, while the ventral hippocampus was unperturbed by the germ-free status of the mice (Ogbonnaya *et al.* 2015). Additional research has since shown that differences in hippocampal neurogenesis of C57Bl/6J germ-free mice were specific to the sex and age of the mouse. Specifically, while male C57Bl/6J germ-free mice showed a reduction in the density of immature hippocampal neurons at 4 weeks of age compared to conventional male C57Bl/6J mice, this difference was not apparent at later ages (Scott *et al.* 2020). Meanwhile, when compared to conventional female C57Bl/6J mice, germ-free female C57Bl/6J mice displayed an increase in the density of immature hippocampal neurons at 8 weeks of age, but not at 4 or 12 weeks, highlighting that the microbiota influences hippocampal neurogenesis in a sex and time specific manner (Scott *et al.* 2020). Intriguingly, bacterial colonization of germ-free male Swiss Webster mice after the time of weaning (P21) was not able to revert the differences observed in adult hippocampal neurogenesis (Ogbonnaya *et al.* 2015), indicating microbially-induced alterations in hippocampal neurogenesis are programmed prior to the period of weaning. In addition to alterations in hippocampal neurogenesis, alterations in several plasticity related genes or genes that regulate hippocampal neurogenesis have been reported in the hippocampus of germ-free mice or rodents whose gut microbiota has been perturbed by probiotics, bacterial transplantation, or prebiotics, including genes encoding for brain-derived neurotrophic factor (BDNF), neurotransmitter receptors (such as $GABA_{B1b}$ and $GABA_{Aa2}$), glucocorticoid receptor, and the Nr1 receptor subunit for N-methyl-D-aspartate (Bravo *et al.* 2011b; Diaz Heijtz *et al.* 2011; Clarke *et al.* 2013; Luczynski *et al.* 2016; Spichak *et al.* 2018; Mao *et al.* 2020). While the germ-free model is useful for understanding the impacts microbes have on their hosts, germ-free rodents are not a translationally relevant model for interrogating the effects of early life gut microbiota disruption on the developing brain.

While adult germ-free mice display numerous alterations in their behaviour and hippocampal gene expression (Luczynski *et al.* 2016; Spichak *et al.* 2018), it is difficult to discern whether disruptions to hippocampal neurogenesis are due to the neurodevelopmental consequences of being reared without the presence of microbes or are compensatory effects secondary to other physiological changes that result, such as immaturity of the immune system. Indeed, germ-free mice have a highly underdeveloped immune system (Spichak & Guzzetta *et al.* 2018), making them not the

most translationally relevant model for interrogating host-immune interactions. On the other hand, the CS mouse model presents itself as a more clinically relevant model for investigating the impact of early life microbiota perturbation on neurodevelopment.

Interestingly, CS birth has been shown to alter the hippocampal transcriptome (Morais *et al.* 2020), and reduce glutamate mGlu1α receptor expression, a receptor previously shown to be sensitive to changes in the gut microbiota (Luo *et al.* 2018; Zuena *et al.* 2021), as well as the binding affinity of mineralocorticoid receptors (Boksa *et al.* 1996) in the hippocampus of male adult offspring, but these studies did not investigate the impact in female offspring. CS also blunts the neonatal production of various circulating cytokines (Malamitsi-Puchner *et al.* 2005), which may influence microglia in the hippocampus (Sei *et al.* 1995), which in turn play a role in the regulation of hippocampal neurogenesis (Kozareva *et al.* 2019a). Yet, whether birth mode impacts hippocampal neurogenesis at any stage of life is currently unknown. Moreover, CS is often accompanied by maternal prepartum or postpartum treatment with antibiotics (Dierikx *et al.* 2021). Previous literature has shown that antibiotic consumption in adult female C57Bl/6 mice decreases hippocampal neurogenesis (Mohle *et al.* 2016). However, the impact of early life antibiotic exposure, and in combination with CS birth, on hippocampal neurogenesis at any stage of life is not yet known.

Therefore, the aim of this study was to examine hippocampal neurogenesis and the gene expression of neuroplasticity markers in mice born vaginally or by CS with or without exposure to antibiotics. The P21 timepoint was chosen as the timepoint for assessment as this is a crucial period for juvenile brain development when gut microbiome is rapidly developing as mice wean onto solid food. The maturation of the gut microbiome during weaning is also crucial for the proper development of the immune system (Al Nabhani *et al.* 2019), which is one of the mechanisms by which the gut microbiome regulates hippocampal neurogenesis. Moreover, previous studies have found that microbial colonization of germ-free mice after P21 was not sufficient to reverse the alternations in adult hippocampal neurogenesis (Ogbonnaya *et al.* 2015), suggesting that P21 or earlier is a key time window during which the gut microbiome influences hippocampal neurogenesis. Translationally, this early life timeframe around weaning appears to be crucial for the future success of an infant; two studies examining the impacts of antibiotic exposure in early life indicate that exposure to antibiotics during the first year of life, but not later, negatively impacts

future cognitive development (Slykerman *et al.* 2017; Slykerman *et al.* 2019), making this early life window an important timeframe for understanding how early life perturbance to the gut microbiome influences ongoing neurodevelopment. To address whether biological sex plays a role in offspring hippocampal neurogenesis following CS birth, hippocampal neurogenesis was examined in both male and female P21 offspring.

5.3 Methods

5.3.1 Animals

All experimental procedures involving animals were approved by the Ethics Committee of University College Cork AEEC #2018-016 and conducted under HPRA project authorization AE19130/P103 in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposes. NIH Swiss mice were used due to their attentiveness as mothers, compared to other strains of mice (Champagne *et al.* 2007). Female and male NIH Swiss mice were ordered from Envigo UK (7-8 weeks), group-housed by sex upon arrival in the Biological Services Unit and allowed to acclimate for at least one week prior to breeding. All animals were kept under a 12:12-h light-dark cycle with controlled temperature and humidity (20 ± 1°C, 55.5%), with food and water provided *ad libitum*.

5.3.2 Caesarean-section surgery

Mice were time-mated, and females were observed daily for a vaginal mucus plug. Upon discovery of a mucus plug, the individual female was removed and single-housed, and plug date was recorded as gestational day 0.5 (G0.5). If no plug was observed, the male and female were kept together for up to 2 weeks prior to removal of the male. At full term (G19.5), pregnant females were euthanized via cervical dislocation. To minimize any potential contamination, the dam positioned on sterile gauze supinely, the abdominal cavity sprayed with 70% ethanol, and incised using sterilized surgical instruments. The uterine horn was then removed and placed onto sterile gauze atop a heating pad to prevent hypothermia of the foetuses within the uterus. Pups were removed by incising the uterine horn and gently releasing the pup using sterile cotton swabs. The umbilical cord was cut, and pups were massaged using sterile cotton swabs until self-regulated breathing was observed.

Caesarean-sectioned pups were given to a foster dam that had given birth the same day. To minimize disturbance to the dam by olfactory cues, the pups were placed in a sterile container and gently mixed with bedding material from the cage of the foster dam. The natural litter of the foster dam was removed, and the caesarean-sectioned pups (CS) placed in their nesting area Some pregnant females were left to deliver vaginally born litters (VB). To control for any potential

effects of cross-fostering on the CS pups, additional vaginally born litters were cross-fostered (CF) at postnatal day 0 (P0). To mimic the common clinical administration of antibiotics to a mother following CS birth, an additional group of foster dams with CS litters were treated with antibiotics (see section 5.3.4) in the drinking water during P2 to P9 (CS ABX). All litters were randomly reduced to a maximum of n = 10 pups at P2 to minimize litter size variance as a potential confounder.

5.3.3 Cross-fostering

Vaginally born P0 litters were cross-fostered by a dam that had given birth on the same day to control for potential time-sensitive differences in milk nutritional properties and to increase likelihood of acceptance of new pups by the foster dam. Similar to the CS pups, to minimize potential disturbance to the dam by olfactory cues, the vaginally born litter was first placed in a sterile container and gently mixed with bedding material from the cage of the foster dam. The litter of the foster dam was removed, and the new litter was gently placed in their previous nesting area. All litters were randomly reduced to a maximum of n = 10 pups on P2 to minimize litter size variance as a potential confounder.

5.3.4 Antibiotic administration

Antibiotics are commonly administered prophylactically to mothers who undergo CS surgery to prevent postpartum infection or other post-surgical complications (Williams *et al.* 2021). Furthermore, these antibiotics, including penicillin, are considered safe for breastfeeding infants, and mothers are encouraged to continue breastfeeding throughout the course of their antibiotic treatment (Kaiser *et al.* 2007; Williams *et al.* 2021). Furthermore, penicillin is the most commonly administered drug to children globally (Leclercq *et al.* 2017). To understand the impact of exposure to antibiotics following CS, the foster dams of an additional group of CS litters were treated with Penicillin V in their drinking water from P2 until P9 (CS ABX). Antibiotic solutions were diluted in drinking water to 100 mg/L, or an estimated 31 mg/kg/day, in line with previous literature (O'Connor *et al.* 2021), and prepared fresh every two days.

5.3.5 Tissue collection

Mice were kept undisturbed, aside from weekly cage cleaning, until P21. At P21, cages were removed from the Biological Services Unit and habituated on the ground floor of the Biosciences Institute for at least one hour prior to culls. Pups were removed individually and weighed prior to undergoing either decapitation followed by intestinal tissue dissection and isolation of hippocampi, or anaesthetic overdose followed by transcardial perfusion and post fixation of brains (described in detail in 5.3.6). Following decapitation, the caecum was weighed, and intestinal length measured. The brain was immediately removed, hippocampi were dissected, and snap-frozen on dry ice and stored at -80 °C until further use. Male and female littermates (n = 6 per sex, per euthanasia method) from 4-6 litters were used for decapitation and for perfusion.

5.3.6 Transcardial perfusion

Sodium pentobarbital (90 mg / kg bodyweight) was administered via intraperitoneal injection using a 27G needle. Loss of consciousness was confirmed by firmly pinching between the toes and upon availing no pedal reflex response, mice were pinned supinely, and the thoracic cavity opened. A 27G needle was inserted into the left ventricle, the right atrium was cut to prevent a pressure build-up, and phosphate-buffered saline (PBS, 10 mM) was pumped through the heart using a peristaltic pump. The body was perfused with PBS until the fluid exiting the right atrium ran clear. 4% paraformaldehyde (PFA) was then perfused for a minimum of 6 minutes or until the body was completely stiff. The mouse was decapitated, brain removed, and post-fixed in 4% PFA for 4 h prior to undergoing a cryoprotecting sucrose gradient of 15% sucrose in PBS overnight, followed by 30% sucrose overnight. Brains were then dried of sucrose solution and snap-frozen in isopentane on dry ice and stored at -80 °C until sectioning.

5.3.7 Immunohistochemistry

For quantification of recently born immature neurons within the dentate gyrus of the hippocampus, doublecortin (DCX) immunohistochemistry was performed. Brains were sectioned on a cryostat at -23 °C in 20-µm sections onto Superfrost Plus slides in a 1:6 series and stored at -20 °C until staining.

DCX immunohistochemistry staining was carried out on hippocampal sections and the whole procedure was conducted at room temperature. For this, slides were brought to room temperature, then washed for 5 min in PBS. Sections were blocked using 10% normal donkey serum (NDS) in 0.3% triton-X 100 in PBS for 2 h. Sections were then incubated with primary antibody; rabbit anti-DCX (1:500, Abcam; ab18723) in 2% NDS and 0.3% triton-X 100 in PBS for 24 hours. Subsequently, sections were washed in 0.3% Triton-X 100 in PBS thrice for 5 min, then incubated with secondary antibody; donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:200, ThermoFisher Scientific; A-21206) in the dark for 90 minutes. Sections were then washed thrice for 5 min in PBS and nuclei stained with 4′,6-diamidino-2-phenylindole (DAPI, 1:20,000) in PBS for 5 min, then washed again in PBS thrice for 5 min per wash. Sections were cover slipped using Florescence Mounting Medium (Agilent Dako; S3023) and stored at 4 °C until imaging.

5.3.8 Microscopy

Sections were imaged using an Olympus BX53 upright fluorescence research microscope. First, the bregma of each section was determined at 2X magnification. The area of the dentate gyrus was determined using 10x DAPI-stained images and ImageJ software. DCX^+ cell bodies were counted at 20x magnification with assistance from ImageJ Cell Counter. The density of DCX^+ cells in the dentate gyrus of each section was determined by dividing the number of DCX^+ cells by the area of the dentate gyrus of the individual hippocampal section. As the hippocampus is still developing during the first several weeks of life (Bayer 1980), the coordinates of each section were extrapolated by comparing the shape of the P21 hippocampus along with other visible brain regions to the known shape and coordinates of the adult mouse brain, and similar coordinates were compared across all individual mice to minimize potential regional effects on neurogenesis. Previous research involving the adult mouse brain defined the dorsal hippocampus coordinates as -0.94 to -2.30 and the ventral hippocampus spanning from -2.46 to -3.80 (O'Leary *et al.* 2018). Therefore, to assess differences in DCX^+ cell density in the dorsal and ventral hippocampus, three sections containing the left and right hemispheres were analysed in the dorsal hippocampus (approximately -1.55 mm to -2.03 mm bregma units) and three sections containing the left and right hemispheres were analysed in the ventral hippocampal regions (approximately -2.79 mm to -3.39 mm bregma units) of each mouse.

5.3.9 Neuroplasticity-related hippocampal gene expression

Immediately following decapitation, the brain was removed from the skull and the whole hippocampus was dissected out of each hemisphere and immediately snap frozen on dry ice and stored at -80 °C until future use. Total RNA was extracted from the right hippocampus using the Invitrogen TRIzol™ Reagent RNA extraction method as described by the provider ThermoFisher Scientific, using 200 µL of TRIzol™ Reagent per sample, with a resuspension in 50 µL RNase-free water. Complimentary DNA was generated from 46.8 ng/mL Total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and utilized in Real-Time PCR (Roche) using the SYBR Green SensiFAST™ SYBR No-ROX Kit (Bioline). Relative mRNA expressions were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001), normalizing against the reference gene β-actin. Genes assessed included *DCX*, *Ki67*, *BDNF*, *BDNF IV*, *GRIN1*, $GABA_{B1a}$, *Nr3c1* and *Nr3c2*. These genes were selected due to their known relevance in hippocampal neurogenesis and/or previously demonstrated sensitivity to gut microbiota manipulations. Primer sequences are described in Supplementary Table 5.1.

5.3.10 Statistical analysis

All data was assessed for normality using the Shapiro-Wilk test. To determine whether cross fostering impacted vaginally born mice, an unpaired t-test, or a Mann-Whitney test in the case of nonparametric data, was performed between male or female VB and CF mice. As there were no differences between VB and CF mice, the effect of CS or CS ABX versus CF was then determined by a one-way ANOVA, post-hoc Fisher's LSD test in the case of normally distributed data, or a Kruskal-Wallis test followed by a Mann-Whitney U test where data was non-parametric. All data is presented as Mean ± SEM. Statistical analysis and graph generation were performed using SPSS software v. 24 (IBM Corp) and GraphPad Prism v. 8.3.0, respectively.

5.4 Results

5.4.1 Caesarean section elicits sex-specific increases in DCX$^+$ cell density in the dorsal but not ventral hippocampus

To determine whether birth mode impacted the number of maturing neurons in the dentate gyrus during the sensitive period of weaning, the quantity of DCX$^+$ cells was determined in the hippocampus of male and female vaginally born and CS-born NIH Swiss mice. A timeline of the study is provided in Figure 5.1A. To determine whether cross fostering alone impacted hippocampal neurogenesis, the difference between the density of DCX$^+$ cells in the total hippocampus of VB and VB CF mice was assessed. There was no effect of cross fostering on the density of DCX$^+$ cells in the whole hippocampus of VB versus VB CF mice (Figure 5.1B; males U = 14, p – 0.5887, females $t(10)$ = 0.7686, p = 0.4599). While CS did not alter the density of DCX$^+$ cells in the whole hippocampus of male offspring ($F(2, 15)$ = 2.729, p = 0.3517), the female mouse hippocampus appeared more sensitive to CS. Female CS mice showed an increase in the density of hippocampal DCX$^+$ cells compared to CF mice, although this was not statistically significant ($F(2, 15)$ = 1.799, p = 0.0195; CF vs CS p = 0.0659). However, caesarean section birth coupled with exposure to maternally administered antibiotics during early life significantly increased the density of hippocampal DCX$^+$ cells in female (CF vs CS ABX p = 0.0062) but not male offspring (CF vs CS ABX p = 0.1944).

Upon segregation into dorsal and ventral regions, the effects of CS on hippocampal DCX$^+$ cells was apparent in the dorsal, but not ventral, hippocampus. Specifically, in the dorsal hippocampus the number of DCX$^+$ cells was significantly increased in female offspring born by CS (Figure 5.1C, D; females $F(2, 15)$ = 5.164, p = 0.0197; CF vs CS p = 0.0289) but not in males ($F(2, 15)$ = 5.140, p = 0.0199; CF vs CS p = 0.2506), indicating that the effect of CS on hippocampal neurogenesis in the dorsal hippocampus was sex-dependent. Interestingly, exposure to penicillin antibiotics via maternal administration during early life increased the number of DCX$^+$ cells in the dorsal hippocampus in both sexes (Figure 5.1C, D; females CF vs CS ABX p = 0.0082; males CF vs CS ABX p = 0.0063). There were no observed effects of cross fostering on DCX$^+$ cells in the dorsal hippocampus vaginally born mice (females VB vs CF $t(10)$ = 0.06528, p = 0.9492; males VB vs CF $t(10)$ = 0.08141, p = 0.9367).

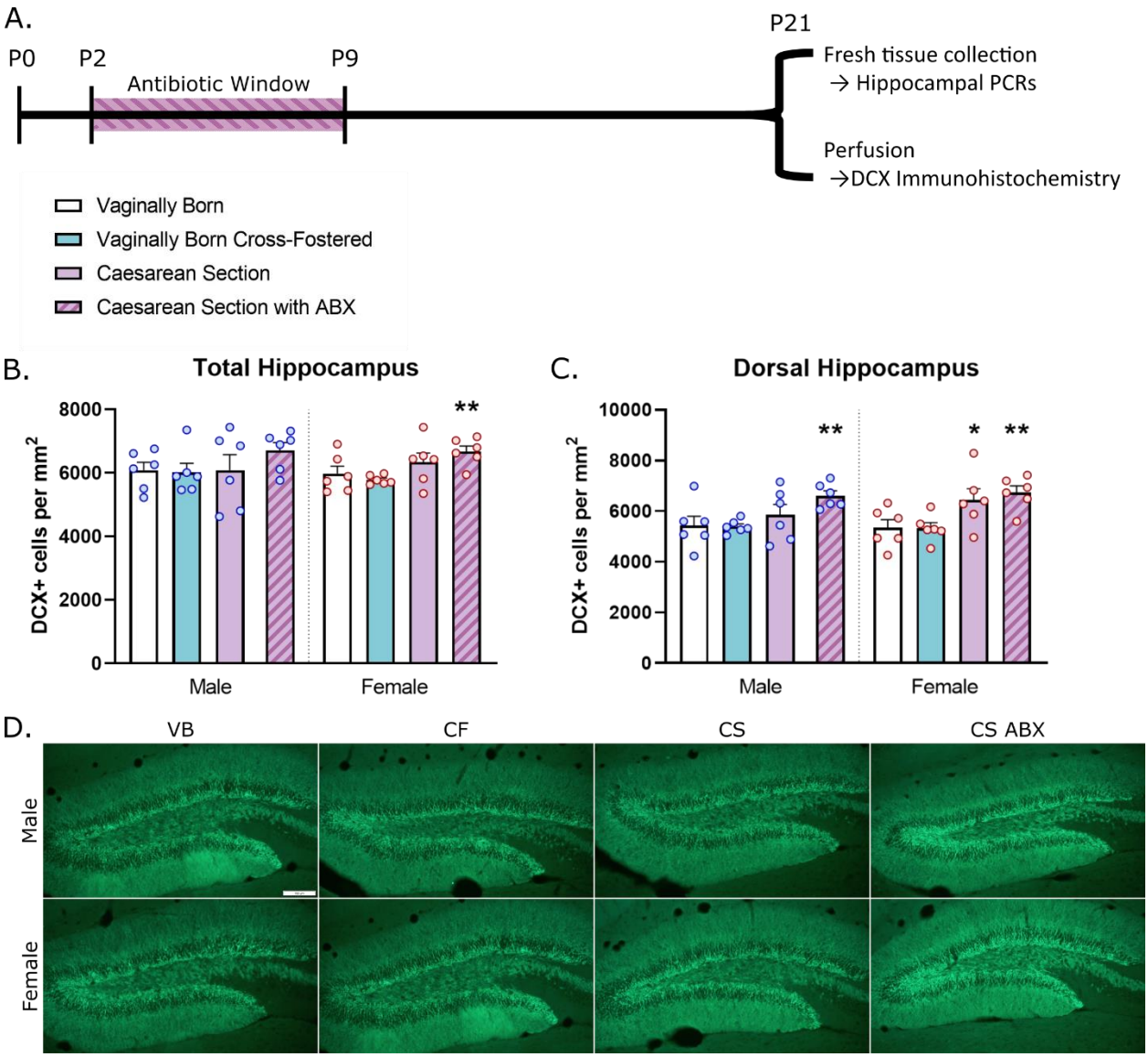


*Figure 5.1. Hippocampal neurogenesis is increased in the dorsal hippocampus of female P21 mice born by CS, and CS-born offspring exposed to early life maternal antibiotics (A) Study overview. At p21, littermates went for either fresh tissue collection or perfusion (n = 6 males, n = 6 females from 4-6 different litters per euthanasia method per group). (B) DCX+ cells per area in the hippocampus of male and female mice (C) DCX+ cells per area in the dorsal hippocampus of male and female mice. Graphs display the Mean ± SEM, n = 6 mice per group. Significant differences are defined as * $p < 0.05$ or ** $p < 0.01$ versus the CF group. (D) Representative images taken at 20X of DCX staining in the dorsal hippocampus of male and female vaginally born (VB), vaginally born and cross fostered (CF), caesarean section born (CS) and CS born with exposure to maternal antibiotics (CS ABX). Scale bar indicates 100 μm.*

In the ventral hippocampus, there were no differences in DCX^+ cells between CF, CS, or CS ABX mice of either sex (Figure 5.2A B; females $F(2, 15) = 0.4694$, $p = 0.6342$; males $F(2, 15) = 0.2028$, $p = 0.8186$) suggesting that the impact of birth mode on hippocampal neurogenesis is specific to the dorsal hippocampus. Cross fostering did not impact the number of DCX^+ cells in the ventral hippocampus of male ($t(10) = 0.1515$, $p = 0.8826$) or female ($t(10) = 0.9629$, $p = 0.3583$) vaginally born mice.

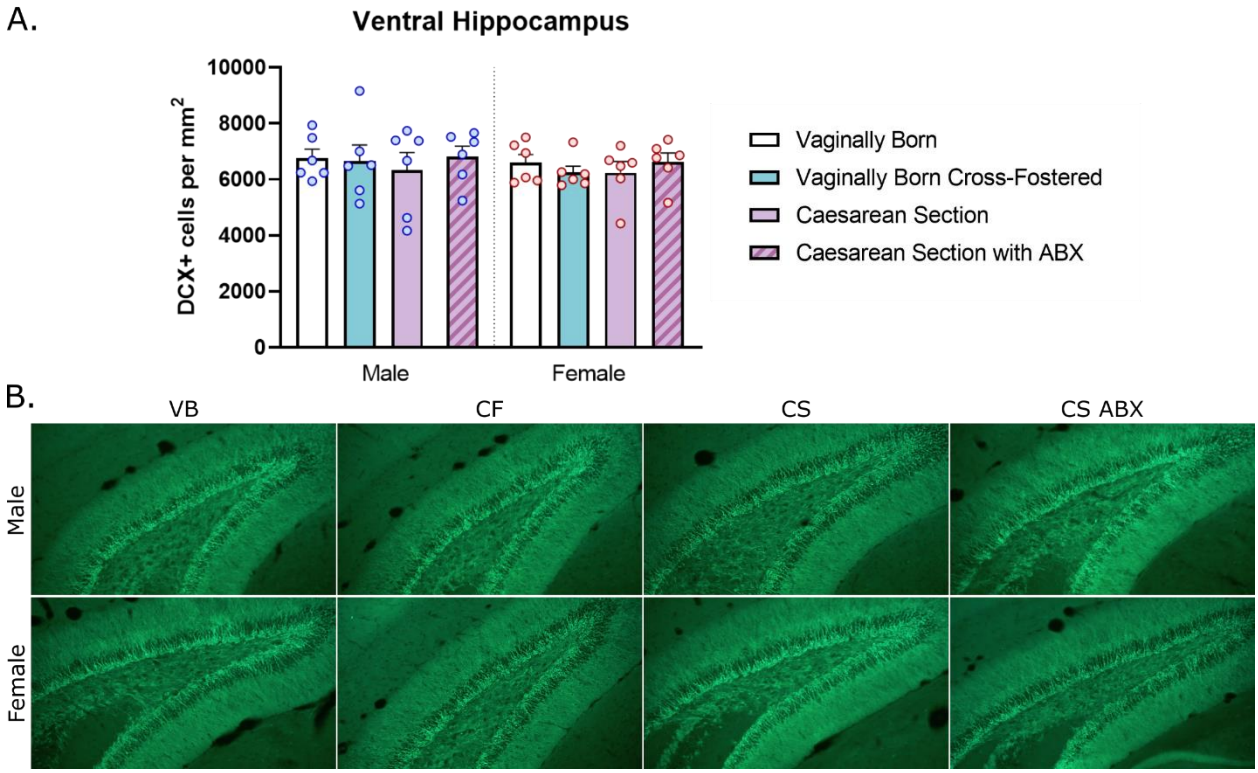


Figure 5.2. Hippocampal neurogenesis is unaffected in the ventral hippocampus of female P21 mice born by CS and CS-born offspring exposed to early life maternal antibiotics (A) DCX+ cells per area in the ventral hippocampus of male and female mice. Graph displays the Mean ± SEM, n = 6 mice per group. (B) Representative images taken at 20X of DCX staining in the ventral hippocampus of male and female vaginally born (VB), vaginally born and cross fostered (CF), caesarean section born (CS) and CS-born with exposure to maternal antibiotics (CS ABX). Scale bar indicates 100 μm.

5.4.2 Expression of neuroplasticity-related genes in the whole hippocampus are unaffected by birth mode

To further evaluate the impact of birth mode and early life antibiotic exposure on neuroplasticity, the hippocampal expression of several neuroplasticity genes, some of which have been shown to be sensitive to gut microbiota alterations, were measured via semiquantitative RT-PCR. DCX is a marker for post mitotic immature neurons and proliferating mitotic neural progenitor cells destined to become neurons, and is reported in immunohistochemical studies to be altered in germ-free mice in a sex and age dependent manner (Scott *et al.* 2020). Furthermore, Ki67 is an endogenous marker indicative of cell proliferation (Kee *et al.* 2002). Neonatal bacterial infection has previously been shown to increase hippocampal Ki67 and DCX in adulthood (Hennessey *et al.* 2021), indicating that early life microbiota can influence Ki67 and DCX expression. Additionally, administration of specific prebiotics can impact $GABA_{B1}$ receptor expression in the hippocampus

(Burokas *et al.* 2017). The hippocampal $GABA_{B1a}$ receptor subunit isoform is a potential target for stress resilience and antidepressant response, perhaps through its ability to impact 5-HT_{1A} receptor responses (Jacobson *et al.* 2017). Brain-derived neurotrophic factor (BDNF) plays a key role in regulating hippocampal progenitor cell proliferation, maturation and survival (Vicario-Abejon *et al.* 1995; Choi *et al.* 2009) and is reduced in the hippocampus of germ-free mice (Sudo *et al.* 2004; Diaz Heijtz *et al.* 2011; Clarke *et al.* 2013). Furthermore, hippocampal BDNF exon IV is sensitive to early life disturbances, including stress and probiotics (O'Sullivan *et al.* 2011). Glutamate Ionotropic Receptor NMDA Type Subunit 1 (Grin1) is crucial for supporting synaptic plasticity and neurodevelopment (Platzer *et al.* 1993; Hasan *et al.* 2013). Meanwhile, nr3c1 encodes for the glucocorticoid receptor that can inhibit the cell cycle to regulate neurogenesis (Shin *et al.* 2015). Knockdown of the glucocorticoid receptor reduces hippocampal neurogenesis (Kronenberg *et al.* 2009), and there is different sensitivity to glucocorticoids reported within different regions of the hippocampus, wherein the ventral hippocampus appears more sensitive to chronic glucocorticoid exposure (Levone *et al.* 2021a). Furthermore, increased expression of mineralocorticoid receptor Nr3c2 was able to mitigate the negative impact of early life stress on neurogenesis (Kanatsou *et al.* 2017), indicating that Nr3c2 is critical for maintaining early life hippocampal neurogenesis. There were no observed differences in mRNA expression between groups in any of the genes assessed (Figure 5.3, statistical data reported in Supplementary Table 5.2).

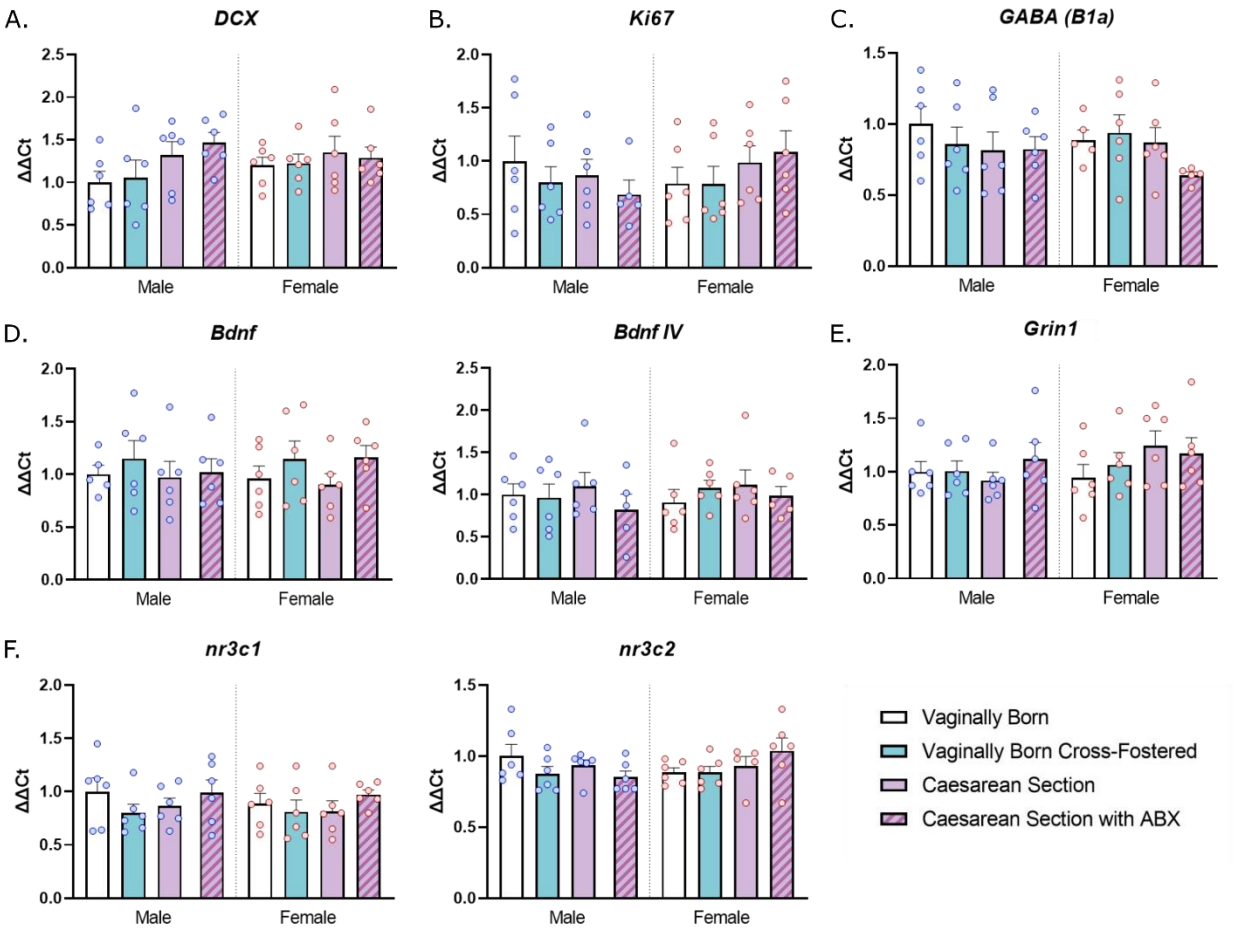


Figure 5.3 Hippocampal gene expression assessed via RT-PCR. Markers assessed included (A). immature neuronal marker Doublecortin (DCX), (B). cell proliferation marker Ki67, (C) inhibitory neurotransmitter Gamma-aminobutyric acid (GABA) receptor subunit B1a (D) Brain-derived neurotrophic factor (Bdnf) and Bdnf exon IV (Bdnf IV, (E) glutamatergic signalling factor Glutamate Ionotropic Receptor NMDA Type Subunit 1 (Grin1), (F) glucocorticoid receptor components Nuclear Receptor Subfamily 3 Group C Members 1 and 2 (nr3c1, nr3c2). Graphs display the Mean ± SEM.

5.4.3 Birth mode leaves lasting impacts on caecal organ weight

Early life disruption to the gut microbiota via antibiotics has been shown to impact weight gain later in life (Cox *et al.* 2014). Furthermore, germ-free mice and mice undergoing treatment with antibiotics are characterized for having enlarged caecal mass (Savage & Dubos 1968; Spichak *et al.* 2018). Therefore, body weight and gastrointestinal metrics including caecal weight and intestine length were assessed.

There were no differences in body weight between CF and CS pups at P21 (Figure 5.4A). Interestingly however, male mice born by CS and exposed postnatally with antibiotics displayed

reduced body weight compared to their CF counterparts ($F(2, 33) = 3.309$, $p = 0.0490$; CF vs CS ABX $p = 0.0154$). This effect was not present in female CS ABX mice ($F(2, 33) = 0.8709$, $p = 0.4280$; CF vs CS ABX $p = 0.2453$). Interesting, although mice born by CS had no significant alterations in body weight, both male and female CS mice displayed significantly lower caecum weights compared to the CF control mice (Figure 5.4B; males $F(2, 15) = 5.323$, $p = 0.0179$; CF vs CS $p = 0.0053$; females $F(2, 15) = 3.916$, $p = 0.0428$; CF vs CS $p = 0.0364$; CF vs CS ABX $p = 0.0230$). This did not reflect in differences in length of the small intestine or colon (Figure 5.4C, D). Cross fostering did not impact any of these metrics in vaginally born mice (statistics not shown).

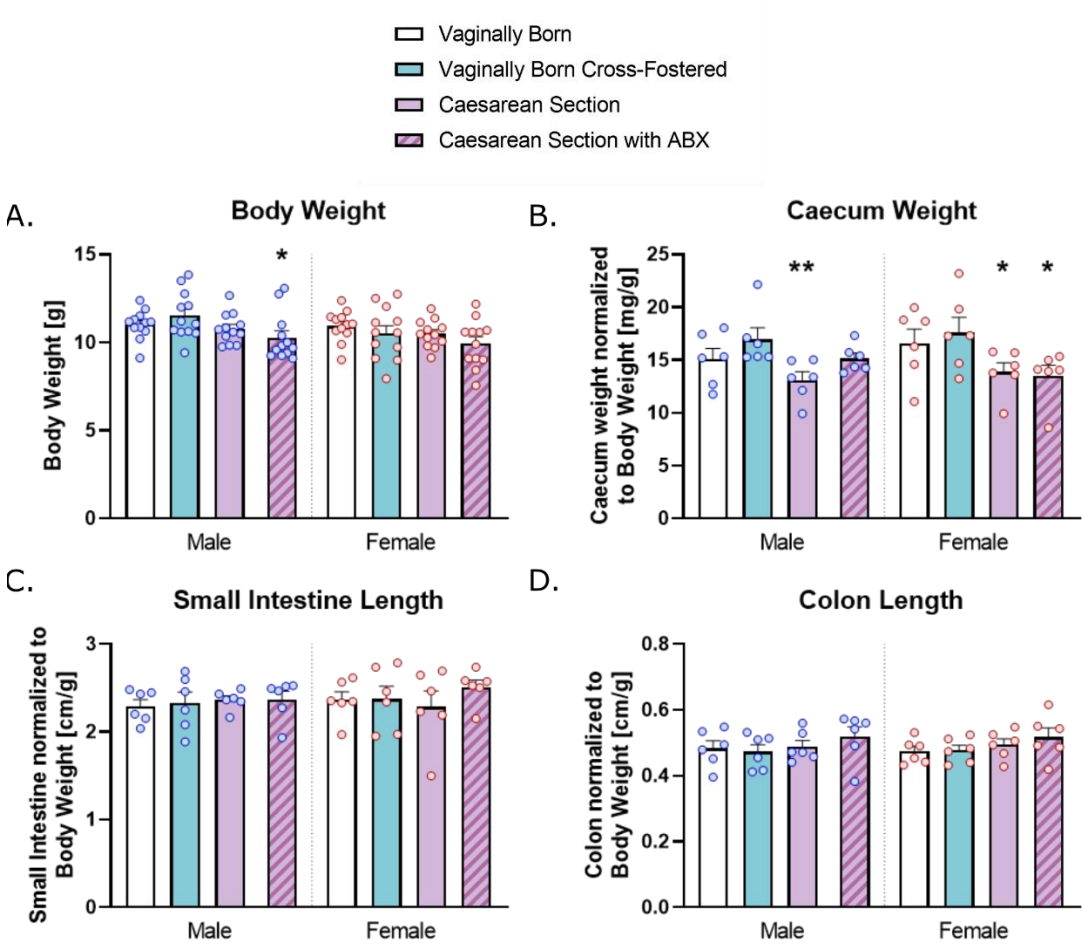


*Figure 5.4. Body and organ weights at the time of culls (A) Body weights of all individuals studied (n = 12 per group). (B) Caecum weight normalized against the body weight. (C) Length of the small intestine normalized against the body weight. (D) Colon length normalized against the body weight. Graphs display the Mean ± SEM. Significant differences are defined as * $p < 0.05$ or ** $p < 0.01$ versus the CF group.*

5.5 Discussion

Hippocampal neurogenesis is a critical process underlying healthy cognition and stress response of the host (Shors *et al.* 2002; Aimone *et al.* 2006; Imayoshi *et al.* 2008; Sahay *et al.* 2011; O'Leary & Cryan 2014). The absence of a gut microbiota has been shown to leave lasting marks on hippocampal neurogenesis, even when a rodent is colonized with bacteria after weaning (Ogbonnaya *et al.* 2015; Scott *et al.* 2020), which may contribute to the behavioural differences observed in adult germ-free animals (Spichak *et al.* 2018), though the relevance of CS-mediated disruption to the gut microbiota on hippocampal neurogenesis has never been investigated. Furthermore, the time period of weaning, around P21 in mice, appears as a highly sensitive period of rapid gut microbial expansion and development (Al Nabhani *et al.* 2019; Morais *et al.* 2020), after which, colonization of germ-free mice was unable to affect hippocampal neurogenesis (Ogbonnaya *et al.* 2015). This research demonstrates, for the first time to our knowledge, that birth mode elicits an effect on neurogenesis in the hippocampus of young animals in a sex-specific manner, whereby female CS born P21 NIH Swiss mice displayed an increased DCX^+ cell density in the dorsal hippocampus. Intriguingly, CS birth coupled with maternal antibiotic administration from the P2 to P9 timeframe increased dorsal hippocampal neurogenesis in both male and female offspring at P21.

Previous research suggests that neurogenesis in the dorsal hippocampus is more sensitive to alterations in the gut microbiota than the ventral hippocampus (Ogbonnaya *et al.* 2015). Herein, we found that the density of immature neurons was only increased in the dorsal, and not ventral hippocampus of CS-born mice treated with antibiotics, further indicating that the dorsal hippocampus may be more responsive to alterations in the gut microbiota. The hippocampus is not homogenous in function or biology (Leonardo *et al.* 2006; Dong *et al.* 2009; O'Leary & Cryan 2014; Levone *et al.* 2021a; Levone *et al.* 2021b). Differences in gene expression are evident when the hippocampus is divided into dorsal and ventral regions, which may trigger region-sensitive differences in hippocampal neurogenesis (Dong *et al.* 2009; Levone *et al.* 2021a). This may relate to the functional differences observed in the dorsal and ventral hippocampus (O'Leary & Cryan 2014). Research involving regional lesions of the hippocampus suggests that the dorsal hippocampus primarily regulates aspects of cognition, including learning and memory, (Ferbinteanu & McDonald 2001; Pothuizen *et al.* 2004) and contextual fear retention (Kim &

Fanselow 1992), while the ventral hippocampus is suggested to be more crucial for emotional response, including stress and anxiety (Henke 1990; Kjelstrup *et al.* 2002; O'Leary & Cryan 2014; Levone *et al.* 2021a; Levone *et al.* 2021b). Nonetheless, there are no published studies that investigate whether regional differences in hippocampal neurogenesis also contribute to specific behaviours and whether birth mode leads to consequences in behaviours mediated by the dorsal hippocampus. Previous research has demonstrated that CS-born mice have persistent heightened anxiety-like behaviour and social memory behaviour deficits (Morais *et al.* 2020; Morais *et al.* 2021), regardless of sex (Nagano *et al.* 2021). Given that CS born mice display heightened anxiety-like behaviour (Morais *et al.* 2020), which is more related to the ventral hippocampus (O'Leary & Cryan 2014), it is interesting that there were no differences observed in the density of DCX^+ cells in the ventral hippocampus of P21 CS-born mice, including CS-born mice who were exposed to antibiotics in early life. However, DCX is only one marker for hippocampal neurogenesis, and differences in neuron proliferation, survival, and synaptic plasticity at the timepoint coinciding with these behaviours has not been assessed. Additionally, the anxiety-like behaviours previously assessed in CS born rodents have not been directly mechanistically linked to changes in hippocampal neurogenesis (Shors *et al.* 2002; Morais *et al.* 2020). To better understand the role of hippocampal neurogenesis-related in anxiety-like behaviour, the novelty-induced hypophagia test or novelty suppressed feeding test should be performed (Santarelli *et al.* 2003). Furthermore, future studies involving CS-born rodents should investigate cognitive functions that rely on the dorsal hippocampus and/or on hippocampal neurogenesis, such as pattern separation, spatial learning and long-term spatial memory in the Morris water maze task (Moser *et al.* 1995; Shors *et al.* 2002; Clelland *et al.* 2009; Sahay *et al.* 2011).

Early life antibiotic exposure from P2 to P9, coupled with CS birth, substantially increased dorsal hippocampal neurogenesis in P21 offspring regardless of sex. Antibiotics, such as penicillin, are commonly administered to mothers who undergo CS to prophylactically prevent postpartum infections or postsurgical complications (Dierikx *et al.* 2021). Furthermore, these antibiotics are deemed safe for the baby's health, and mothers nursing their babies are recommended to continue to breastfeed throughout the course of their antibiotics (Kaiser *et al.* 2007; Williams *et al.* 2021). Nonetheless, antibiotic exposure in early life leaves striking scars on the developing gut microbiome (Cox *et al.* 2014; Leclercq *et al.* 2017; O'Connor *et al.* 2021), and early life antibiotic consumption has been related to increased symptomatology of depression and behavioural

difficulties in toddlerhood (Slykerman *et al.* 2017). While no previous research has studied how early life antibiotics impact neurogenesis in the developing brain or later in life, administration of antibiotics to adult mice for seven weeks was reported to reduce the number of proliferating neural precursor cells in the hippocampus (Mohle *et al.* 2016). Interestingly, co-treatment with probiotics was able to ameliorate these effects (Mohle *et al.* 2016). While CS also remarkably alters the early life gut microbiome (Dominguez-Bello *et al.* 2010; Bokulich *et al.* 2016; Morais *et al.* 2020), the double-hit of CS coupled with early life antibiotic exposure may be more consequential to the gut microbiota, therefore leading to more remarkable differences in hippocampal neurogenesis regardless of sex. Furthermore, it has previously been shown that early life microbiota-targeted treatments can reverse behavioural impairments caused by CS birth (Morais *et al.* 2020), but whether probiotics can reverse the alterations caused by birth mode and early life antibiotic administration on hippocampal neurogenesis remains unknown.

Interestingly, female, but not male, mice born by CS displayed increased dorsal hippocampal neurogenesis at P21. Perhaps sex-specific differences in the gut microbiome may underlie the sex-specific effects of birth mode on hippocampal neurogenesis (Jaggar *et al.* 2020), although additional research would need to be conducted to better characterize the relationship between early life sex-specific alterations in the gut microbiome and hippocampal neurogenesis.

Based on hippocampal gene expression data, the effects of CS and early life antibiotic exposure on hippocampal neurogenesis were not accompanied by alterations in *BDNF*, $GABA_{B1a}$, glutamatergic signalling factor *Grin1*, or differences in glucocorticoid receptors *nr3c1* or *nr3c2*. However, these findings are limited because gene expression was assessed in the whole hippocampus. Meanwhile, immunohistochemistry data indicates that neurogenesis is perturbed in the dorsal, and not ventral, hippocampus. Therefore, it is possible that the lack of changes observed in hippocampal gene expression may be due to a dilution of effects caused by using the entire hippocampus, rather than assessing dorsal and ventral regions separately. The heterogeneity of gene expression within the hippocampus should be considered in future research (Leonardo *et al.* 2006; Thompson *et al.* 2008; Dong *et al.* 2009; O'Leary & Cryan 2014; Levone *et al.* 2021a).

The mechanistic underpinnings of birth mode-driven alterations in hippocampal neurogenesis are unknown. However, it has been reported that birth mode-driven behavioural alterations may involve oxytocin (Morais *et al.* 2021) and specific bacteria (Morais *et al.* 2020). Indeed, oxytocin

supplementation restored deficits in social memory and some aspects of anxiety-like behaviour in mice (Morais *et al.* 2021). Of relevance to the data observed here, oxytocin is known to stimulate hippocampal neurogenesis (Leuner *et al.* 2012; Lin *et al.* 2017). The gut microbiota of mice and humans born by CS is noticeably different into infancy (Dominguez-Bello *et al.* 2010; Bokulich *et al.* 2016; Morais *et al.* 2020). Interventions targeting the gut microbiota, including probiotic or prebiotic administration, as well as co-housing with vaginally born mice, rescued deficits in the behaviour of mice born by CS (Morais *et al.* 2020), making it tempting to speculate that such microbiota alterations might also contribute to effects observed on hippocampal neurogenesis. However, this remains to be investigated in future studies.

Interestingly, the caecum weight of mice born by CS was significantly reduced compared to vaginally born cross-fostered counterparts three weeks after birth, while the body weight of these pups was unaffected. The weight of the caecum is highly sensitive to perturbations in the gut microbiota; germ-free rodents have substantially enlarged caecums (Spichak & Guzzetta *et al.* 2018), the size of which are reduced following microbial colonization (Van Eldere *et al.* 1988). This data hints that alterations in the gut microbiota due to CS may persist into P21. Furthermore, previously published research has shown that alpha diversity of caecal microbiota assessed by Chao1 was significantly reduced in P21 mice born by CS (Morais *et al.* 2020), which may relate to these findings. Given that adult hippocampal neurogenesis is regulated by gut microbiota-directed immune pathways (Mohle *et al.* 2016) and vagus nerve signalling (O'Leary *et al.* 2018), which can relay gut microbiota-derived signals to the brain (Bravo *et al.* 2011b; Fulling *et al.* 2019), whether changes in the gut microbiota caused by caesarean-section birth elicit effects on hippocampal neurogenesis through these pathways should be investigated.

While increased hippocampal neurogenesis in adulthood is often considered beneficial, due to its role in antidepressant action and cognitive performance (Santarelli *et al.* 2003; Ziv *et al.* 2006), the functional relevance of the temporal dynamics of early life hippocampal neurogenesis is largely unknown. Indeed, early life hippocampal neurogenesis appears critical for allowing an individual to forget less important existing information in favour of forming new memories (Akers *et al.* 2014). However, neurogenesis is also increased following injury to the nervous system, such as stroke (Arvidsson *et al.* 2002), so an increase in neurogenesis may indicate that the brain is responding to a harmful pathological event (Scharfman & Hen 2007). Furthermore, new neurons

must develop, migrate, and integrate appropriately into hippocampal circuitry. Failure to do so contributes to seizures in animal models and is hypothesized to underly issues related to epilepsy (Jessberger & Parent 2015), although no link between CS and epilepsy in the offspring has been established. Furthermore, little is known about the temporal dynamics of early life hippocampal neurogenesis, or whether aberrant early life hippocampal neurogenesis leads to functional consequences later in life. Whether the observed differences in hippocampal neurogenesis caused by birth mode and maternal antibiotic consumption have functional implications needs to be investigated further.

In the developing rodent brain, neurogenesis of granule cells peaks around two weeks post-birth (Bayer 1980), and undergoes a gradual decline throughout the process of aging, with little neurogenesis occurring into old age (Klempin & Kempermann 2007). In germ-free C57Bl/6J mice, the temporal dynamics and amplitude of change in the density of DCX^+ hippocampal cells is disrupted, whereby 4 week old male germ-free mice show a reduction in DCX^+ cells at 4 weeks compared to conventional males, but not later timepoints, and female germ-free mice have increased DCX^+ cells at 8 weeks, but not 4 or 12 weeks compared to conventional females, indicating that the gut microbiota is pivotally involved in regulating the dynamics of hippocampal neurogenesis (Scott *et al.* 2020). Given that CS birth disrupts the initial colonization and developmental trajectory of the gut microbiota, and that the gut microbiota influence the trajectory of hippocampal neurogenesis, it might be suspected that the trajectory of hippocampal neuron maturation may also be perturbed by CS birth mode, though this remains to be investigated. Future studies should examine effect of CS on hippocampal development and neurogenesis at earlier and later timepoints.

The focus of this study was to investigate whether birth mode, additionally coupled with the common maternal administration of antibiotics postnatally, impacted hippocampal neurogenesis during early life. There are several limitations and additional considerations that future research should consider to further understand the relevance of birth mode-induced alterations to hippocampal neurogenesis and discriminate the underlying mechanistic causes. For instance, previous research using germ-free mice has revealed that microbiota-related disruptions in hippocampal neurogenesis may be time-sensitive, whereby male and female germ-free mice have altered hippocampal neurogenesis at different times during development (Scott *et al.* 2020). The

persistence of the increase in hippocampal neurogenesis observed in the current study should be examined at additional timepoints. Another question to ponder is whether antibiotic administration to the dam altered maternal behaviour. It has previously been shown that hippocampal neurogenesis is effected by early life stressful experiences (Tanapat *et al.* 1998), including lack of maternal care (Mirescu *et al.* 2004) and social isolation stress (Lu *et al.* 2003), leading to an increase in cellular proliferation in the dentate gyrus of P21 male mice (Nair *et al.* 2007). From the current study, it is impossible to rule out whether there were alterations in the offsprings' perceived stress due to changes in maternal care triggered by, for instance, maternal antibiotic consumption. Furthermore, this model of CS is confounded by the fact that caesarean pups are born approximately a half-day earlier than vaginally born pups. Premature birth has been shown to supress neurogenesis in rabbits (Malik *et al.* 2013), though any consequences of being born one half-day early on hippocampal neurogenesis in mice is unknown. Furthermore, due to the nature of this model, CS pups likely do not receive the same initial colostrum breast milk due to the need to cross-foster to dams that have already birthed. It is impossible to decipher whether these features of the CS model confound results. Finally, maternal antibiotic administration alone during the early life window from P1 to P8 has been shown to alter pup behaviour and hippocampal gene expression in adulthood (O'Connor *et al.* 2021). In this study, antibiotics were administered to the mother from P2 to P9. Whether the antibiotic-induced effects we observed were exacerbated by CS birth is impossible to elucidate in our study design as a limitation of this study is that no antibiotic treated VB and CF groups were included. However, antibiotics are commonly administered prior to or following CS birth, and therefore in this study, they were considered as part of the process of CS.

One caveat of this study is that it is completely unknown whether the differences in hippocampal neurogenesis observed herein are also observed in human babies born by caesarean section. The global rate of CS birth has risen globally over the last several decades, resulting in roughly 21% of women giving birth via caesarean section between 2010-2018, and is predicted to continue to grow (Betran *et al.* 2021), meaning that it is increasingly important to understand the relationship between birth mode and hippocampal neurogenesis-related behavioural outcomes in humans. However, there has historically been insufficient research on cognitive development in CS-born babies in comparison to babies born vaginally (Blake *et al.* 2021). Accumulating epidemiological evidence suggests that babies born by CS have significantly reduced cognitive performance during

childhood (Al Khalaf *et al.* 2015; Curran *et al.* 2017; Polidano *et al.* 2017; Hanrahan *et al.* 2020), although not all studies have replicated these findings (Li *et al.* 2011; Smithers *et al.* 2016), perhaps due to differences in assessment type, corrections for confounders. Interestingly, one study that considered biological sex differences in outcomes observed that female CS-born infants had higher rates of delayed communication skills compared with males (Hanrahan *et al.* 2020), which suggests that the impact of CS on the developing brain is sex-specific. Nonetheless, more clinical research should be conducted to uncover whether there are functional or structural differences caused by birth mode within the developing human brain, as well as potential long-term consequences.

In summary, these findings demonstrate that birth mode increases dorsal hippocampal neurogenesis in female, but not male, P21 NIH Swiss mice. It is imperative to further understand the consequences and mechanisms underlying how CS birth clinically impacts baby neurodevelopment, and how sex factors into the susceptibility of offspring to these birth mode-related consequences. In doing so, novel therapeutics may be discovered to alleviate these outcomes, and support the development of the postnatal brain.

5.6 Supplementary Material

Supplementary Table 5.1. Sequences of SYBR Green Mouse PCR Primers. F indicates forward primer, R indicates reverse primer.

Target mRNA	**Froward Primer 5'-3'**	**Reverse Primer 5'-3'**
β-actin	CTT TCC CTG TAT GCC TCT G	ATG TCA CGC ACG ATT TCC
DCX	CAC CCA ACC AGC AGA AAA C	GCC AGC AAC GCA TCA AAA C
KI67	AAA ACT TGC CTC CTA ATA CAC C	CTT CCT GCC TGC TTT CTT G
Bdnf	AAG GAC GCG GAC TTG TAC AC	CGC TAA TAC TGT CAC ACA CGC
Bdnf IV	GGA CCG GTC TTC CCC AGA GCA	TCA TGG GCG CCG CCT TCA TG
GRIN1	GCT GGA GGA GCG TGA GTC	AGC AGA GCC GTC ACA TTC TT
GABA(B1a)	TGT TCT TGC TGT TGT CTG TC	TCC TCC ATT CCT TCT TCT CC
Nr3c1	TTT CTG CGT CTT CAC CCT C	CAT TCC CCA TCA CTT TTG TTT C
Nr3c2	GAA ACA ACA AAA TCA ACC CCA G	AAA GAA AAG TAC GAG CCA TCC

Supplementary Table 5.2. Statistical outputs from PCR results. Results are displayed as the overall ordinary one-way ANOVA between CF, CS, and CS ABX male or female P12 mice. Outliers were removed, final n = 5-6 per group.

	Male			**Female**		
Target mRNA	F	DFn, DFd	p-value	F	DFn, DFd	p-value
DCX	1.605	2, 15	0.2335	0.1927	2, 15	0.8267
KI67	0.3502	2, 14	0.7105	0.7771	2, 15	0.4774
Bdnf	0.3595	2, 15	0.7039	1.172	2, 15	0.3365
Bdnf IV	0.6529	2, 14	0.5357	0.2563	2, 14	0.7775
GRIN1	0.7835	2, 15	0.4746	0.4575	2, 15	0.6414
GABA(B1a)	0.04681	2, 15	0.9544	2.203	2, 14	0.1473
Nr3c1	1.035	2, 15	0.3792	1.095	2, 15	0.3599
Nr3c2	0.9045	2, 15	0.4257	1.325	2, 14	0.2972

Chapter 6

General Discussion

6.1 General Summary

The overall goal of my thesis was to explore the relationship between various gut microbiota manipulations and host hippocampus-associated cognitive function, hippocampal neuroplasticity and hippocampal neurogenesis throughout the lifespan, led by my hypothesis that perturbance of the gut microbiota will induce alterations in hippocampal neurogenesis, neuroplasticity, and cognitive function.

I explored this hypothesis through various preclinical studies involving faecal microbiota transplantation from young to aged mice (Chapter 2), administering microbial consortia associated with cognitively-impaired or health elderly patients into healthy adult rats (Chapter 3), dietary and antidepressant interventions within the rat model of early life stress-induced gut microbiota disruption (Chapter 4), and the caesarean section birth mode model of neonatal gut microbiota disruption coupled with antibiotics in mice (Chapter 5). These results are summarised in Table 6.1.

In Chapter 2, we transferred the faecal microbiota (FMT) from young mice or aged mice into aged mice and found that FMT from young mice into aged mice reversed aspects of aging in the hippocampal metabolome, transcriptome, and microglia, as well as some aging-related phenotypes in peripheral immunity. Furthermore, aged mice that received FMT from younger mice showed improvements in spatial learning and memory in the hippocampus-depended Morris water maze behavioural test. While aging induced an expected decline in the survival of newly born hippocampal neurons, FMT from young into aged mice was unable to rescue this deficit, highlighting the limitations of FMT on adult hippocampal neurogenesis in aging.

In Chapter 3, we administered consortia of bacteria with identified bioactive potential which were either highly abundant in cognitively impaired elderly or elderly humans with healthy cognitive function into adult rats. We expected to observe a transfer of the cognitive impairment phenotype to rats which received microbiota consortia derived from cognitively impaired elderly, and assessed multiple parameters of cognition, anxiety-like, and depressive-like behaviour, as well as metrics indicative of gut health. However, neither consortium of bacteria was able to alter performance in any of the assessed behaviours, although noradrenaline appeared to be slightly reduced in the colon of rats that received the microbial consortia derived from cognitively impaired elderly. This research indicates that aging-related cognitive deficits in humans are not able to be

conferred via select microbial transplantation to healthy adult rodents, although there may be several other factors that may mask potential capabilities of these microbes to exert effects on cognition, such as the age and health of the host receiving the bacteria, bacterial community interactions, and interspecies differences in gastrointestinal microbial ecology.

In Chapter 4, we examined whether dietary interventions with fish oil or various polyphenols could reverse early life stress-induced alterations in the adult gut microbiome or hippocampal neuroplasticity related genes. We found that early life stress induced by maternal separation left lasting impacts on the gut microbiome, such as altered α-diversity and β-diversity and decreased relative abundance of *Christensenellaceae* and *Rothia* and increased abundance of *Lachnospiraceae NK3A20 group* and *Colidextribacter*, ultimately leading to changes in the predicted functions of the gut microbiome upon the gut-brain axis. Furthermore, neuroplasticity-related hippocampal gene expression was affected by early life stress in adulthood, resulting in increased *BDNF* and *Grin1a* gene expression and lower *Ki67* gene expression. While all dietary supplementations tested, including fish oil, quercetin, phlorotannin's, and xanthohumol exerted effects on the gut microbiome in maternally separated rats, treatment with fish oil appeared to highly restructure the gut microbiome as well as its predicted functional ability within gut-brain communication. Furthermore, this research revealed that the relative abundance of several bacterial taxa correlated with the expression of neuroplasticity-related genes. For instance, a larger relative abundance of *Bifidobacterium* or *Faecalibaculum* were related to lower expression of *BDNF* and *Ki67* in the hippocampus, while higher abundance of *Anaerovorax* significantly associated with increased hippocampal *DCX* expression. This research highlights the potential capacity for dietary supplementation to reverse or alter stress-induced phenotypes in hippocampal neuroplasticity, perhaps through remodelling the gut microbiome and its predicted function.

Chapter 5 focussed on understanding the relationship between birth mode, which is one of the main factors that influences the postnatal gut microbiota, and hippocampal neurogenesis in male and female mice at the timepoint of weaning, during which the gut microbiota is rapidly expanding. By assessing the number of immature neurons in the dentate gyrus of mice born vaginally or by caesarean section, I revealed that caesarean section exerts a sex-specific effect on hippocampal neurogenesis. Female caesarean section born mice displayed greater quantities of DCX+ cells in the dorsal hippocampus than vaginally born female mice, while this effect was not present in male

mice born by caesarean section, nor in the ventral hippocampus of either sex. Furthermore, the addition of early life antibiotic exposure (which is commonly administered prophylactically to mothers undergoing caesarean section), via penicillin in the mother's drinking water significantly increased the number of DCX+ cells in the dentate gyrus of male caesarean section born pups and exacerbated the effect of caesarean section birth in females. However, no significant differences were observed in the expression of neuroplasticity-related genes in the whole hippocampus between any groups. This research highlights a potential consequence of birth mode on the developing brain, which may underly previously observed alterations in hippocampus-related behaviour in caesarean section mice.

The data presented herein showed that some, but not all, gut microbiota manipulations may be able to influence hippocampus-associated cognitive function, hippocampal neuroplasticity, and hippocampal neurogenesis at different stages throughout life. Furthermore, this research highlights potential windows within the lifespan where gut microbiota-targeted treatment may or may not be useful interventions to modulate hippocampal functions. A summary of the findings within this thesis are presented in Table 6.1. Altogether, I suggest that the gut microbiota should be further considered as a potential target for improving the disruption in hippocampal neurogenesis and related behavioural deficits that may occur as a consequence of aging, stress, or birth mode.

Table 6.1. General summary of results.

Chapter	Microbiota Manipulation	Hippocampal neurogenesis and plasticity	Additional readouts
Chapter 2	FMT from young to aged mice	↓ new-born neuron survival in aged mice was not reversed with FMT from young mice	↑ spatial learning and memory in the Morris water maze; ↓ anxiety-like behaviour, restructured hippocampal metabolome and transcriptome, ↓ hippocampal microglia cell body size, rejuvenated aspects of peripheral immunity
Chapter 3	Consortia transfer from cognitively impaired or health elderly into young rats	Not measured due to lack of behavioural phenotype differences	No differences observed in behaviour, faecal water content or gastrointestinal transit. ↓ colonic noradrenaline levels
Chapter 4	Early life stress Dietary supplementation with the antidepressant fluoxetine, fish oil, or polyphenols	Altered expression of neuroplasticity genes following early life stress and dietary supplementation with fluoxetine, quercetin and xanthohumol	Stress and dietary supplementation altered α- and β-diversity and composition of the gut microbiome, restructuring microbiota predicted functions involved in the gut-brain axis
Chapter 5	Caesarean section (CS) birth and early life maternal antibiotic consumption	↑ immature neurons in CS ♀ dorsal hippocampus ↑ immature neurons in the dorsal hippocampus of CS ♂ and ♀ pups exposed to maternal antibiotics	CS pups had reduced relative caecum weight at P21, which persisted in ♀ but not ♂ pups exposed to early life maternal antibiotics CS male pups exposed to antibiotics showed reduced birth weight

6.2 Neurogenesis, the gut microbiota, and the aging brain – Where are we now?

The decline in hippocampal neurogenesis with aging is well evidenced, and clinically correlates to the development and trajectory of aging associated neurological diseases, including Alzheimer's Disease (Moreno-Jimenez *et al.* 2019; Tobin *et al.* 2019), as well as aging-associated cognitive decline in rodents (Haughey *et al.* 2002; Drapeau *et al.* 2003). For these reasons, the ability to increase hippocampal neurogenesis has been highlighted as a potential critical attribute for future treatments of aging-associated cognitive impairment, including Alzheimer's Disease (Moreno-Jimenez *et al.* 2019; Essa *et al.* 2022).

Given that the gut microbiota can orchestrate changes in adult hippocampal neurogenesis (Ogbonnaya *et al.* 2015; Mohle *et al.* 2016; Kim *et al.* 2021; Wei *et al.* 2021), the gut microbiome presents as an accessible potential target for improving the aging-associated detriments to hippocampal plasticity via maintaining hippocampal neurogenesis at beneficial levels, and cognition. Novel probiotic therapeutics, including *Bifidobacterium breve A1 (MCC1274)*, have shown promise in improving cognition in older patients with suspected mild cognitive impairment, a precursor to Alzheimer's Disease (Xiao *et al.* 2020). In a rodent model of Alzheimer's Disease, *Bifidobacterium breve MCC1274* administration increased the quantity of synaptic proteins in the hippocampus and quenched the activation status of microglia (Abdelhamid *et al.* 2022), indicating that it may harness the ability to influence hippocampal neurogenesis. However, based on evidence presented in this thesis, the capability for the gut microbiota to remodel hippocampal neuron survival in the aged mouse brain seems limited. Chapter 2 of this thesis demonstrated that faecal microbiota transplant from young to aged mice was able to rescue aspects of aging-induced alterations in peripheral immunity, hippocampal microglia activation, the hippocampal metabolome and transcriptome, and hippocampus-dependent cognitive behaviour. Furthermore, aging led to a clear reduction in the survival of newly born hippocampal neurons, measured by co-staining of NeuN and BrdU (Chapter 2). Nonetheless, FMT from young mice into aged mice was not sufficient to have any impact on the aging-induced decrease in the survival of newly born neurons (Chapter 2). While other aspects of hippocampal neurogenesis were not measured in this study, such as neuronal proliferation and maturation of newly born neurons, this inability of FMT to impact the survival of newly born hippocampal neurons in the aged mouse may highlight the limitations of gut microbiota-targeted interventions on the aging brain.

The future for gut microbiota-based drug discovery to rejuvenate the aging brain relies on the ability to identify novel potential probiotics or other microbiota-targeted treatments. While some research has identified potential probiotics (Xiao *et al.* 2020; Abdelhamid *et al.* 2022), prebiotics (Casadesus *et al.* 2004), and postbiotics (Mossad *et al.* 2021) that may be able to restore some neurocognitive and neurological deficits that occur during normal aging, the frontier of therapeutic probiotic discovery must continue to be pushed by the development and assessment of novel microbiota-based therapeutics. Chapter 3 of this thesis was based on the concept of identifying microbes which may elicit effects on cognition and may serve as potential therapeutic targets for aging-associated cognitive decline. By examining a large database of gut microbiome samples from healthy and cognitively-impaired elderly patients, two novel consortia of gut bacteria were developed by collaborators based on the abundance of a bacterium within either population, and its known or predicted ability to influence aspects of the gut-brain axis. Ultimately, the aim of this chapter was to decipher whether either of the microbial consortia (identified from cognitively impaired or cognitively healthy elderly humans) harness the ability to alter neurocognitive functioning in a young adult rat model (Chapter 3). While neither of the consortia proved effective in altering behaviour, this type of research, guided by bioinformatics and existing knowledge of microbiota-host interactions and seeded in preclinical investigations, is necessary on the current frontier of probiotic discovery. As the body of knowledge on microbiota-host dialogue grows larger with each day, there is constant promise for a novel breakthrough in targeting the gut microbiota to improve host brain aging.

6.2.1 Transitioning towards microbiome-based interventions in clinical aging trials

The movement from preclinical and clinical drug discovery isn't always successful. Although research outcomes from Chapter 2 provide a valuable preclinical foundation for FMT as a means to rejuvenate aspects of brain aging, including cognitive deficits, there are leaps and bounds that must be made before clinical trials should be conducted, including examination of off-target consequences and clinical feasibility in an elderly, fragile population. While clinical FMT is already being used to treat chronic microbial infections and some gastrointestinal disorders, a recent death of an immunocompromised patient following FMT to treat *C. difficile* infection (U.S. Food and Drug Administration 2020) raises questions as to whether the elderly population are viable candidates for FMT treatment. The process of aging coincides with immune dysregulation

and physical frailty which may mean that FMT into elderly individuals who are already inherently immunocompromised could do more harm than good. Furthermore, rectal administration of FMT, the common administration route, may not be as feasible in physically frail patients. Additionally, the advancement of this treatment in an aging population raises important questions as to who resembles the appropriate faecal donor, including what age; children, young adults, or healthy aged adults. If faecal material is required from donors who are minors, ethical and regulatory dilemmas will arise regarding medical and bodily autonomy, consent, and financial compensation for their donation.

Given the potential limitations and hazards of FMT, the selection of specific strains of bacteria may be more beneficial and straightforward, allowing the oral introduction of these beneficial bacteria into patients. However, the development of such probiotics relies on the identification of key bacteria that have been shown to promote or improve cognitive ability in aging, especially within clinical trials. Currently, there is some, albeit limited, clinical evidence suggesting some strains of *Bifidobacterium breve* can improve declining cognition assessed by Mini-Mental State Examination in elderly people with mild cognitive impairment (Kobayashi *et al.* 2019b; Xiao *et al.* 2020). While there have been additional studies investigating the clinical impacts of specific probiotics on cognitive function in elderly patients with impaired cognition (Den *et al.* 2020), several of these studies are limited by using insufficient cognitive assessments, and should focus on using the Mini-Mental State Examination, or other widely accepted cognitive rating scales (Den *et al.* 2020).

Large-scale studies investigating whether microbial manipulation by dietary intervention in elderly humans can benefit cognitive health in the aging brain are currently underway. For instance, the NU-AGE study is a year-long trial which enrolled over 1,000 participants aged 65-80 years old across five European countries in an attempt to determine the benefits of a Mediterranean diet on multiple aspects of health in an aged population (Berendsen *et al.* 2018; Marseglia *et al.* 2018; Ghosh *et al.* 2020b). Indeed, elderly individuals who maintained high adherence to a Mediterranean diet showed significantly improved global cognition and episodic memory (Marseglia *et al.* 2018) which correlated to alterations in their gut microbiome (Ghosh *et al.* 2020b), suggesting that dietary intervention may present an additional less invasive strategy than FMT for altering the gut microbiota towards improved cognition in elderly people. However, not

every individual might be willing to give up their unhealthy eating habits and may instead favour flavour over health outcomes, making diet prescriptions perhaps less feasible than a probiotic pill or FMT. Furthermore, by identifying key nutrients within diets shown to support healthy cognitive ability in aging, it may be possible to refine these dietary interventions to prebiotic supplements, rather than requiring a full dietary change, although there is currently insufficient research around the ability for prebiotics to clinically improve cognitive ability in aging (Bialecka-Debek *et al.* 2021).

6.2.2 Aging perspectives: Is it all in the microbial composition?

One of the largest limitations of the current sequencing techniques used to assess the gut microbiome noninvasively is the inability to determine the metabolic and functional profile of the microbiome, especially within key niches along the gut. While the more expensive sequencing option, shotgun metagenomic sequencing, can inform us about the functional potential of the microbiome, and functional profiling can be predicted with the more cost-friendly 16S rRNA sequencing, neither of these options can inform the exact ongoing functions of microbiota, or the dynamics of these functions within the gut.

For instance, both intermittent fasting and caloric restriction with nutrient maintenance have been shown to improve cognition of aged rodents and primates, including humans (Witte *et al.* 2009; Dal-Pan *et al.* 2011; Kesby *et al.* 2015; Pifferi *et al.* 2019; Chakraborty *et al.* 2020; Ooi *et al.* 2020; Rojic-Becker *et al.* 2021), implicating a potential for perturbing hippocampal neurogenesis. Indeed, rodents who were fasted intermittently displayed increased adult hippocampal neurogenesis (Ahn *et al.* 2019; Baik *et al.* 2020), while aged rodents with calorie restricted diets did not show improvements in the aging-related decline in adult hippocampal neurogenesis (Bondolfi *et al.* 2004), although this may be due to lack of nutrient maintenance. Both of these dietary patterns have been shown to extend lifespan in mammals (Ramsey *et al.* 2000; Pifferi & Aujard 2019), and leave lasting effects on the composition of the gut microbiome (Ozkul *et al.* 2020; Zou *et al.* 2020). Furthermore, the gut microbiota plays a crucial role in the homeostasis of the host metabolic state, and indeed contribute to the beneficial metabolic changes observed in obese individuals who fasted intermittently (Rong *et al.* 2021). However, it is unknown whether the beneficial functional state of the gut microbial community, which may promote hippocampal neurogenesis, still remains when the external pressure of diet-induced energy scarcity is removed.

An additional critical study has shown that gut microbiota from aged mice lead to different outcomes when transplanted into young or old germ-free mice. Counterintuitively, young mice who received microbiota from aged mice displayed increased hippocampal neurogenesis and signs of prolongevity (Kundu *et al.* 2019), while the same effects did not occur in aged germ-free mice. This research highlights the importance of the phenotype of the recipient, and not solely the composition of the gut microbiome. This study raises questions about whether the microbial consortia identified in elderly with healthy or impaired cognition would induce different effects if they were transplanted into a different host, such as an aged rat, or a rat expressing signs of cognitive decline. Likewise, research involving FMT from depressed or healthy patients into rats that were bred to either show increased sensitivity or resistance to the induction of depressive-like behaviour has shown that the genetics of the host is a strong factor shaping whether an individual responds to a gut microbiota manipulation (Knudsen *et al.* 2021). While it is beyond the scope of this PhD, the phenotype of the host appears critical in the function of the microbiota and must be considered in gut microbiota interventions.

Furthermore, a critical aspect of research is the ability to reproduce findings. It is becoming clear that there is converging evidence around the ability of the gut microbiome to influence host brain health in aging. Recently, the findings from Chapter 2 have been replicated by another lab group who similarly found that microbiota from young mice can rescue impairments in learning and memory that occur with aging (Mossad *et al.* 2021). This reproducibility confirms our findings around the importance of the gut microbiota in the brain aging process, and additionally suggests that the gut microbiota-derived metabolite δ-valerobetaine, plays a mechanistic role in this ability (Mossad *et al.* 2021). It will be exciting to see whether future research can replicate these findings in additional animal models, and potentially eventually clinically.

6.3 Early life gut microbiota: Priming for future brain health

6.3.1 Back to the start: Implications of birth mode on outcomes of brain health

At the other spectrum of life, the development of the gut microflora during early life appears to set the stage for the future health of the host, including brain health. Consequentially, perturbance of the gut microbiota at birth via caesarean section birth mode has been shown to leave lasting deficits

on anxiety and social behaviour as well as neurochemistry in mice (Morais *et al.* 2020; Cabre *et al.* 2022), although the underlying mechanisms by which birth mode disrupts host neurobiology is largely unknown. Chapter 5 of this thesis uncovered that caesarean section birth mode increases the number of immature neurons in the dorsal hippocampus of female, but not male, mice during the period of weaning (i.e. three weeks after birth). Furthermore, the maternal consumption of antibiotics during early life mice delivered by c-section increased the number of immature neurons in the dorsal hippocampus regardless of sex, displaying the sensitivity of host hippocampal neurogenesis to early life gut microbiota insults.

The global rate of caesarean section birth, especially elective caesarean section, has grown dramatically over the last several decades and is projected to continue to rise (Figure 6.1) (Betran *et al.* 2021). Therefore, the accumulating preclinical evidence that mode of birth disrupts host neurodevelopment, as shown in this thesis, and behaviour (Morais *et al.* 2020; Morais *et al.* 2021), is a potential concern. Clinical studies on the short- and long-term impacts of caesarean section on neurodevelopment and neurological disease susceptibility are few in number and largely reliant on statistical association, rather than randomized controlled trials. This is further complicated by the fact that caesarean section birth is often required due to medical necessity, such as pregnancy complications, breech delivery, foetal distress, or maternal chronic health conditions, making it difficult to isolate whether caesarean section birth mode is specifically the culprit of any noticeable differences in offspring outcomes, or if any noticeable differences in offspring outcomes are due to other confounding effects. Nevertheless, clinical research indicates that caesarean section birth mode alters the stress response in early life with caesarean section-born 8-week old babies showing lower salivary cortisol concentrations and crying response (Taylor *et al.* 2000), and that this disparity persists into adulthood, wherein adults born by caesarean section demonstrated heightened vulnerability to acute stress (Dinan *et al.* 2022). Beyond an altered stress response, caesarean section birth mode has been linked to intellectual disability in childhood in four clinical studies (Khadem & Khadivzadeh 2010; Li *et al.* 2011; Polidano *et al.* 2017; Hanrahan *et al.* 2020), which may implicate altered hippocampal neurogenesis in caesarean section-born humans, though these findings have been disputed (Al Khalaf *et al.* 2015; Smithers *et al.* 2016; Curran *et al.* 2017). Whether birth mode impacts susceptibility to aging-associated neurodegenerative conditions related to altered adult hippocampal neurogenesis in people is yet to be observed.

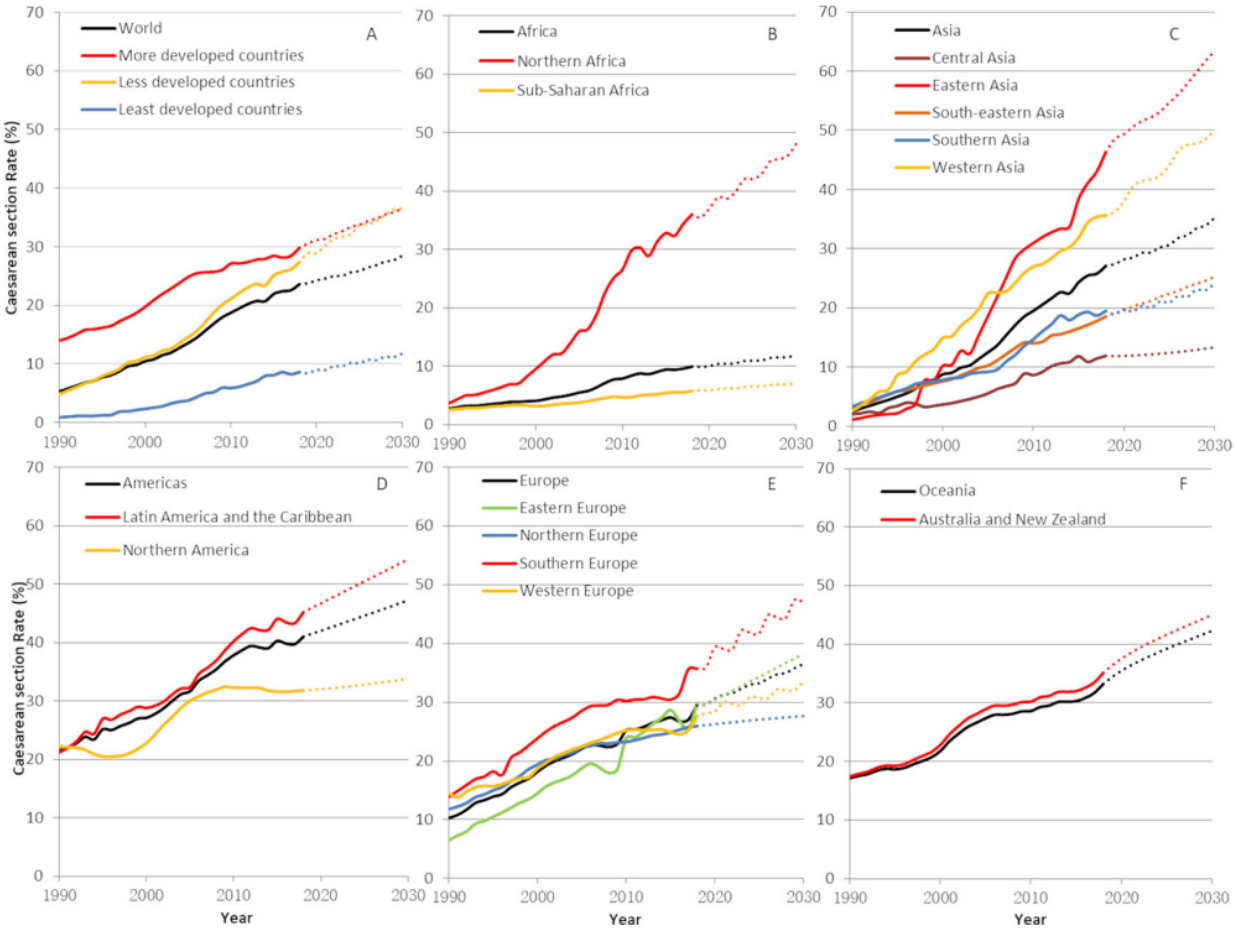


Figure 6.1. Rates of caesarean section birth have grown globally between 1980-2018 and are projected to continue to rise. A) World; (B) Africa; (C) Asia; (D) Americas; (E) Europe; (F) Oceania. Rates and projections for Melanesia, Micronesia, and Polynesia were not calculated due to low data availability. Solid lines represent trend estimates and dotted lines display projections. Figure from (Betran et al. 2021).

6.3.2 Potential mechanisms underlying caesarean section-induced differences in hippocampal neurogenesis

The underlying mechanism(s) by which caesarean section birth and early life antibiotic exposure alters host neurogenesis are largely a mystery. Microbiota-targeted interventions, such as prebiotics, probiotics, and cohousing (Morais *et al.* 2020), have proven successful in restoring the behavioural deficits observed in caesarean section born mice. Still, the pathways by which the early life gut microbiota acts upon the gut-brain axis to influence these changes, or whether gut microbiota-targeted interventions mitigate the observed effects of caesarean section and early life antibiotic exposure on hippocampal neurogenesis, remain unknown. Disruptions to microbiota-dependent vagus nerve signalling or altered microbially driven metabolite synthesis and

degradation are both highly plausible given the vast differences in the early life gut microbiome composition. For instance, vitamin B_6, an enzymatic cofactor in the kynurenine pathway, can be synthesized by most bacteria classified as *Actinobacteria*, *Bacteroidetes*, or *Proteobacteria* (Uebanso *et al.* 2020), and has been implicated in supporting hippocampal neurogenesis (Zysset-Burri *et al.* 2013) while vitamin B_6 deficiency leads to impaired social behaviour (Li *et al.* 2020b). Indeed, caesarean section has been shown to reduce the functional ability of the new born human gut microbiome to metabolise vitamin B_6, a feature which lasted the length of the three month study (Long *et al.* 2021).

Furthermore, the gut microbiota is extremely important for host immune system priming, especially during early life. Delays or disruptions to the typical trajectory of the mouse gut microbiome in the first few weeks of life, induced by altering the weaning period or antibiotic exposure, leads to differences in immune system imprinting, notably upon regulatory T cell induction, and can influence the host's susceptibility to pathological inflammation, including later in life (Al Nabhani *et al.* 2019). As discussed several times throughout this manuscript, various components of the immune system, including T cells, are critical for maintaining healthy hippocampal neurogenesis. Therefore, it is highly likely that the disrupted early life gut microbiota observed in caesarean section born offspring, who do not receive their initial gut microbiota seeding from the vaginal microbes, triggers abhorrent immune system functioning, which may then incur consequences to the brain. Future research involving flow cytometry analysis of immune cell populations within the periphery and brain, immunohistochemical staining of microglia within the hippocampus, and the use of genetic knock-out mouse models or adaptive transfer of specific immune cells would be very useful for elucidating the immune system's involvement in the disruption to hippocampal neurogenesis observed following caesarean section birth and early life antibiotic exposure.

6.3.3 Are caesarean section-induced alterations in the early life gut microbiota a concern for developing neurodevelopmental disorders?

Hippocampal neurogenesis is a critical process regulating cognitive and emotional health. The increase in the quantity of immature neurons observed in female mice born by caesarean section relative to those born vaginally is concerning, given that early life neurogenic processes in the hippocampus set an individual up for future success in cognitive and emotional processes. While

in young adult animals, increased adult hippocampal neurogenesis has been shown to be required for alleviating depressive symptoms (Santarelli *et al.* 2003) and positively correlates to better spatial memory in aged rodents (Drapeau *et al.* 2003), the timeframe and conditions under which hippocampal neurogenesis is elevated may not relate to beneficial outcomes. For instance, 10-week-old male germ-free Swiss Webster mice displayed increased hippocampal neuronal survival compared to conventional controls (Ogbonnaya *et al.* 2015). Meanwhile, a separate study involving male C57BL/6J germ-free mice found decreased hippocampal immature neurons at 4 weeks old, which was normalized at both 8 weeks and 12 weeks of age (Scott *et al.* 2020). In this same study, female germ-free mice from the same litters only show elevated hippocampal immature neurons at 8 weeks old, and not 4 weeks or 12 weeks (Scott *et al.* 2020). Various strains of adult germ-free mice, including NMRI, Swiss Webster, C57BL/6, BALB/c and MK strains, show deficits in anxiety and cognitive behaviours related to hippocampal neurogenesis and hippocampal plasticity (Spichak & Guzzetta *et al.* 2018). Therefore, although the differences in hippocampal neurogenesis in germ-free mice may have subsided by 12 weeks of age, persistent effects of early life disrupted hippocampal neurogenesis might still lead to lasting consequences on host behaviour. Furthermore, studies examining the effects of early life stress on hippocampal neurogenesis found increased hippocampal cell proliferation after the stressor, which was associated with a decreased hippocampal stem cell pool in adulthood (Youssef *et al.* 2019). Nonetheless, whether the effects of caesarean section on hippocampal neurogenesis normalizes in adulthood are unknown. Further research should be conducted that investigates this question, as well as whether caesarean section birth leads to deficits in hippocampal neurogenesis-associated behaviour, including in the Morris water maze, pattern separation, and novelty-suppressed feeding.

The deficits in social and anxiety-like behaviours observed in mice born by caesarean section have been likened to the pathology of autism in humans (Nagano *et al.* 2021). Furthermore, rodent models of autism show disrupted hippocampal neurogenesis (Stephenson *et al.* 2011; Zhong *et al.* 2020). While hippocampal neurogenesis is difficult to assess in human tissue, one study involving tissue from 13 autistic individuals found evidence of disrupted hippocampal neurogenesis (Wegiel *et al.* 2010). Interestingly, patients with autism show distinctly unique gut microbiomes (Yap *et al.* 2021), although it is unclear whether these alterations in the gut microbiome underly the disease pathology or are a side-effect of the limited dietary preferences associated with autism (Yap *et al.* 2021). FMT trials attempting to treat symptoms of autism appear promising for improving

gastrointestinal symptoms as well as behavioural symptoms of the disorder (Li *et al.* 2021). However, whether gut microbiota-derived disruptions to early life hippocampal neurogenesis underly autism susceptibility in humans is unclear.

6.3.4 Postnatal stress and the developing brain: Are microbes our buffer?

Stressful events during early life leave lasting impacts on the host, including on the gut microbiome and neuroplasticity-related genes in the hippocampus (Chapter 4). While it is currently unknown the exact mechanism by which stress induces hippocampal plasticity changes, the gut microbiome is emerging as a very likely mediator between the two. Recently, research has shown that the elevation of proinflammatory cytokines and carbonyl (a measure of oxidative stress) within the hippocampus of adult mice subjected to chronic mild stress can be alleviated following FMT from unstressed mice, or induced by FMT from stressed mice to naïve mice (Li *et al.* 2019b; Marcondes Avila *et al.* 2020). These proinflammatory cytokines have previously been shown to disrupt hippocampal neurogenesis (Monje *et al.* 2003), which is a potential pathway through which the gut microbiome influences stress-related behaviour. The decrease in stress-induced elevated hippocampal proinflammatory cytokines following FMT was not reversible via vagotomy (Marcondes Avila *et al.* 2020), indicating that non-vagal microbiota-gut-brain communication pathways, such as metabolite-driven or immune cell mediated mechanisms, may be more important for inducing a stress-derived proinflammatory phenotype in the hippocampus.

6.3.5 Critical early life windows for microbiota-based interventions?

The research presented in this thesis disrupted the development of the early life gut microbiota via either caesarean section birth mode (Chapter 5) or maternal separation stress from postnatal day 2 (P2) – P12 (Chapter 4). While early life stress appeared to impact the expression of genes related to neuroplasticity in the rat hippocamps, no effects were observed on gene expression of these same genes in caesarean section mice. This dichotomy begs the question as to why differences were observed in the effects of early life gut microbiota disturbance on the expression of genes related to hippocampal neuroplasticity.

One of the key differences between these studies was the species of animal in which they were carried out: Chapter 4 was conducted in Sprague Dawley rats while Chapter 5 was conducted in

NIH Swiss mice. While mice and rats share common ancestry with both belonging to the order Rodentia, differences have been observed in brain development between species, including in the hippocampus (Ellenbroek & Youn 2016). Indeed, is has been observed that rat new-born hippocampal neurons expressed markers of maturation 1-2 weeks earlier than in mice and did not undergo cell death as frequently as new-born neurons of mice (Snyder *et al.* 2009), which may contribute to the differences observed in the responsiveness of hippocampal neuroplasticity markers to microbial perturbations between the two chapters. Furthermore, there were differences in the time of intervention as well as the time of assessment of hippocampal gene expression between the two chapters. In Chapter 5, the gut microbiota of mice was disrupted from birth via caesarean section birth mode, and tissue was collected around the time period of weaning (at P21). Meanwhile, in Chapter 4, stress was administered to rats between P2 – P12, and hippocampal neuroplasticity related gene expression was assessed at 16 weeks of age. While no changes in whole hippocampal gene expression were observed at P21 in caesarean section and vaginally born mice, caesarean section born female mice showed increased dorsal hippocampal immature neurons, an effect which was present in both male and female caesarean section born mice that were exposed to early life antibiotics between P2-P9. Meanwhile, adult rats subjected to early life stress between P2-P12 display increased expression of *BDNF* and *Grin1a* and decreased expression of *Ki67* in their whole hippocampi compared no non-stressed rats (Table 6.2). These differences in the time window of intervention and tissue collection time, along with differences in the species of the model organism, may account for the disagreement in how early life gut microbiota perturbation affected hippocampal neuroplasticity.

Furthermore, the enduring impact of early life antibiotic exposure or early life stress on hippocampal neurogenesis or neuroplasticity-related markers may suggest that the early life window between P2 until P9 or P12 is a critical window during which gut microbiota disruption may be more consequential to host hippocampal neurodevelopment later in life, especially given that only female mice were affected by microbial disruption at P0 (ie. Caesarean section birth mode). However, further research would need to be conducted to determine if this is the case. For instance, it would be very interesting to use antibiotic exposure at various timepoints of neurodevelopment, including starting at birth, to determine whether hippocampal neurogenesis is more sensitive to microbiota manipulations at a specific time window of early life.

Additionally, in Chapter 4, dietary supplementation with fish oil, fluoxetine, or polyphenols occurred between 8 to 16 weeks of age. The potency of these microbiota-targeted treatments administered in early adulthood to impact hippocampal neuroplasticity-related genes, as well as reverse some of the effects of stress on adult behaviour (as previously published) (Donoso *et al.* 2020; Egerton *et al.* 2020) indicates that the window of opportunity to intervene on early life gut microbiota disruption does not close in adulthood.

*Table 6.2: Changes in hippocampal neurogenesis and neuroplasticity-related gene expression following microbiota-targeted interventions in early life. CS = Caesarean section born mice compared in vaginally born cross fostered mice, CS ABX = caesarean section born mice with penicillin antibiotics administered in the mothers' drinking water from P2 – P9 and compared to vaginally born cross fostered mice, MS = maternal separation stressed rats from p2 – p12, compared to non-stressed control rats. MS + dietary treatment comparisons denote significant differences between MS rats and MS + dietary treatment rats. CS mice were 21 days old at the time of tissue collection while MS rats were 16 weeks old at the time of tissue collection. * indicates a trend towards significance (p < 0.07).*

Density of Immature Hippocampal Neurons										
Intervention	Sex	Total Hippocampus			Dorsal Hippocampus			Ventral Hippocampus		
CS	♂	-			-			-		
	♀	-			↑			-		
CS ABX	♂	-			↑			-		
	♀	↑			↑			-		
Hippocampal Gene Expression Differences in the Total Hippocampus										
Intervention	Sex	*BDNF*	*BDNF IV*	*DCX*	*Ki67*	*Grin1a*	*Grin2a*	*$GABA_{B1a}$*	*Nr3c1*	*Nr3c1*
CS	♂	-	-	-	-	-	-	-	-	-
	♀	-	-	-	-	-	-	-	-	-
CS ABX	♂	-	-	-	-	-	-	-	-	-
	♀	-	-	-	-	-	-	-	-	-
MS	♂	↑	-	-	↓	↑	-	-	-	-
MS + Fluoxetine	♂	-	-	-	↑ *	-	-	↓	-	-
MS + Fish Oil	♂	↓	-	↑	-	-	-	-	-	-
MS + Phlorotannins	♂	-	-	-	-	-	-	-	-	-
MS + Quercetin	♂	↓	-	-	↑ *	-	-	-	-	-
MS + Xanthohumol	♂	-	-	-	↑	-	-	-	-	-

6.4 The diet-microbiota-gut-brain axis: Towards novel interventions

Chapter 4 of this thesis set out to decipher whether microbiota-targeted dietary supplementations could ameliorate the effects of early life stress on hippocampal plasticity and the gut microbiome diversity, composition, and predicted functional capacity within the microbiota-gut-brain axis. While only some of the effects of stress on hippocampal neuroplasticity-related gene expression and microbiome features could be rescued by specific dietary treatments, including fish oil, quercetin, and xanthohumol, this research highlights the critical role that diet plays in shaping host brain function, and how it may be utilized to effectively remodel features within the gut and brain. Furthermore, this research begs the question of whether a diet with insufficient nutrients or prebiotics may underly unhealthy mental states or perhaps susceptibility to disorders and diseases.

An individual's diet plays a huge role in dictating the daily patterns of change within their gut microbiome (Johnson *et al.* 2019), as well as establishing the long-term phenotype of the gut microbial ecosystem (Wu *et al.* 2011). The food an individual consumes provides essential nutrients to their own cells, but also for the gut microbial cells. Therefore, the ability to restructure the gut microbiome using the non-invasive, natural tool of dietary supplementation appears to be highly beneficial for reshaping the gut microbiome to improve hippocampal plasticity and potentially, overall brain health.

It is currently unclear as to whether the beneficial outcomes of these dietary supplements on hippocampal neuroplasticity are driven by mediation of the gut microbiome, moderation of the microbiota-gut-brain axis, direct effects of nutrients on the host, or a combination of all three (Figure 6.2). Some nutrients, such as fibre, are known to be digested via microbial fermentation into short-chain fatty acids, which are known to act within the gut-brain axis. Apart from supplementation, the deficiency in specific nutrients may also be detrimental to the gut microbiota. For instance, a diet deficient in magnesium was shown to restructure the gut microbiome, which correlated with increased depressive-like behaviour and hippocampal cytokine interleukin-6, suggesting an involvement in the gut microbiota in modulating depressive-like behaviour during nutrient deficiency (Pyndt Jorgensen *et al.* 2015; Winther *et al.* 2015). Meanwhile, whether fish oil-derived ω-3 fatty acids or polyphenols act directly on the brain or solely through microbiota-directed mechanisms would require further exploration, such as nutrient labelling and tracing with

^{13}C or deuterium. Experiments involving germ-free mice have shown that polyphenols require the gut microbiome to support their bioavailability and potentially bioactive function (Espin *et al.* 2017; Pasinetti *et al.* 2018). Furthermore, baseline differences in the gut microbiome may mean that some individuals respond differently to dietary interventions than others, or don't respond at all (Berding *et al.* 2021b), underscoring that the translational road towards gut microbiota-directed dietary interventions is just beginning.

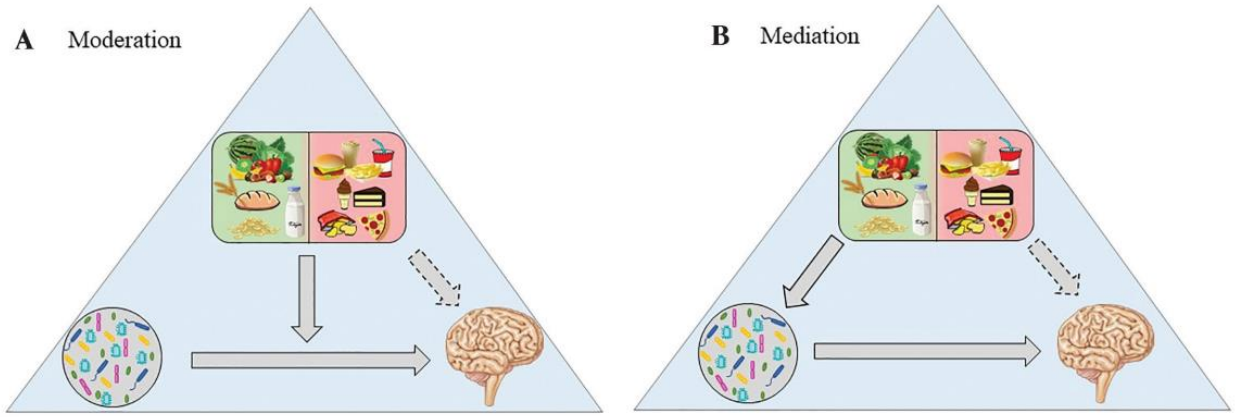


Figure 6.2. Moderation versus mediation of diet within the gut-brain axis. Two potential mechanisms exist whereby diet may influence the microbiota-gut-brain axis: (A) Moderation of ongoing microbiota-gut-brain axis communication, and/or (B) Mediation of the gut microbiota which would then indirectly affect gut-brain communication. The potential for diet to directly affect the brain is depicted with the dashed lines. Figure from (Berding et al. 2021b).

6.5 The future of microbiota-based therapeutics for improving disrupted brain plasticity

Our understanding of the roles that the gut microbiota plays in brain health has rapidly expanded over the past decade (Figure 6.3), making this an exciting time to investigate microbiota-host interactions and hypothesize the future potential that microbiota-based therapeutics have in improving brain function.

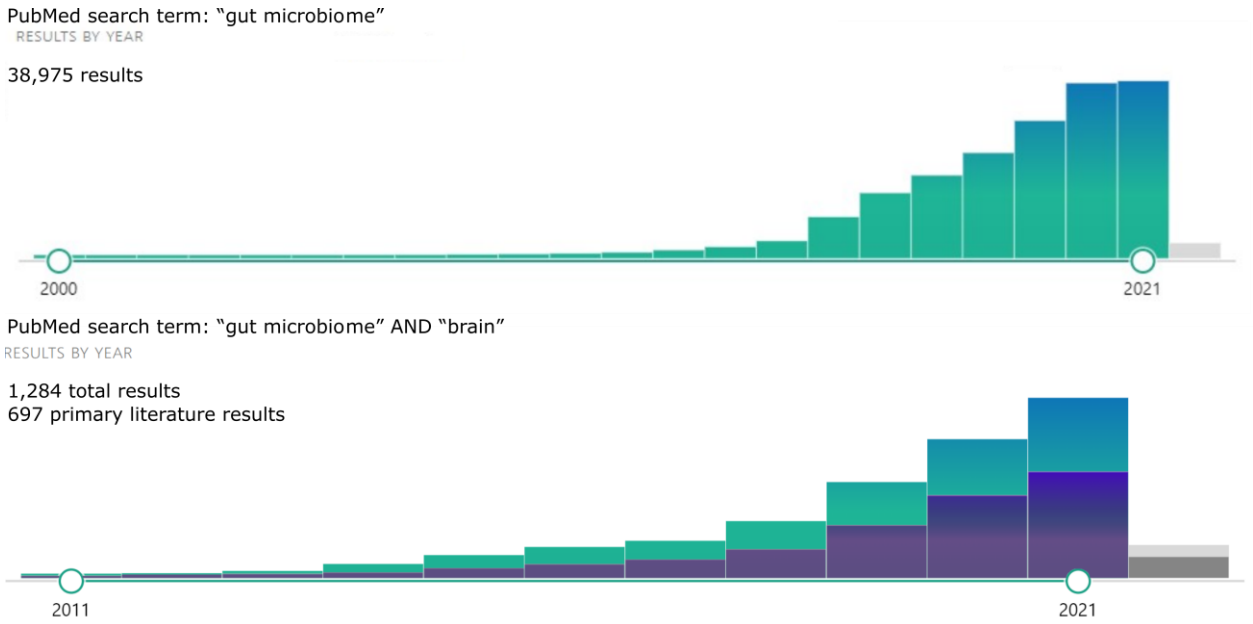


Figure 6.3. The last decade has brought a rapid increase in the number of scientific publications related to the search term "gut microbiome" in the PubMed server. Furthermore, when narrowing findings to "gut microbiome" AND "brain", there is a noticible lag in the evolution of gut microbiome research to progress towards including the microbiota-gut-brain axis. In the lower panel, teal indicates all publications belonging to the pubmed seach "gut microbiome" AND "brain" while the overlaid purple bars indicate only primary literuature, and not review articles.

This thesis has demonstrated that manipulating the gut microbiota can influence hippocampal plasticity following early life stress (Chapter 4), hippocampus-dependant spatial learning and memory in aging (Chapter 1), and hippocampal neurogenesis in early life (Chapter 5), suggesting that microbiota-targeted therapeutics may be beneficial for improving hippocampus-related brain functions at all stages of life.

6.5.1 The next generation of microbiota-targeted therapeutics

Chapter 2 demonstrated that FMT from young to aged mice was sufficient to improve aging-associated deficits in spatial learning and memory in aged mice, which indicates that it may be a novel therapeutic intervention for aging-related cognitive decline in humans, such as dementia or Alzheimer's Disease. However, as discussed earlier, the practice of clinical faecal transplantation is currently limited to treating gastrointestinal infection or disorders, though its applications for treating autism and depression are being trialled. Therapeutic FMT also presents logistical and feasibility questions, especially when operating within a community that has a higher incidence of frailty and immunodeficiency (Marquez *et al.* 2020).Therefore, the ability to move beyond faecal transplantation to remodel the microbiome-gut-brain axis would be highly beneficial.

The identification an individual microbe or consortia of microbes that are acting upon the gut-brain axis to improve neuroplasticity and/or cognitive performance could minimize the potential risks and logistical dilemmas around FMT in humans, such as donor requirements and in-hospital care. For instance, an individual bacterial strain previously demonstrated to improve stress-induced depressive behaviour in rodents, likely through 5-hydroxytryptophan synthesis (Tian *et al.* 2019a) was recently shown to also improve depression scores in humans (Tian *et al.* 2021). Alternatively, if specific microbes can be identified that are causing harm to hippocampal neuroplasticity, the selective elimination of these microbes via pathogen-specific antibiotic consumption, or microbe-targeted bacteriophage therapy could be critical tools for perturbing the gut microbiota without the need for FMT.

Unfortunately, the discovery of neuroprotective or detrimental bacteria has been slow, and often relies on correlation analysis between gut microbiome and neuroplasticity readouts or inferring functional traits that may work within the microbiota-gut-hippocampus axis. The frontier of this science therefore relies on both *in silico* modelling and *in vivo* screening to attempt to elucidate these abilities. Chapter 3 of this thesis highlighted that this approach does not always work. Despite utilizing one of the most comprehensively studied human cohorts on aging and the gut microbiome (Wilmanski *et al.* 2021) coupled with existing knowledge of bacterial functional potential within the gut-brain-cognition axis (Valles-Colomer *et al.* 2019), the identified consortium of bacteria that were implicated in healthy or cognitively-impaired aging were not able to impact any aspect of cognitive, depressive-like, or anxiety-like behaviour examined (Chapter 3). Therefore, it seems too early to rely on the strengths of *in silico* modelling to predict the ability of microbes to impact host hippocampal neuroplasticity.

As discussed previously, the diet-microbiota-gut-brain axis is increasingly proving to be a valuable tool to reshape the gut microbiota towards improved health. Indeed, various dietary components, including prebiotics, probiotics, and personalized diets have been suggested as a means to improve brain function without directly influencing the gut microbiota, as in FMT (Figure 6.4). Chapter 4 demonstrated that dietary supplementation with fish oil or various polyphenols was able to alleviate some of the effects of early life stress on the gut microbiome and hippocampal neuroplasticity-related gene expression. While diet has a heavy impact on shaping the gut microbiome (Johnson *et al.* 2019), it is currently unclear whether diet alone could be relied upon

to remodel the gut microbiota as a therapeutic in cases with extremely disrupted hippocampal neuroplasticity. Nonetheless, dietary reshaping of the gut microbiome may be able to prevent hippocampal neurogenesis-related issues or prolong health. Indeed, dietary supplementation with fish oil was recently strongly associated with a greatly reduced risk of developing dementia in elderly cohorts (Liu *et al.* 2022). The ability of fish oil supplementation to remodel the gut microbiome and alter neuroplasticity-related markers after early life stress (Chapter 4) suggests that fish oils may be promoting healthy cognition in aging via the gut microbiome, although this link would need to be established.

Ultimately, the continued elucidation of pathways by which the gut microbiota can remodel hippocampal neuroplasticity, as well as a greater understanding of how existing microbiota-targeted interventions shape host cognition and hippocampal plasticity will continue to drive new microbiota-based therapeutics. Advances in the development and understanding of probiotics, pathogenic bacteria, microbial species-specific antibiotics (including selective bacteriophages), and dietary interventions may allow medical treatments to move beyond the limitations and potential hazards of clinical FMT to improve host neuroplasticity and cognition.

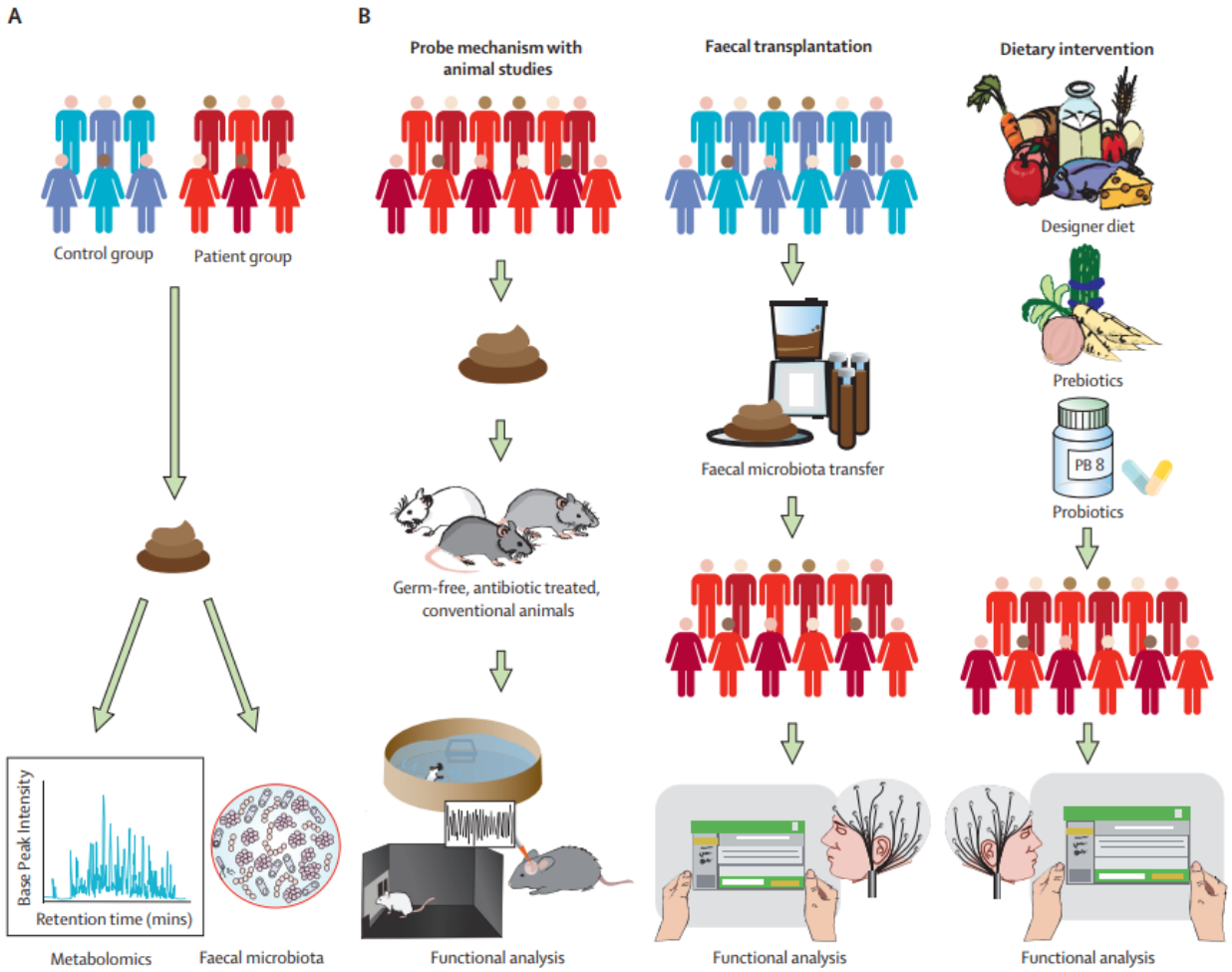


Figure 6.4. Potential strategies to harness the human gut microbiome towards hippocampal neuroplasticity-related discoveries. (A) The faecal microbiome of patient and control groups can be analysed for differences in microbial composition and the metabolome. (B) Three potential ways to study mechanisms of diseases and effects of potential treatments utilizing the gut microbiome. Microbial mechanisms of disease pathology may be probed by transferring the faecal microbiota of patients into naïve rodent recipients and assessing differences in hippocampus-depended cognitive behaviour and molecular and imaging techniques. If differences in the gut microbiome are implicated in the patients' condition, faecal transplants from healthy donors to patients, or transfer of a defined microbial consortia, could be beneficial for cognitive outputs. Dietary interventions through a designed diet, prebiotics, or probiotics could remodel the gut microbiota and/or the microbiota-gut-brain axis towards improved cognitive outcomes. Figure from (Cryan et al. 2020).

6.5.2 The importance of personalization

Every individual harbours a unique microbial ecosystem which exists in a dynamic state of flux, appearing to rely highly on the interplay of daily diet and host genetics (Johnson *et al.* 2019), but also by environment and lifestyle factors (Cryan *et al.* 2019b) making it highly important to understand the interactions between microbes and their host at an individual level. Differences in the gut microbiota function or malleability between individuals may therefore underly differences

in treatment response and lead to variability in outcomes of microbiota-based manipulations on the host (Ejtahed *et al.* 2017). Similar to how an organ transplant procedure requires similarities in the donor's and recipient's anatomy and physiology, the same receptiveness of an individual's body to a microbiota transplant or microbiome-based therapy exists. The idea of faecal microbiota biobanking, or cryogenically storing poo to later use as an FMT in the same individual to support health following gut microbiota disruption, is an interesting concept. This autologous (self-to-self) faecal transfer from a time when an individual mouse was calorically restricted has already shown to be more potent than a heterologous (donor-to-separate recipient) FMT at mitigating the effects of a high-fat diet on weight gain and adipose tissue (Perez-Matute *et al.* 2020). This data suggests that biobanking faeces from a time of health could be extremely valuable later in life, and potentially following the onset of cognitive decline or stress-induced decline in hippocampal neuroplasticity, although whether autologous faecal transfer has the ability to rescue the effects of stress or aging on hippocampal plasticity or related behaviours is currently unknown. Furthermore, given the ability to transfer specific phenotypes, such as depression, weight, or cognitive performance through gut microbiota transfer (Kelly *et al.* 2016; Kootte *et al.* 2017; Li *et al.* 2019b; Perez-Matute *et al.* 2020; Boehme *et al.* 2021; Knudsen *et al.* 2021), it would be extremely crucial to properly assess the state of the individual prior to biobanking faecal material.

Furthermore, the baseline state of the gut microbiome may hold great bearing on health outcomes following microbiota-based treatments. For instance, the gut microbiota composition has proven to dictate the response of individuals with metabolic syndrome to faecal microbiota treatment (Kootte *et al.* 2017), although whether this holds true in patients with disrupted hippocampal functioning is currently unknown. The responsiveness of the composition of the gut microbiome following insults or interventions (ie. volatility) may reveal insights into what approaches may be more viable in specific individuals (Bastiaanssen *et al.* 2021).

6.5.2.1 Considering the host organism: translating preclinical findings to personalised clinical practice

Assessing complex mechanistic interactions between the gut microbiome, hippocampal neurogenesis, and behaviour in clinical trials is currently impossible to do without preclinical research. Therefore, mice and rats have become the model of choice for research investigating the microbiota-gut-brain axis, including for trailing potential novel microbiota-based therapeutics to

combat neurodevelopmental and neurodegenerative ailments. However, the gut microbiome differs between species, and even strains, of animals (Goodrich *et al.* 2016), and taxonomic overlap at the strain level of the rodent and human gut microbiome appear as low as 10% (Kieser *et al.* 2022), which can hinder the translation of therapeutics from preclinical to clinical success. A multitude of factors are known to contribute to the differences observed in the gut microbiome between species, including differences in genetics, physiology, age, sex, diet, and other lifestyle and environmental factors, all of which can crucially impact microbe-host dialogue (Nguyen *et al.* 2015). For these reasons, not all microbe-host interactions revealed by rodent research translates to humans (Kelly *et al.* 2017). Moreover, heterogeneity within these host factors can influence how an individual responds to a microbial intervention. For instance, A/J and C57BL/6J mice respond differently to the consumption of a high-fat diet, and microbiota transplantation between strains is unable to overcome the impact of host genetics on these metabolic differences (Safari *et al.* 2020). Therefore, more attention needs to be given to the relationships between genetic and physiological host factors and their role in shaping host-microbe communication.

6.5.2.2 The relevance of biological sex in developing novel microbiota therapeutics

As highlighted in Chapter 5, wherein only female juvenile mice born by caesarean section displayed increased hippocampal neurogenesis, biological sex can be a determining factor in how perturbances to the gut microbiome effects hippocampal plasticity. Given that differences between sexes are noticeable in the gut microbiome (Jaggar *et al.* 2020), it is crucial to consider the importance of biological sex as a factor in microbiota-targeted therapeutics for hippocampal plasticity-related conditions.

Biological sex yields differences in circulating hormones, brain chemistry and function, and the immune system (Marquez *et al.* 2020; Kurth *et al.* 2021), which may influence mechanisms of microbiota-gut-brain signalling. Furthermore, many disorders and diseases have shown different incidence rates and symptomology within each sex, including depression and Alzheimer's Disease (Mazure & Swendsen 2016; Eid *et al.* 2019), which also have relevance for hippocampal neurogenesis and the gut microbiome, as previously discussed. Therefore, understanding how biological sex shapes differences in the gut microbiome and neuroplasticity, and whether this underlies differences in disease pathology or treatment responsiveness may be useful for deciphering potential novel therapeutics.

6.5.3 Does microbial engineering have a place for improving hippocampal neuroplasticity through the gut microbiome?

The discovery of the CRISPR-Cas9 complex revolutionized precise gene editing of microbial cells, including within the gut microbiome. Research published in the last year has shown that CRISPR-based technology can knock out specific microbial strains and deliver or inhibit functional genes within the gut microbiome *in vivo* (Lam *et al.* 2021; Jin *et al.* 2022). This novel technology can be applied to investigate unknown functions of bacteria and their genes *in vivo*, opening the door to a plethora of microbial discoveries. Therefore, applications of microbial genetic engineering techniques could revolutionize the diagnostics and treatments of disorders and diseases, including those in which hippocampal neuroplasticity or related cognition is disrupted. For instance, individuals diagnosed with major depressive disorder show lower level of serum tryptophan metabolite 5-hydroxytryptophan (Colle *et al.* 2020). Interestingly, symptoms of depression were improved in patients treated with the probiotic *Bifidobacterium breve M2CF22M7 (Tian et al. 2021),* which can synthesize 5-hydroxytryptophan (Tian *et al.* 2019a). While the mechanism(s) by which *Bifidobacterium breve M2CF22M7* may impact hippocampal neurogenesis are not understood, the mediation of antidepressant action by hippocampal neurogenesis (Santarelli *et al.* 2003) raises the possibility that increased hippocampal neurogenesis might be involved in the ability for *Bifidobacterium breve M2CF22M7* to attenuate depression symptoms. With this knowledge, it would be interesting to separately determine whether the depletion of gut microbial genes required for 5-hydroxytryptophan synthesis is sufficient to induce a depressive phenotype and alter hippocampal neuroplasticity, and alternatively, whether different bacteria engineered with CRISPR-based technologies to harness the ability to synthesize 5-hydroxytryptophan can enhance hippocampal neurogenesis and improve depressive symptoms. The recent cutting-edge advancements in *in vivo* microbiome genetic manipulation (Lam *et al.* 2021; Jin *et al.* 2022) offers exciting potential for mechanistic discovery and novel therapeutics within the future of the microbiota-gut-brain axis field.

Nonetheless, when considering genetic modification, it is critical to be mindful of the environmental implications that novel microbial strains could have. The possibility that genetically modified pathogenic bacteria, or bacteria with unknown harmful effects could leak from a lab and be consequential for humans, other animals, or environmental health necessitates that research within this scope must be stringent. Furthermore, the potential off-target effects of novel gene

modification technologies must be deeply understood as they are developed to target the gut microbiota. Therefore, while microbial engineering will continue to prove useful in understanding the interactions between the gut microbiota and its host, realising its full potential will take time due to the need to mitigate potential environmental hazards.

6.6 Perspectives and conclusions

In summary, the research presented within this thesis utilizes several preclinical approaches to further understand the relationship between the gut microbiota and hippocampal neuroplasticity, with an emphasis on hippocampal neurogenesis, throughout the lifespan. This research highlights the potential for gut microbiota-targeted interventions for rejuvenating aspects of the aging brain, including within the hippocampal transcriptome and metabolome, and hippocampus-dependent spatial learning and memory, although the consequence of aging on the survival of newly born hippocampal neurons was irreversible with the FMT. Furthermore, this thesis screened microbial consortia which were newly identified *in silico* to potentially contribute to cognitive health or decline in aging, but found these microbial consortia had no impact on behaviour when transplanted into a young rat recipient, highlighting the limitations of our existing knowledge of the microbiota-gut-brain axis and host cognitive behaviour. This thesis investigated the lasting impacts of early life gut microbiota perturbance via maternal separation stress on hippocampal neuroplasticity markers in adulthood, and the gut microbiome and its predicted functional ability within the scope of the gut-brain axis and found lasting impacts of stress on hippocampal *BDNF*, *Ki67,* and *Grin1a* gene expression. Meanwhile, targeting the gut microbiota via fish oil, fluoxetine, or polyphenol dietary interventions was shown to reverse or differentially alter the expression of key hippocampal genes related to neuroplasticity, as well as features of the gut microbiome, following early life stress. By correlating the effects observed on hippocampal gene expression with the composition and predicted function of the gut microbiota, data within this thesis highlights key bacterial genera which may be relevant to hippocampal neuroplasticity and stress. Finally, this thesis demonstrated for the first time that hippocampal neurogenesis is disrupted in female mice born by caesarean section – a sex-specific effect which was prevalent in both male and female caesarean section born mice that were also exposed to early life maternal antibiotics, highlighting a potential mechanism by which disruption of the postnatal gut microbiota via caesarean section may be able to impact host behaviour.

Overall, the research conducted within this thesis highlights the importance of the gut microbiota in maintaining hippocampal functioning, hippocampal neurogenesis and neuroplasticity, and lays the foundation for novel microbiota-targeted therapeutic discoveries in early life and aging. The next five years will lay the inroads for understanding the deeper mechanisms for how individual microbes influence neuroplasticity and brain function, and pave the way for translating preclinical finds towards medical human interventions.

"*As for the future, your task is not to foresee it, but to enable it."*

- Antoine de Saint Exupery

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