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Investigations into the Role of Microbiota-Gut-Brain Axis on Behaviour and Physiology in the Mouse

Thesis presented by

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Microbiome Ireland
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Contents

Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

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

Chapter 2: Dr. Gerry Moloney (UCC) performed cDNA extractions and PCR analysis of hippocampal samples.

Chapter 3: Dr. Emanuella Morelli (UCC) performed initial behavioural tests as well as FITC permeability tests along with Dr. Anna Golubeva (UCC). Dr Kiera Murphy (Teagasc) performed DNA extraction and shotgun sequencing of gut microbiota samples, Thomaz Bastiaansen (UCC) undertook bioinformatic analysis of gut microbiota data.

Chapter 4: Dr Ana-Paula Ventura-Silva performed behavioural tests in my absence, Dr. Marcel Van De Wouw (UCC) performed flow cytometry analysis of tissue and subsequent data analysis.

Chapter 5: Dr. Ken O'Riordan (UCC) and Dr. Henry Darch (UCC) undertook electrophysiological recordings and data collection and assisted with analysis of data.

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Publications and presentations

Published Manuscripts relevant to the thesis

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Cryan JF, O'Riordan, KJ, Cowan CSM, Bastiaanssen TFS, Boehme M, Codagnone MG, Cussotto S, Fulling C, Golubeva AV, Guzzetta KE, Long-Smith CM, Lyte JM, Martin J, Moloney GM, Morelli E, Morillas E, **O'Connor R**, Pereira J, Peterson VL, Rea K, Ritz NL, Sandhu KV, Sherwin E, Spichak S, van de Wouw M, VenturaSilva AP, Wallace-Fitzsimons SE, Hyland N, Clarke G, Dinan TG. (2019). The Microbiota-Gut-Brain Axis. Physiological Reviews *99*, 1877-2013

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Manuscripts in preparation relevant to the thesis

O'Connor, R. Moloney, G. Fulling, C. O'Riordan, K. Fitzgerald, P. Schellekens, H. Dinan, TG. Cryan, JF. Maternal Antibiotic Administration During a Critical Developmental Window Has Enduring Neurobehavioural Effects in Offspring Mice. To be submitted to Behavioural and brain function.

Other published Manuscripts

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Conference posters and Oral Presentations:

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Abstract

The growing recognition of the influence of the intestinal microbiota over the brain and behaviour has led to a shift in the world of neuroscience. Recent developments in the understanding of the microbiota-gut-brain axis have highlighted the importance of this bidirectional system of communication as well as its potential as a therapeutic target in psychiatric and neurological disorders. There is therefore growing interest in understanding the mechanisms that underpin the behavioural changes that have been observed and an increased knowledge of these mechanisms will be pivotal in the development of future therapeutic compounds that target the microbiota-gut brain axis.

In this thesis we explore the effects of modulation of the gut microbiota in early life through two distinct mechanisms. Firstly we assess the impact of maternal antibiotic administration on behaviour and physiology throughout the lifespan, comparing the effects of a single antibiotic and a cocktail of antibiotics. We observed effects on behaviour both in early life as well as in adulthood including tests of sociability, cognition, and anxiety-like behaviour. As regards to an underlying mechanism, alterations in hippocampal expression of BDNF were also observed following this treatment.

Next, we explored behavioural and microbiota alterations following Caesarean section delivery in a mouse model. Behavioural changes were found in anxiety-like behaviour, learning and memory, and antidepressant-sensitive behaviour. We also assessed impact of additional microbiota disruption in these animals through the administration of maternal antibiotics, finding that the further disruption led to exacerbation of behavioural effects as well as additional alterations in microbiota composition. Finally, we assessed whether administration of psychobiotics could reverse these behavioural disruptions, finding that antidepressant-sensitive behaviours were a particularly sensitive to these compounds.

In addition to assessing behavioural alterations after early-life modulation of the gut microbiota, we also studied behavioural differences in an aged model of autism spectrum disorder, the BTBR mouse, which is known to have an altered gut microbiota composition. Here we found that many of the behavioural alterations observed in these animals in youth are maintained in ageing, and that these sustained alterations may be linked to an increased immune system activation.

Finally, we sought to determine whether exposure of hippocampal slices to various concentrations of the gut microbiota metabolites short-chain fatty acids, can lead to alterations in synaptic signalling, using a multi-electrode array apparatus. It was observed that perfusion with a high concentration of sodium acetate (700μM) lead to a disruption in long-term potentiation (LTP) that is not seen in lower concentrations of the compound. This presents a potential link between gut microbiota metabolites and synaptic function in the brain that may shine a light on a key mechanism underpinning microbiota-gut-brain axis communication.

Overall these results highlight the importance of gut microbiota composition throughout the lifespan and particularly during key developmental windows such as early life. Our results also suggest that targeting the microbiota with psychobiotic compounds may be a viable therapeutic tool in cases where disruption of microbiota composition has occurred. Finally, we suggest that alterations in synaptic plasticity may be a key mechanism leading to the observed alterations in behaviour, providing an excellent target for future investigations into the microbiota-gut-brain axis.

Chapter 1. General Introduction

1.1. Bacteria and the brain

A link between bacteria and the brain has been recognised in medicine for many years, with examples including cases of neurosyphilis, where bacterial infection has been linked to cognitive and behavioural changes (Toptan et al., 2015) and also in the ability of antibiotic treatment to reverse cognitive deficits observed in hepatic encephalopathy (Wijdicks, 2016). Furthermore, in nature, instances such as *Orphiocordyceps unilateralis* have been noted. This fungus causes ants to risk life and limb, by hijacking the central nervous system and manipulating their behaviour, resulting in a more efficient spread of spores (Mongkolsamrit et al., 2012). Each of these examples demonstrates the relationship that exists between microorganisms and their hosts. They do not exist in isolation, but myriad interactions occur at sites throughout the body, driving the observed changes in behaviour and physiology.

As more has been learned about the microbiota, the trillions of bacteria that occupy every nook and cranny of the human body, the symbiotic relationship between host and organism has become apparent. While infection with pathogenic bacteria can have disastrous consequences, there also exists a large community of bacteria that are beneficial, and even necessary for healthy development (Sommer and Bäckhed, 2013). Our commensal bacteria shape our immune systems (Belkaid and Hand, 2014), allow us to extract nutrients from food that would otherwise be inaccessible (Clarke et al., 2014a) and protect against invasion from pathogens (Belkaid and Hand, 2014). The composition of the microbiota in the gut, the largest collection of microorganisms in (or more accurately, on) the human body (Belkaid and Hand, 2014) is particularly important for these effects

Research shows that the makeup of our gut microbiota also plays an important role in behavioural development (Cryan et al., 2019a; Desbonnet et al., 2015a; Diaz Heijtz et al., 2011; Fröhlich et al., 2016; Gareau et al., 2011; Hoban et al., 2018; Park et al., 2013). When this population is disrupted in animal models by antibiotic administration or raising animals in a sterile environment, for example, alterations in sociability, stress response or cognition are observed (Desbonnet et al., 2015a; Fröhlich et al., 2016; Guida et al., 2018b). Early investigations provided evidence that the microbiota can influence the brain and behaviour (Rex et al., 2006), without probing the mechanisms underpinning these effects. Recent studies have begun to focus on these mechanisms: routes of communication between the

gut and the brain (Guida et al., 2018b; Savignac et al., 2013), changes in gene expression in the brain (Blau et al., 2012; Desbonnet et al., 2015a; Distrutti et al., 2014a; Stilling et al., 2018), and more recently on how dendritic structure in the hippocampus may be altered by microbial signalling in the gut (Luczynski et al., 2016).

Through utilisation of various techniques for modulation of the microbiota, we will attempt to examine how differences in gut microbiota composition can lead to significant changes in brain and behaviour at discrete stages throughout life. Furthermore, we will examine whether administration of pre and probiotics may be able to positively modulate these effects. An particular area of interest is the potential role that the gut microbiota may play in synaptic plasticity whereby connections between neurons in the brain can be altered in strength in an experience-dependent manner. This introduction will pay particular attention to studies that suggest that disruption to the makeup of the microbiota through mode of delivery, antibiotic administration or prebiotic and probiotic administration can alter behaviour with links to alterations in synaptic plasticity.

1.1.1. The gut microbiota through the lifespan

The gut microbiota is the community of bacteria and other micro-organisms (archaea, fungi & viruses) that reside within the gastrointestinal tract and consists of 10^{13}-10^{14} organisms (Rinninella et al., 2019) – a figure in the same order as the number of human cells in the body (Bianconi et al., 2013). The gut microbiota consists of over 1000 species comprising more than 7000 strains (Ley et al., 2006a) of bacteria and, according to a study examining the human faecal microbiome of over 1200 individuals (Li et al., 2014) contains approximately 9.9 million genes. The metabolic products of these bacteria, along with the other by-products of their normal function are multitudinous, and have the potential to interact with the host and influence physiology (Sommer and Bäckhed, 2013).

Far from being exclusively a reservoir of pathogens, the gut microbiota plays a key role in a number of essential processes both during development as well as throughout life. Gut microbiota composition influences healthy maturation of the immune system (Fulde and Hornef, 2014; Olszak et al., 2012a; Round et al., 2010), protects against pathogen overgrowth (Kamada et al., 2013), maintains gut barrier function (Kelly et al., 2015) and intestinal endocrine function (Neuman et al., 2015) as well as nutrient absorption, fat distribution (Ley et al., 2006b) and synthesis of vitamins (LeBlanc et al., 2013). While gut microbiota composition remains relatively stable in adulthood, a number of factors can influence its

makeup. These include: diet (Murphy et al., 2015), antibiotic use (Fröhlich et al., 2016), drug use (Cussotto et al., 2019; Jackson et al., 2015), mode-of-delivery (Backhed et al., 2015), probiotics (Butel, 2014) and prebiotics (Burokas et al., 2017) Not only do these factors affect microbiota composition on a daily basis, but changes also occur across the lifespan.

Overall diversity, presence of particular species, and relative abundance of different phyla, all contribute to altered intestinal environments, as well as different microbiota-host interactions. For example; Bacteroidetes and Firmicutes are the major phyla observed in the human gut microbiota (Eckburg et al., 2005); differences in the relative ratios of these phyla lead to different energy extraction from the diet and have been linked to obesity (Ley et al., 2006b).

1.1.2. The gut microbiota in early life

Initial microbiota exposure establishes an early-life microbiota that forms a symbiotic relationship with the host and is pivotal in many aspects of normal development. The precise timing of this initial contact, however has been the subject of recent debate. Traditionally the *in utero* environment has been regarded as sterile and a neonate's initial colonisation thought to be determined following exposure at birth. This view was challenged with the discovery of bacterial species in the placenta of healthy mothers (Aagaard et al., 2014; Aagaard et al., 2012) and the amniotic fluid of preterm infants (DiGiulio et al., 2010) with both displaying low diversity and a proteobacteria-dominant profile. These studies stop short, however, of definitive evidence of a strict microbiota (i.e. a live, persistent and functional community of microorganisms) and indeed a recent study found that that the human placenta is devoid of a microbiota (though it can contain potential pathogens) (de Goffau et al., 2019). In either case, mode of delivery remains a key factor in initial colonisation with microbiota (Bäckhed et al.; Dominguez-Bello et al., 2010b). Vaginally-born babies have a microbiota resembling the vaginal microbiome, high in *Lactobacillus* and *Prevotella* species (Dominguez-Bello et al., 2010b). C-section delivered neonates have a "skin-like" microbiota dominated by staphylococcus, Corynebacterium, and Propionibacterium (Dominguez-Bello et al., 2010a). Other factors such as antibiotic use, breast-feeding or processed formula milk, will play a role in gut microbiota composition in early life (Borre et al., 2014b). In infancy, following the introduction of solid food, there is a decrease in the prevalence of *Bifidobacteria*, and an increased richness of diversity (Eckburg et al., 2005)

1.1.3. The gut microbiota in adulthood

Gut microbiota composition becomes more stable during childhood and at the age of around 10 the number of taxa and functional genes are approximately equivalent to what is seen in adulthood (Heiman and Greenway, 2016). A healthy adult microbiota composition is relatively stable across individuals (Huttenhower et al., 2012). In adulthood, lifestyle factors such as exercise (Mailing et al., 2019) , diet (Oriach et al., 2016), and antibiotic use (Langdon et al., 2016) are key in shaping the composition of the bacterial composition of the gut. Distinct differences in microbiota composition are noted in those with inflammatory bowel conditions such as Crohn's disease (Manichanh et al., 2006) and ulcerative colitis (Sokol et al., 2009), however and targeting the microbiota as a treatment for these conditions is a key aspect of gut microbiota research in adulthood.

1.1.4. The gut microbiota in ageing

With increasing age comes an increased prevalence of disease as well as an overall deterioration of homeostatic function (Lopez-Otin et al., 2013). In humans 'older persons' are regarded as those over 60 years of age, equating to approximately 20 months in an rodent model (Prenderville et al., 2015). In addition to alterations in telomere length, oxidative stress and dysregulated immunity among many other cellular and molecular alterations (Lopez-Otin et al., 2013) changes in gastrointestinal physiology are present in old age leading to compositional and functional changes of the microbiota (Konturek et al., 2015).

The relative stability of the adult microbiome deteriorates with increasing age (Claesson et al., 2011). Diet and exercise, two modulators of microbiota composition mentioned previously have an exaggerated effect in older individuals (Vauzour et al., 2017). Decreased gut microbiota diversity, generally associated with health has been linked to ageing (Biagi et al., 2010) and age-related impairments like frailty in humans (Claesson et al., 2012; Jackson et al., 2016). Surprisingly, however, aged (24-month old) mice have been shown to exhibit increased diversity compared to younger adult mice (Scott et al., 2017a). Studies in semi-supercentenarians (people between 105-109 years of age) have found specific taxa such as *Akkermansia* to be more abundant, suggesting distinct gut microbiota changes at this extreme of life might be promoting healthy aging and longevity (Biagi et al., 2016; van der Lugt et al., 2018). As a result of these findings the elderly population is at the core of research for prebiotic and probiotic interventional studies (O'Toole and Jeffery, 2015). The variations in gut microbiota composition throughout the lifespan mean that the function of the bacteria

between these periods will also vary. The huge number of microbial genes present in the gut results in a large array of metabolic products (Sridharan et al., 2014) with many of these products having the capability to interact directly or indirectly with the host (Sridharan et al., 2014), leading to suggestions that the microbiota effectively acts as an additional endocrine organ (Clarke et al., 2014b). Among the compounds known to be produced by the microbiota are short-chain fatty acids (SCFAs) (MacFabe, 2012; Macfarlane and Macfarlane, 2012), gastrointestinal hormones (Holzer et al., 2012), cortisol (Dinan and Cryan, 2012), precursors to neuroactive compounds, as well as neurotransmitters themselves (including GABA (Komatsuzaki et al., 2005), serotonin (Yano et al., 2015a), and noradrenaline (Lyte, 2013)). While these neurotransmitters are unlikely to reach the brain to directly affect central neuronal signalling, they may influence signalling in the local, enteric nervous system (Clarke et al., 2014b). Other products of the microbiota are capable of communicating with the brain via various routes, as will be discussed in a subsequent section.

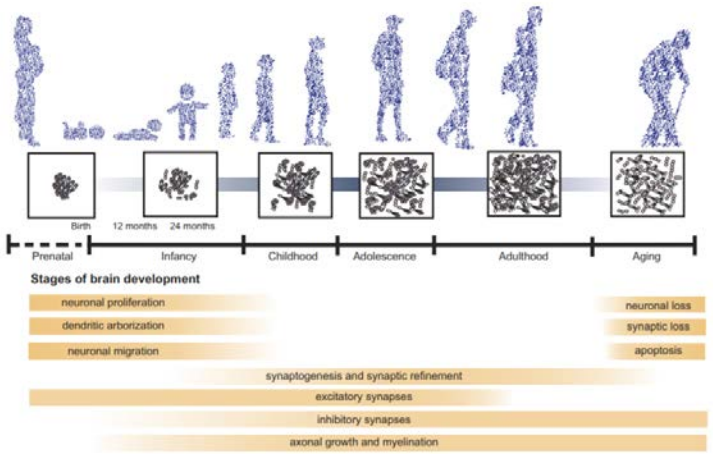

Figure 1: The gut microbiota across the lifespan. *The timeline graph indicates changes in microbial diversity across the human lifespan, from birth through to ageing, including infancy, childhood, adolescence and adulthood. This is accompanied by typical changes in neural development indicating concomitant neuronal processes occurring during specific stages of life. Figure taken from Cryan at al. (2019).*

1.2. Studying the Microbiota-gut-brain axis

How alterations in microbiota composition can translate into differences in the central nervous system and in behaviour is a critical question being addressed by much of the current research into the microbiota-gut-brain axis. Communication between the gut and

the brain occurs via multiple routes (Forsythe et al., 2014b; Mayer, 2011) which may be nervous (vagus nerve and enteric nervous system), humoral, or immune. A significant feature of each is that communication is bi-directional (Cryan and Dinan, 2012c). Disruptions in microbiota composition and subsequent changes in bacterial metabolites etc. lead to an altered gastrointestinal environment which can in turn influence the brain. Altered psychological states signal to the gut (Karl et al., 2018) which may lead to a modulation of factors such as gastric motility (Park et al., 2013), with the potential to effect microbiota composition. The routes of communication between the gut and the brain will be discussed in detail in a subsequent section (1.3)

1.2.1. The influence of the gut microbiota on behaviour

Preclinical models of behaviour are an essential aspect of determining the effects of alterations of the microbiota on the brain. By modifying the gut microbiota through various routes including the use of germ-free animals (Neufeld et al., 2011a), antibiotic administration (Hoban et al., 2016b), probiotic administration (Messaoudi et al., 2011), prebiotic administration (Burokas et al., 2017) and faecal microbiota transfer (FMT) (Kelly et al., 2016b) effects on behaviour can be studied in animals.

In order to undertake these assessments, a number of behavioural tests have been developed in animals that allow various aspects of behaviour to be studied and we will now discuss the behavioural tests used in this thesis in greater detail.

1.2.1.1. Behavioural tests in early life

Behavioural tests carried out in early life allow for assessment during a key developmental window, while animals are still under the care of their mother. One such test is isolation-induced ultrasonic vocalization (USV). In this test the number and frequency of vocalizations are measured following separation from their parents.

In neonates, these vocalizations are used to attract adult animals (Motomura et al., 2002). The USV can be used as a measure of early communicative behaviour and as an aversive affective measure of separation stress (Scattoni et al., 2009b). They also serve as a measure of social development (Grimsley et al., 2011). When the microbiota from high-fat-diet-fed mice was transplanted to mothers prior to breeding and the USV characteristics of the offspring measured, it was found that these mice vocalized less upon maternal separation than pups from control dams (Bruce-Keller et al., 2017). This indicates that microbiota composition can affect sociability at this early stage.

A second behavioural test carried out during early life is the homing test that measures attachment to maternal bedding. This behaviour establishes basic parameters for later social interactions. The homing test paradigm serves as a test to assess social behavioural deficits in early life. It is a measurement of neonatal social recognition and motivation towards a relevant social stimulus. The premise of this test is that recognition of maternal odours is vital for mother-offspring pairing through facilitating the establishment of social behaviours. Mouse pups at P10 have demonstrated the ability to recognize pertinent social stimuli and move towards their mother's nest when removed from it (Macri et al., 2010). This pairing between mother and offspring is important for the development of social behaviours (Brennan and Kendrick, 2006). Preference for maternal bedding has been shown to be reduced following c-section delivery (Morais, 2018)

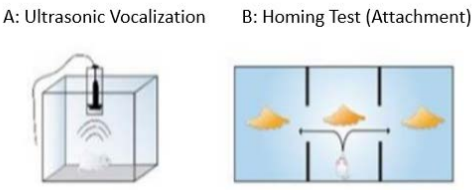

Figure 2: Early-life behavioural paradigms used in this thesis. *(A) Ultrasonic vocalization apparatus (B) Homing test apparatus.*

1.2.1.2. Tests of anxiety-like behaviour

Multiple tests have been developed in order to attempt to model aspects of human anxiety in rodents. While many of these induce a fearful response through an aversive event or anticipation of an aversive event, others utilise an approach-avoidance conflict in order to influence an ongoing behavioural characteristic for the animal (such as the contrasting tendency of mice of mice to engage in exploratory activity or social investigation against the aversive properties of an open, brightly lit, or elevated space) (Buccafusco, 2008). Here we utilise both of these properties across various behavioural tests to determine effects on anxiety-like behaviour.

The defensive marble-burying test (MBT) measures compulsive, repetitive and anxious behaviours, with a greater number of marbles buried representing increasing levels of anxiety, regarded as neophobic behaviour (Wolmarans et al., 2016). Behaviour in this test

can be influenced by the administration of prebiotics and probiotics (Kantak et al., 2014; Nishino et al., 2013; Savignac et al., 2014).

The elevated plus maze is perhaps the most frequently used test for anxiety-like behaviour (Rodgers and Dalvi, 1997). The EPM is named as such because the raised apparatus consists of 4 arms that form a cross or 'plus' shape, with 2 'open arms' facing each other, and 2 'closed' arms facing each other. Closed arms have walls, whereas open arms do not. An increased in the amount of time spent in the 'open' arms is regarded as indicating a reduction in anxiety-like behaviour (Treit et al., 1993), an increased number of entries to the open arms can be similarly regarded (Pellow et al., 1985). Alterations in this test have been linked to almost every aspect of gut microbiota modulation including germ-free (Diaz Heijtz et al., 2011) and antibiotic-treated mice (Frohlich et al., 2016).

The open field test was originally designed to evaluate locomotor activity and involves placing an animal in an unknown environment with surrounding walls. It has been adapted, however to include measures of anxiety and stress reactivity to a novel environment (Ennaceur and Chazot, 2016). Animals that display higher levels of anxiety tend to stay on the periphery of the apparatus and avoid the centre of the arena. In order to generate a more aversive environment, a bright light can be applied, this exacerbates fear as rodents feel more vulnerable to predators in brightly lit, open environments (Godsil et al., 2005). Anxiety-like behaviour in this test can be reduced following prebiotic administration (Burokas et al., 2017).

The light-dark test acts as a measure of anxiety-like behaviour and again exploits the conflict between the tendency of mice to explore novel environments, and tendency to avoid open, brightly-lit areas (Bourin and Hascoet, 2003). Here mice are placed into a bright arena with a dark chamber at one side and the amount of time spent in each arena, the number of exploratory rearings, and the number of transitions between the arenas measured. More time spent in the dark chamber is indicative of increased anxiety-like behaviour (Takao and Miyakawa, 2006). The effects of microbiota modulation are uncertain in this test and when it has been undertaken no changes were observed (Gareau et al., 2011).

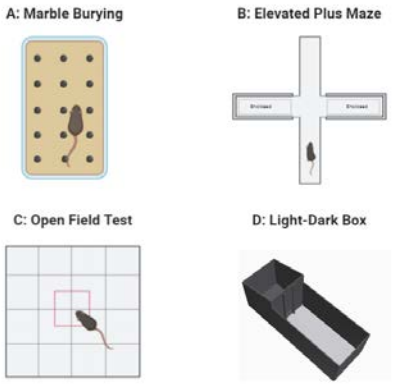

Figure 3: Tests for various aspects of anxiety-like behaviour used in this thesis. *(A) Defensive marble burying apparatus (B) Elevated plus maze apparatus (C) Open field test apparatus (D) Light-dark box apparatus.*

1.2.1.3. Tests of learning and memory

Rodent behavioural tests assessing learning and memory form an integral part of measuring the potential for treatments to improve cognition, or to slow the rate of cognitive decline. The novel-object recognition test is used to determine hippocampal-dependent memory (Lueptow, 2017). Initially, animals are allowed to become familiar with the arena in order to reduce impaired performance due to neophobia. Following this, animals are then allowed to explore the same arena with two objects placed in adjacent corners. The final part of the test harnesses the inherent preference of nice for novelty (Au - Denninger et al., 2018) through assessing the time spent with the novel and familiar objects.

Numerous studies of antibiotic disruption of the microbiota have found this intervention to adversely affect performance in the novel object recognition test (Desbonnet et al., 2015a; Fröhlich et al., 2016; Guida et al., 2018a; Möhle et al., 2016).

The Y-maze test of spontaneous alteration is a test of the willingness of a mouse to explore a new environment. Rodents typically prefer to visit a new arm of the maze than return to one that was previously visited. It acts as a test of hippocampal-dependent spatial memory (Sarter et al., 1988). A probiotic mixture of two *lactobacillus* strains has shown to reverse age-dependent reductions in spontaneous alternations (Jeong et al., 2015).

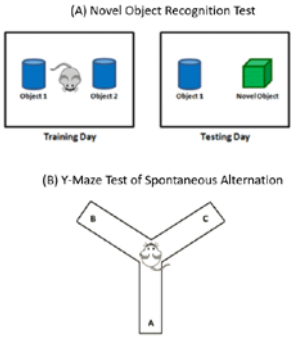

Figure 4: Tests of learning and memory used in this thesis *(A) The apparatus used in the novel object recognition test (B) The apparatus used in the y-maze test of spontaneous alternation.*

1.2.1.4. Tests of sociability

The three chamber test was originally proposed to evaluate social approach behaviours relevant to social disorders such as autism spectrum disorder, social phobias, anxiety and schizophrenia (Moy et al., 2004a). It consists of a three-chambered apparatus where mice are given the choice between spending time in a chamber containing an age-matched mouse of the same strain, a chamber containing an object, or in an empty compartment (Crawley, 2007). Rodent tendency to spend more time with another rodent as compared with an object or in an empty compartment represents sociability. An important aspect of social behaviour that can also be investigated in the three-chamber test is social novelty (Kaidanovich-Beilin et al., 2011). Normally, when rodents are given the choice between spending time with a novel and familiar mouse, they express a preference for the novel. Therefore, in animal models that mimic social deficits (such as ASD) these animals usually display a deficit in sociability and/or decreased preference for social novelty (Crawley, 2012).

Assessment of sociability using this test has highlighted the pivotal role that the microbiota plays in development of normal social behaviour. The absence of a microbiota from birth corresponds with impaired sociability and social memory in adulthood (Desbonnet et al., 2014). Modulation of the microbiota using antibiotics can also lead to significant effects on sociability with perinatal maternal antibiotic administration leading to deficits in sociability in the offspring (Leclercq et al., 2017b) while antibiotics in adulthood can disrupt the ability to distinguish social novelty (Guida et al., 2018b). Probiotic treatment can restore normal

social behaviour in animals where is disrupted, further highlighting the significant role for the microbiota in these behaviours (Buffington et al., 2016).

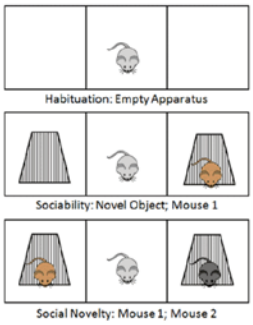

Figure 5: The three chamber test for social behaviour. *Initially animals are allowed to habituate to the apparatus (top figure), following this they are given the choice between interacting with a mouse, an object or an empty chamber – testing for sociability (middle figure). Finally, the object is replaced with a novel mouse – this tests for social novelty preference (bottom figure).*

1.2.1.5. Tests of antidepressant-sensitive behaviour

The forced-swim test (FST) acts as a measure of antidepressant-sensitive behaviour in rodents. The test is based on the observation that rodents placed in an enclosed cylinder filled with tepid water will initially engage in vigorous escape-orientated movements, followed by increasing bouts of immobility. It is one of the most widely used behavioural paradigms and was initially developed for the screening of novel antidepressant therapies. Indeed, the pre-administration of antidepressants reverses the immobility phenotype in rodents (Cryan and Holmes, 2005). In addition to attenuating FST-induced immobility, specific antidepressants have also been demonstrated to recover HPA axis response in rats (Connor et al., 2000) when this is measured following the test.

Certain probiotic and prebiotic interventions have also been shown to reduce antidepressant-sensitive behaviors in rat and mouse models in this test, in addition to ameliorating inflammatory responses (Bravo et al., 2011b; Burokas et al., 2017; Desbonnet et al., 2010b; Tillmann et al., 2018)

Fig 6: *Apparatus used in the forced-swim test*

1.2.2. Neurochemical analysis

Neurochemical analysis has contributed greatly to our understanding of the changes that occur in the brain following modulation of the gut microbiota and how these may be driving behavioural alterations.

Brain-derived neurotrophic factor (BDNF) is perhaps the most ubiquitous neurochemical analysed in studies of the microbiota-gut-brain axis. This protein acts to support neuronal survival, growth, and differentiation of neurons (Huang and Reichardt, 2001) as well as playing an important role in memory (Bekinschtein et al., 2008). Levels of BDNF have been shown to be reduced in the brain of germ-free mice in regions including the cortex, hippocampus (Sudo et al., 2004c), amygdala (Neufeld et al., 2011b) and the hypothalamus (Schele et al., 2013) highlighting how commensal bacteria may contribute to learning and memory. Antibiotic treatment can reduce hippocampal BDNF levels (Desbonnet et al., 2015a; Guida et al., 2018b) while probiotic treatment can increase hippocampal BDNF (Lee et al., 2018a). This is not an exhaustive list, and other studies have seen opposing effects on BDNF levels in the brain (Gareau et al., 2011), however, it serves to illustrate the impact that alteration of the microbiota can have over a key neurochemical messenger in the brain.

Levels of neurotransmitters including noradrenaline, dopamine, serotonin (Burokas et al., 2017; Diaz Heijtz et al., 2011; Liang et al., 2015) are altered in animals where gut microbiota composition is disrupted compared to controls. These measurements are made postmortem, therefore results represent changes at the timepoint at which animals are culled. An interesting development in this area may be the use of microdialysis to assess neurotransmitter levels at specific points during treatment or following an infusion of microbiota metabolites (SCFAs, for example). This technique allows for rapid analysis of the neurotransmitter state of a given brain area at a particular time (Chefer et al., 2009) for example, following infusion of SCFAs to the gut, or directly following a probiotic gavage –

vastly improving knowledge about short-term alteration in response to gut microbiota modulation.

1.2.3. Brain imaging

A variety of different brain imaging techniques have been used to understand the microbiota-gut-brain axis. Using magnetic resonance spectroscopy (MRS), it has been shown that the bacterial strain *L. rhamnosus*- JB-1 was capable of increasing the neurotransmitter glutamate and its precursor glutamine in addition to N-acetyl aspartate and GABA (Janik et al., 2016). In a recent study, diffusion tensor imaging was used to identify global changes in white matter structural integrity occurring in a diet-dependent manner in rats (Ong et al., 2018); whilst not surprising, microbiota analysis indicated changes in bacterial populations as a function of diet.

1.2.4 Organoid and in-vitro models

While the use of animal models is invaluable to the progression of knowledge regarding the microbiota-gut-brain axis, there are ethical concerns with the use of the large number of animals that are required to generate sufficiently powered studies. As such there has been a drive to improve data that is generated from cellular and *in-vitro* models.

To date, the use of *in vitro* models in the study of the microbiota-gut-brain axis has generally focused on aspects such as exposure of cell lines to microbiota metabolites, for example to screen for the effects on G-protein coupled receptors (Colosimo et al., 2019). The application of butyrate to a cell culture has been shown to increase production of serotonin from colonic enterochromaffin cells, for example (Yano et al., 2015b). Additional *in vitro* studies have assessed the antimicrobial activity of non-antibiotic drugs (Gunics et al., 2002; Gunics et al., 2001; Kruszewska et al., 2000, 2002).

An exciting recent development is the advent of the 'organ on a chip' in the context of the gut brain axis. These systems are miniature bioreactors of millimetric dimensions that combine existing 'lab on a chip' technology with 3D organotypic cell culture under constant perfusion (Bhatia and Ingber, 2014). In particular, the 'MINERVA' (MIcrobiota-Gut-BraiN EngineeRed platform to eVAluate intestinal microflora impact on brain functionality) technology aims to represent all the key elements of the microbiota-gut-brain axis *in vitro* for the first time. This system uses five individual 'organ on a chip' devices to account for the key components of the axis (the gut microbiota, the gut epithelium, the immune system, the BBB, and the brain) (Raimondi et al., 2019). While still in its infancy and serving as a vast simplification of the *in vivo* environment, such technologies allow for high-throughput

screening of novel probiotic cocktails or the effects of microbiota metabolites before moving to animal models, of particular use in neurodegenerative disorders such as Alzheimer's disease or Parkinson's disease (Raimondi et al., 2019).

1.2.5: Electrophysiology

Another area that is starting to be explored is the potential impact that gut microbiota composition may exert over neuronal signalling in the brain (Distrutti et al., 2014a). Since electrical signalling between neurons controls activity in circuits throughout in the brain no behavioural changes can occur without a change in synaptic signalling.

When patterns of synaptic activity result in changes in synaptic strength, the process is referred to as 'synaptic plasticity' and it is known to play a role in learning and memory as well as other aspects of behaviour (Duman et al., 2016; Neves et al., 2008). The physical alterations underpinning synaptic plasticity occur both presynaptically and postsynaptically, and these effects may be monitored using numerous techniques. These range from observation of structural alterations including changes in hippocampal spine density, to monitoring expression of plasticity-related genes in areas of the brain known to control behaviours of interest. The gold-standard method for measuring synaptic plasticity is electrophysiological recordings. These provide real-time, highly-sensitive measurements of communication between neurons in living tissue (Koch, 2006). Developments in this technology in this field have meant that it is now possible to record from multiple electrodes simultaneously, vastly increasing the quantity of data that can be recorded at a single time, an example of this being multi-electrode array (MEA) recordings.

These tools consist of organizations of electrodes arranged such that information can be gathered from the entirety of a brain slice (Shaban et al., 2017a). The high-frequency recording that this allows for over multiple channels (up to 252) as well as being able to record from many sites at variable distances and across multiple circuits and networks (including the hippocampus and the hypothalamus – fig 7). MEAs can be used to record activity in both cultured neurons and organotypic brain slice preparations as well as ex-vivo slices (Ness et al., 2015; Obien et al., 2015) and allow for the investigation of activity dynamics of large groups of neurons at both high temporal and spatial resolution (Franke et al., 2012; Rolston et al., 2010). Multi-electrode arrays have been used to probe cellular and molecular correlates of a wide range of neurophysiological processes including the neuronal basis of pain, odour perception, hypothermia, as well as synaptic plasticity mechanisms (le Feber et al., 2010; Li et al., 2007; Ling et al., 2010; Rubinsky et al., 2007) . In addition, MEAs

have been successfully used to investigate functional dynamics of the cerebellar cortical networks (Nicholson and Llinas, 1975), the neocortex (Lu et al., 2013) and hippocampal circuits (Chong et al., 2011; Tominaga et al., 2001). To date, however, this technology has not been used to address the link between the gut microbiota and synaptic activity the brain. We aim to harness it in order to assess alterations in synaptic signalling following exposure to gut microbiota metabolites.

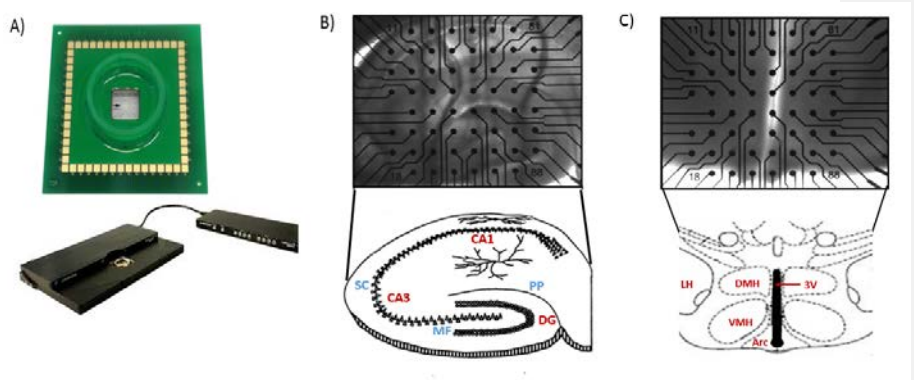

Figure 7: In vitro multi-electrode array (MEA) system. *A) Top: Glass MEA chip (MEA60-200-30-3D,Qwane Biosciences) Bottom: Multi-electrode array setup, consisting of headstage (amplifier and stimulator) & interface board (MEA2100, multichannel systems). (B) Micrograph image displaying a hippocampal brain slice (C) hypothalamic slice mounted on the MEA chip (in an 8 x8 arrangement) with schematic representation. Abbreviations: CA1, CA3, and dentate gyrus (DG) regions within the hippocampus and its associated pathways: Schaffer collateral (SC), mossy fiber (MF), and perforant pathway (PP), Lateral hypothalamus (LH), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), arcuate nucleus (Arc) and the 3rd ventricle (3V)*

Strong evidence linking gut microbiota composition to cognition (Frohlich et al., 2016; Proctor et al., 2017) suggests that gut bacteria can affect signalling in the brain, particularly in the hippocampus, the area of the brain playing the most significant role in learning and memory. Results such as these have spurred on the rush to provide concrete links between the microbiota and synaptic plasticity.

Studies in germ-free (GF) animals, have been shown to have physical differences in brain structure that may relate to synaptic plasticity (Luczynski et al., 2016). GF animals display morphological changes in both hippocampus and amygdala (Luczynski et al., 2016) while also having alterations in dendritic length and spine density in the hippocampus. Interestingly,

when animals were administered a high-fat diet, a factor known to alter gut microbiota composition (Turnbaugh, 2017) reductions were observed in hippocampal spine density, with these effects being reversed following probiotic administration (Chunchai et al., 2018b). In addition to physical alterations affecting synaptic plasticity, GF animals have been shown to have altered expression of genes involved in synaptic transmission, plasticity and synaptic morphology in the hippocampus (Chen et al., 2017) and the amygdala.

More recently a number of studies have started to look at direct effects on electrophysiological parameters following microbiota modulation using various methods of manipulation. An early study used a probiotic mixture as a treatment. When VSL#3 (a commercially-available mixture of eight gram positive bacteria) was administered to aged rats (20-22 months) for six weeks, the age-related decrease in LTP in control rats was reversed to levels similar to those of young animals (Distrutti et al., 2014a). Furthermore, treatment with this probiotic mix was able to modulate the expression of plasticity-related genes in the cortex leading to increases in BDNF and synapsin levels (Distrutti et al., 2014a). A similar study demonstrated that treatment with a probiotic mixture of *Lactobacillus acidophilus*, *Bifidobacterium lactis*, and *Lactobacillus fermentum* was able to rescue a loss of LTP observed in a rat model of diabetes (Davari et al., 2013). In both of these studies, LTP measurements were carried out '*in vivo*' thereby presenting a direct readout of synaptic function in an anaesthetized animal.

Since gut microbiota composition is hugely influenced by diet, administration of different diets and various dietary components has been widely used as a method to modulate gut microbiota composition. Following the administration of a high-fat diet to rats, hippocampal LTP was impacted (Chunchai et al., 2018b). A twelve week treatment with either a probiotic (L. paracasei), a prebiotic (Xylooligosaccharide, XOS) or a symbiotic combination of the two was able to successfully reverse these deficits in synaptic plasticity, while also reversing inflammation in the CNS (Chunchai et al., 2018a) . This study, along with a study of antibiotic administration in a mouse model of colitis find that gastrointestinal inflammation is correlated with reductions in LTP (Riazi et al., 2015a) and that modulation of the gut microbiota in these inflamed states correlated with reduced inflammation as well and a concomitant improvement in LTP traces (Chunchai et al., 2018b; Riazi et al., 2015a). The negative effects of high-fat diet on LTP and gut microbiota composition have also been demonstrated in mice (Hwang et al., 2010; Karimi et al., 2013; Liu et al., 2015). These effects of diet have been observed across generations, with administration of a high-fat diet to mothers during pregnancy leading to impaired synaptic plasticity in the ventral tegmental

area of offspring compared to offspring whose mothers received a normal diet (Buffington et al., 2016). Furthermore this diet led to alteration of gut microbiota composition, impaired social behaviour and decreased oxytocin expression in the paraventricular nucleus of the hypothalamus in maternal high fat diet offspring. These abnormalities could be reversed, however, following the administration of a single probiotic strain, *Lactobacillus reuteri* (Buffington et al., 2016).

As mentioned in section 1.1.2, mode of delivery is a key determinant of microbiota composition in early life. A recent study aimed at determining whether term, or preterm caesarean section delivery caused any long-term synaptic effects in mice (Chiesa et al., 2018) found that c-section delivery does not alter the frequency or amplitude of glutamatergic or GABA-ergic postsynaptic currents in hippocampal pyramidal neurons (Chiesa et al., 2018), however.

Short-chain fatty acids are the most widely studied gut microbiota metabolite, and will be discussed in detail in section 1.3.5.1. These compounds have also been studied in relation so synaptic plasticity. Beneficial effects of the SCFA, butyrate on LTP induction have been demonstrated (Levenson et al., 2004a). This study demonstrated that both butyrate as well as the histone deacetylase inhibitor, trichostatin A, were able to enhance induction of long-term potentiation. Subsequent research has highlighted the positive impact of histone deacetylase inhibitors on synaptic function (Basu et al., 2019) in disease models where normal signaling activity is disrupted.

In our study we aim to utilize the multi-electrode array in order to probe the effect of short-chain fatty acid application to hippocampal slices. We look to determine whether different SCFAs have the ability to exert different effects on these slices as well as assessing whether any observed effects are dose-dependent. Multi-electrode array technology will then be utilized to determine whether different aspects of signaling, including basal synaptic transmission, input-output, short-term plasticity and long term plasticity are affected following SCFA application.

1.2.6. Other Model organisms

While studies in rodents account for the vast majority of research undertaken assessing the microbiota-gut-brain axis various other organisms have also proven useful in shedding light on the topic.

One example of such a model organism is the zebrafish (*Danio rerio*). When these animals are raised in a germ-free environment, this leads to alterations in locomotor activity and

social behaviour (Davis et al., 2016). Similarly, exposure to antibiotics has been shown to increase anxiety-like behaviours as well as decreasing social behaviour(Wang et al., 2016). Treatment of zebrafish with probiotics has been shown to anxiety-related behaviour (Davis et al., 2016) as well as altering social behaviour and BDNF expression (Borrelli et al., 2016). That many of the changes see in rodent models are also present in zebrafish speaks to the significance of the microbiota across the animal kingdom. Advantages of this model organism are that due to its environment, it is very easy to control for factors such as age, sex, diet, and treatment compliance. Furthermore, it has a very easily manipulated genome.

Another non-mamalian model that has proven useful to the study of the microbiota-gut-brain axis is the fruit fly (*Drospohila melanogaster*). In these animals microbiota composition has been linked to preference in selecting a mate (Damodaram et al., 2016; Sharon et al., 2010). Advantages of the fruit fly are the ease of controlling for age, sex, diet and treatment compliance. Furthermore, evolutionarily conserved neurological pathways are easily isolated in these animals. Once again they also have an easily manipulated genome making rapid assessments of genetic alterations possible (Pandey and Nichols, 2011). A major caveat with fruit flies is that they have a vastly different gastrointestinal tract to mammals, while also having a vastly different adaptive immune system (Flemming, 2017). While these models do provide useful knowledge regarding the brain, behaviour and links to the microbiota-gut-brain-axis their limited translational ability is a significant disadvantage, and, as such their use will be mentioned only sparingly during the subsequent discussion.

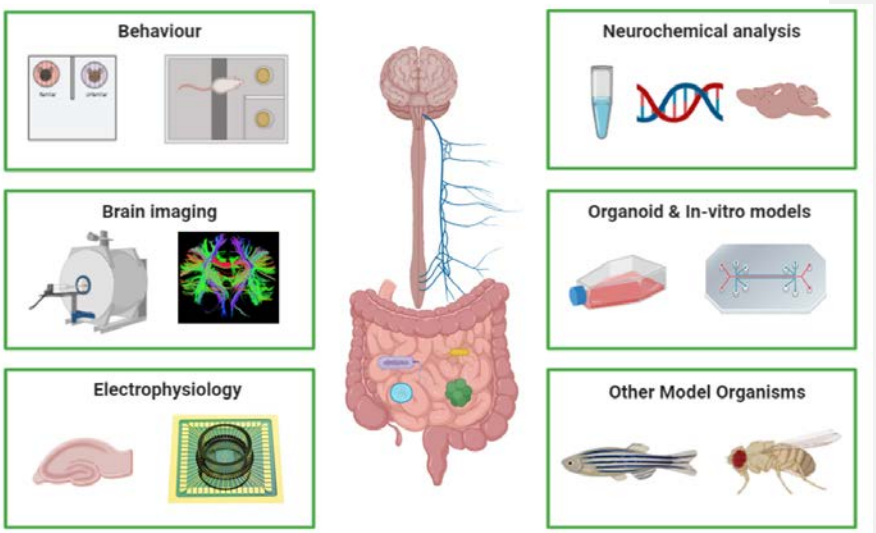

Figure 8: Methods to study the microbiota-gut-brain axis preclinically*. Behaviour can be assessed in rodent models following microbiota modulation. Neurochemical analysis can assess chemical and genetic expression in the brain. Brain imaging provides information on alterations in brain structure and activity. Organoid & in-vitro assessments can address effects of bacteria and their metabolites on cellular and organoid systems. Electrophysiology measures alterations in plasticity and electrical signalling in brain slices or in-vitro. Other model organisms can provide additional information on the gut-brain-axis following microbiota modulation.*

1.3. Microbiota-gut-brain axis routes of communication

1.3.1: Vagus nerve

The tenth cranial, or vagus nerve descends from the brainstem to the distal intestines (Berthoud and Neuhuber, 2000a). The vagus nerve is the principal component of the parasympathetic nervous system and is a mixed nerve, composed of 80% afferent and 20% efferent fibres (Bonaz et al., 2018). It extends widely around the periphery exerting effects on the heart, lungs and gut among other visceral organs (Berthoud and Neuhuber, 2000a). The vagus nerve transmits information from these organs to the brain (bottom-up signalling) as well as providing feedback from the brain (top-down signalling) (Breit et al., 2018).

Within the gastrointestinal tract, the vagus has neuronal terminals in each of the intestinal plexa as well as afferent fibres within the small intestine and mucosa which are able to identify the presence of chemical messengers in the gut (Berthoud, 2008; Berthoud and Neuhuber, 2000b). Chemosensors of the vagal afferents have been shown to be sensitive to multiple molecules, including GLP-1 (Berthoud, 2008; Berthoud and Neuhuber, 2000b), IL1B

(Berthoud and Neuhuber, 2000b) 5HT (Berthoud, 2008; Berthoud and Neuhuber, 2000b) somatostatin, CCK & PYY (Berthoud, 2008; Berthoud and Neuhuber, 2000b).

Signals from vagal afferents are integrated at the nucleus tractus solitarius (NTS) of the dorsal vagal complex in the brainstem (Berthoud, 2008), and are further transmitted to the hypothalamus, amygdala and insula, where they can exert an effect over more widespread brain signalling and behaviour (Bauer et al., 2016). The activation of neurons from the NTS associated with the afferent vagal system is dependent on NMDA receptors (Bauer et al., 2016), which are known to be influenced by the microbiota (Savignac et al., 2013) and to play a key role in synaptic plasticity (Fleischmann et al., 2003).

Vagal nerve integrity has been shown to be essential for modulations of gut microbiota to exert their effect over behaviour. Anxiety induced by colitis was shown to be reversible following gavage with the probiotic *B. longum* (Bercik et al., 2011b). In a group that had undergone vagotomy, however, this effect was no longer observed. Similarly, ingestion of a lactobacillus strain resulted in reduced anxiety-like behaviours and blunted stress-induced corticosterone release, effects that were absent in vagotomised mice (Bravo et al., 2011a).

The role of the vagus nerve in learning and memory, and synaptic plasticity has been examined both in animal models and humans. Vagal nerve stimulation can enhance hippocampal long-term potentiation in rats (Zuo et al., 2007b) while also influencing parameters of adult hippocampal neurogenesis. Vagal nerve stimulation has also been shown to aid in the enhancement of the extinction of fear memories in mice (Alvarez-Dieppa et al., 2016), to improve memory retention performance of rats in an inhibitory avoidance task (Clark et al., 1995; Clark et al., 1998), and to lead to enhanced recognition memory in humans (Clark et al., 1999).

Removal of the influence of the vagus nerve on the other hand may result in deleterious effects on the central nervous system as has been demonstrated in preclinical studies. Subdiagraphramatic vagotomy can decrease adult hippocampal neurogenesis (O'Leary et al., 2018) and increase microglial activation in the dentate gyrus of the hippocampus (Ronchi et al., 2012). Both of these factors have been shown to impact synaptic plasticity (Bruel-Jungerman et al., 2007; Muzio et al., 2016) with both also having been observed in various psychiatric disorders (Hanson et al., 2011).

Other compounds which act though their influence on the gut microbiota have been shown to depend on the vagus nerve for their activity. Human milk oligosaccharides (HMOs) are a

group of structurally diverse unconjugated glycans, unique to human breast milk, which act as prebiotics. Through this prebiotic activity, HMOs have a beneficial effect on gut microbiota composition (Musilova et al., 2014). In addition, they can have beneficial effects on learning and memory. Rats receiving HMO supplementation demonstrated improved performance in behavioural tests for cognition while also displaying more robust LTP in-vivo (Oliveros et al., 2016b; Vázquez et al., 2015). Following subdiaphragmatic vagotomy, the effects were no longer present (Vazquez et al., 2016) again highlighting the importance of this mode of gut-brain communication.

The vagus is clearly an important communication pathway, relaying microbial signals to the brain. Further research is required to determine how specifically the vagus is activated at the level of the gut, how it is regulated, and indeed how its activity modulates synaptic plasticity.

1.3.2: The Enteric Nervous system

In addition to the vagus nerve, another neuronal system plays a key role in the interface between the microbiota and host. The enteric nervous system (ENS) can interact both directly and indirectly with the microbiota and its metabolites. Structurally, the ENS is separated into two ganglionated plexi, the submucosal and myenteric plexus and plays a significant role in the coordination of gastrointestinal functions including motility and control of fluid movement (Furness, 2012).

The ENS also possesses the ability to communicate with the central nervous system via intestinofugal neurons to sympathetic ganglia with sensory information traveling via extrinsic primary afferent neurons that follow spinal and vagal afferent routes (Furness, 2012). These intrinsic and afferent neural pathways provide opportunities for factors derived from the gut lumen, and therefore potentially the microbiota, to influence not only gut function but also the CNS.

There are various methods through which the microbiota might influence the ENS. One suggested route is through serotonin (5-HT) (De Vadder et al., 2018). Antibiotic administration has also been used as a method to determine the effects of microbiota composition on ENS. Microbiota depletion via this route was shown to result in wide-ranging effects on ENS architecture (neuronal and glial), neurochemistry and function (Caputi et al., 2017). Furthermore, glial cells which are located within enteric ganglia were decreased in number following antibiotic treatment (Caputi et al., 2017). Evidence from GF animals also

suggests a more specific role for the microbiota in modulating mucosal enteric glial cell migration (Kabouridis et al., 2015).

While links between microbiota composition, stress and ENS abnormalities have been made, it should also be noted that these factors may be altered independent of one another as observed in offspring of dams exposed to prenatal stress in which the most significant stress-induced changes in the gut microbiota failed to correlate with gut physiological and ENS parameters (Golubeva et al., 2015). Therefore, one must exert a degree of caution in interpreting concomitant changes in the microbiota and ENS as being intrinsically linked.

Specific strains of bacteria, or even components thereof have also been shown to influence ENS function. It was observed that not only do different bacterial strains differentially influence enteric nerve activity but that they may do so by different mechanisms (Mao et al., 2013). The same lab were also able to show that the microbial-derived SCFA, butyrate, and epithelial-applied 5-HT, could affect ENS activity providing some further insight into additional mechanisms by which the microbiota may interact with the ENS (Kunze et al., 2009). Functional studies support that different microbial strains can differentially affect neurally-driven secretomotor responses (Lomasney et al., 2014). The ENS is also central to facilitating changes in motility consequential to diet-microbe interactions (Dey et al., 2015).

Hence the mechanisms that link the gut microbiota and ENS are multiple and varied. Evidence now also suggests that there may be a reciprocal relationship between the ENS and the gut microbiota. Thus far, we have considered the impact of the microbiota on the ENS. However, the ENS appears to be able to exert control of the microbiota (Rolig et al., 2017). In zebrafish lacking an ENS, a 'pro-inflammatory' microbiota profile developed, the effects of which could be ameliorated through transplantation with ENS precursors or by introduction of 'anti-inflammatory' microbes (Rolig et al., 2017).

Enteric nervous system abnormalities have also been linked with serious gastrointestinal disorders (Gariepy, 2001). In addition, the ENS has now also been implicated in disorders of the CNS, including ASD, Alzheimer's disease and Parkinson's disease, generally considered primary disorders of the CNS (Rao and Gershon, 2016). ASD is particularly noteworthy given the high level of comorbid GI symptoms observed (Rao and Gershon, 2016). Further studies are needed to fully appreciate the relative contribution of the microbiota in shaping the pathological effects of ENS dysfunction.

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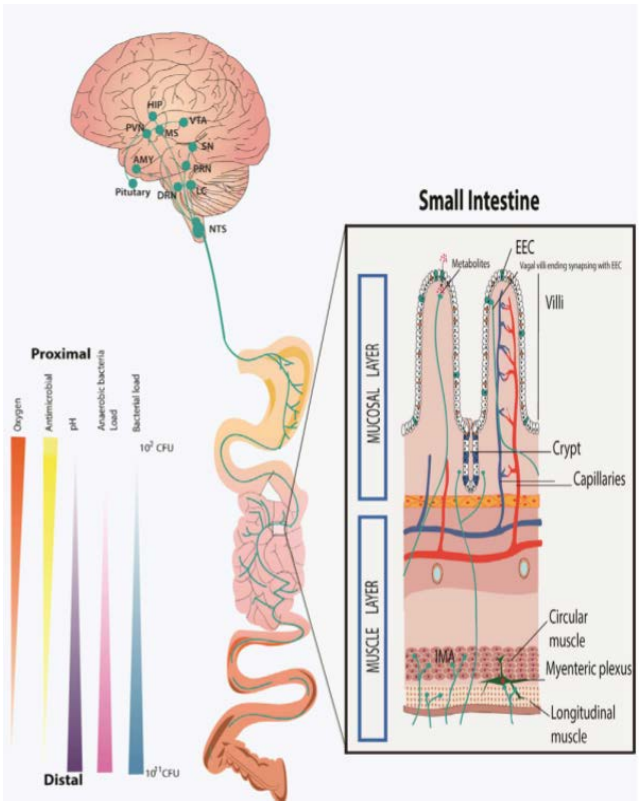


Fig 9 Nervous communication between the gut and brain*: Gradients of pH, oxygen, antimicrobial peptides, and bile salts determine the density and diversity of microbial species along the gastrointestinal tract. Communication between these bacteria and their metabolites can occur through the vagus nerve which descends from the NTS in the brainstem to the lower reaches of the small intestine (shown in green). The enteric nervous system innervates the various plexi of the gastrointestinal tract and is shown in the inset box. Figure taken from Fülling et al. 2019.*

1.3.3: Immune system signalling

Signalling through the immune system is another mode of communication between the gut and the brain. The immune cells of the gastrointestinal tract are in constant communication with the gut microbiota, either through physical contact with bacteria or through interaction with the compounds they produce (Sommer and Bäckhed, 2013) The intestinal epithelium consists of a single cell layer that lines the gastrointestinal tract and forms the barrier between the gut interior and visceral organs. Goblet cells within the epithelial layer secrete a protective mucous which forms an interface layer where the majority of host-microbe interactions occur (Elson and Alexander, 2015). Exchange of molecules between bacteria and this mucous layer allows priming of the immune system as well as acting as a location of antigen presentation (Elson and Alexander, 2015). Furthermore, a diverse gut microbiota enables the development of a healthy immune system (Elson and Alexander, 2015; McDermott and Huffnagle, 2014).

As well as acting as a physical barrier to bacteria, the intestinal epithelium contains numerous cell types (including enterocytes, secretory cells, chemosensory cells and gut-associated lymphoid tissue (GALT)) (Pott and Hornef, 2012). Enterocytes express innate immune receptors and can release cytokines and chemokines (Pott and Hornef, 2012). GALT enables a specific immune response using lymphocytes (Pott and Hornef, 2012). Epithelial pattern recognition receptors recognize molecular patterns unique to bacteria and other microorganisms (pathogen-associated molecular patterns, or PAMPs) (Duerkop et al., 2009; Vaishnava et al., 2008) of which the Toll-like receptor family are the most studied. Once activated, PAMPs can recruit inflammatory mediators and induce cytokine production as well as chemokine-mediated recruitment of acute inflammatory cells (Takeda and Akira, 2004).

Current hypotheses suggest that these microbiota-host interactions at the level of the gut release cytokines, chemokines, neurotransmitters, neuropeptides, endocrine messengers and microbial by-products that can infiltrate the blood and lymphatic systems, or influence neural messages carried by the vagal and spinal afferent neurons to communicate with the brain and update health status, as well as possibly regulate mood and behavior (Clarke et al., 2014a; Mayer, 2011). Disruption of immune signalling in the gut has been linked to inflammatory disorders such as Crohn's disease (Rogler et al., 2018) or ulcerative colitis (Abreu, 2010). In such cases local inflammation may lead to systemic reactions and effects of inflammation on the central nervous system, potentially through a 'mirror' inflammatory response in the brain. These conditions, often characterized by altered microbiota

composition have even been shown to compromise hippocampal synaptic plasticity (Riazi et al., 2015b).

Disruption of the normal microbiota composition can have effects on the immune function regulation of the nervous system as well. For example, treatment with an antibiotic cocktail has been shown to reduce the severity of EAE, the experimental model of multiple sclerosis (Ochoa-Reparaz et al., 2009; Yokote et al., 2008). The reduction in severity of the condition is believed to be linked to the action of antibiotic treatment on immune system function including a reduction of the in the pro-inflammatory T_H1/T_H17 response (Lee et al., 2011). Furthermore, IL-10 producing $Foxp3^+$ cells that accumulated in antibiotic-treated mice were able to protect against transfer of naïve mice (Ochoa-Reparaz et al., 2009). Additionally, administration of an antibiotic cocktail over a prolonged period in adulthood has been shown to alter immune function through $Ly6C^{hi}$ monocytes (Mohle et al., 2016) with knock on effects on cognitive behaviour and adult hippocampal neurogenesis (Mohle et al., 2016). This demonstrates that antibiotic modulation of the gut microbiota can influence immune function with consequent effects on functioning of the central nervous system. Again, this highlights the important role that gut microbiota plays in the normal functioning of these systems.

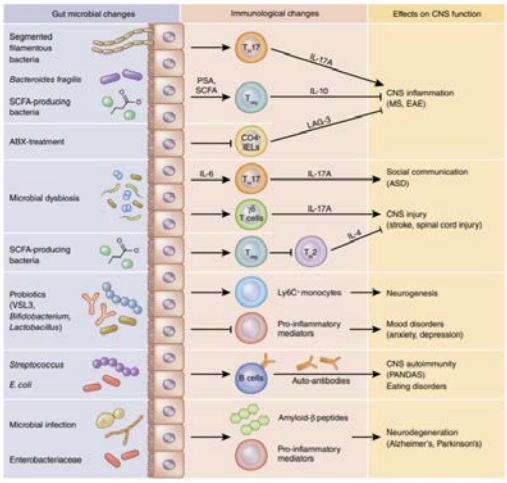


Figure 10: Effect of the microbiota on peripheral immune cells and CNS function. *Intestinal microbes of the gastrointestinal tract regulate peripheral immune responses, CNS function and behaviour. The presence of bacteria and bacterial metabolites in the above graph lead to alterations in the immune system and potential effects on central nervous system function. Image taken from Fung et al. 2017*

1.3.4. The HPA Axis

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine signalling system in the human body. The activation state of this axis is the primary determinant of how the body responds to stress, controlling the widely-known 'fight-or-flight' response (Mayer, 2000). When a stressor is encountered, paraventricular neurons in the hypothalamus release corticosterone releasing factor (CRF) which causes adrenocorticotrophic hormone (ACTH) release in the anterior pituitary. This ACTH is released into systemic circulation, eventually leading to glucocorticoid release from the adrenal cortex (de Wied et al., 1993). Glucocorticoid release also causes a negative-feedback response in the pituitary and the hypothalamus, preventing overworking of the system. In the brain, glucocorticoids interact with high-affinity mineralocorticoid receptors as well as lower affinity glucocorticoid receptors (Herman et al., 2016; Smith and Vale, 2006; Tsigos and Chrousos, 2002).

Early germ-free mouse studies were the first to link the microbiota to the HPA axis. In a seminal study, germ-free mice were observed to display an exaggerated HPA axis response to restraint stress when compared to specific-pathogen free (SPF) controls (Sudo et al., 2004c). Recolonization with a specific pathogen free microbiota or a single bacteria (*B. infantis*) was capable of reversing this effect. Interventions needed to be performed during a critical window in early life in order to be successful, highlighting the importance of microbiota composition during this period (Sudo et al., 2004c). Disruption of microbiota composition through administration of antibiotics during this time has also been shown to disrupt the normal stress response (Desbonnet et al., 2015b).

Alterations in hippocampal N-methyl D-aspartate (NMDA) and serotonin$_{1A}$ (5-HT$_{1A}$) receptor mRNA expression have also been recorded in germ-free mice (Neufeld et al., 2011a). Both of these receptors are known to influence CRF release from the hypothalamus (Owens et al., 1990; Patchev et al., 1994) thus changes their expression may explain altered HPA function in germ-free animals. Importantly, blocking these receptors has been shown to inhibit hippocampal synaptic plasticity of serotonergic receptors in novel environments (Sanberg et al., 2006). This may go some way to explaining behavioural differences in germ-free mice, who have exhibited deficiencies in the novel object recognition test (Gareau et al., 2011).

Mode of delivery is again an important factor in optimal functioning of the HPA axis. While mode of delivery affects gut microbiota composition, with c-section born animals having a different microbiota to vaginally born (Backhed et al., 2015), vaginal birth also acts directly as a primer of the HPA axis (Ochedalski and Lachowicz, 2004). As such mode-of-delivery has

been shown to influence stress response both in clinical studies (Taylor et al., 2000) as well as in animal models (Daniel et al., 1999)

The HPA axis also interacts with other routes microbiota-gut-brain communication. Stimulation of the vagus nerve has been shown to increase both plasma levels of corticosterone and ACTH (Hosoi et al., 2000) as well as alter CRF mRNA in the hypothalamus (Hosoi et al., 2000). Furthermore, the immune system can play a role in activation of the HPA axis. Increasing levels of stress can increase gastrointestinal permeability, thereby increasing the likelihood that microbes and their metabolites will cross the gut wall, resulting in an increased immune activation state (Demaude et al., 2006). Widespread exposure to these bacteria and their antigens leads to a generalized immune response, the release of pro-inflammatory cytokines, and eventual increase in the activation of the HPA axis. Levels of toll-like receptors (TLRs) are significantly decreased in germ-free mice, leading to a disrupted response to bacteria and an altered activation of the HPA axis (O'Hara and Shanahan, 2006). In TLR4 knockout mice, activation of the HPA axis in response to Gram-negative bacteria is absent (Gosselin and Rivest, 2008).

The importance of each of these modes of communication as well as the intercommunication between each, for the normal functioning of the microbiota-gut-brain axis is clear. They transmit information about gut microbiota composition to the brain while also requiring a healthy microbiota for optimal function, highlighting the complex web of interactions occurring along the microbiota-gut-brain axis. Some of the key components in the activation of each of the routes of communication are compounds produced as by-products of the bacteria themselves and this will be discussed in the next section.

1.3.5. Microbial metabolites as signalling molecules

A further mode through which the gut microbiota is able to influence homeostatic function of the host is through the activity of its metabolites as they act in the gut as well as following their release into systemic circulation.

Microbial metabolites found in the gut are widespread and include gastrointestinal hormones (Holzer et al., 2012), cortisol (Dinan and Cryan, 2012), precursors to neuroactive compounds, and neurotransmitters themselves (including GABA (Komatsuzaki et al., 2005), serotonin (Yano et al., 2015a), and noradrenaline (Lyte, 2013)). Some of these compounds are released into circulation where they can relay messages to the brain, such as cortisol

acting via the HPA axis (Dinan and Cryan, 2012) or cytokines acting via the immune system (Schirmer et al., 2016). Neurotransmitters are unlikely to exert any direct effect on the brain due to an inability to cross the blood-brain-barrier. Instead, they can affect signalling locally via the enteric nervous system (Clarke et al., 2014b; Soret et al., 2010). Disruption of the microbiota can lead to knock on effects via the impact of these metabolites, with administration of an antibiotic cocktail having been shown to alter the tryptophan/kynurenine pathway, (Desbonnet et al., 2015a) a factor with potential knock on effects on brain function and behaviour (Desbonnet et al., 2015b). Perhaps the most ubiquitous and widely studied microbial metabolite that can signal to the brain are short-chain fatty acids.

1.3.5.1: Short-chain Fatty acids:

Short-chain fatty acids (SCFAs) are the major products of the bacterial metabolism of host-indigestible carbohydrates and protein in the gut (Macfarlane and Macfarlane, 2012). They include butyrate, propionate and acetate (van de Wouw et al., 2018a) and are generally produced in the proximal large colon, by *Bacteroides*, *Biffidobacteria* and *lactobacillus* (Macfarlane and Macfarlane, 2012). They can exert their effect through activity at GPCRs (FFAR2/ FFAR3) as well as by interfering with energy processing in the metabolic cycles (Frost et al., 2014a). Receptors and transporters for SCFAs are present in the gut where they have been shown to modulate both serotonin secretion (O'Mahony et al., 2015) and PYY release (Holzer et al., 2012).

Following their production by bacteria, the majority of colonic SCFAs are absorbed by the host epithelium where they serve as a source of energy for colonocytes, particularly butyrate (Clausen and Mortensen, 1994; Hamer et al., 2008). The vast majority of butyrate and propionate that do make it to systemic circulation are then metabolized by hepatocytes. This means that plasma concentrations are as low as 1-15µmol/l for butyrate and propionate with much higher concentrations of acetate (100-200µmol/l) (Bloemen et al., 2009; Cummings et al., 1987; Peters et al., 1992). This higher plasma concentration of acetate is likely responsible for it being the only SCFA that has been detected in human cerebrospinal fluid (CSF) at approximate concentrations of 35µmol/l (Nagashima et al., 2010). As such there is debate over whether these compounds will be able to exert direct effects in the brain, or whether the majority of effects will utilise other modes of interaction.

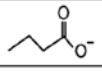
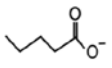
Short-chain fatty acid	Chemical formula	Chemical structure
Formate	CH_1O_2	
Acetate	$C_2H_3O_2$	
Propionate	$C_3H_5O_2$	
Butyrate	$C_4H_7O_2$	
Valerate	$C_5H_9O_2$	

Table 1: Chemical formula and structure of all short-chain fatty acids

Short-chain fatty acids have been implicated in a number of host processes including GI function (Gill et al., 2018), blood pressure regulation (Pluznick, 2017), circadian rhythm (Tahara et al., 2018), and neuroimmune function (Erny et al., 2017). While still in early stages of study, links between short-chain fatty acids, the brain, and behaviour have started to emerge. Decreased SCFA concentrations have been reported in various disorders in which physiology and behaviour are altered. These include anorexia nervosa (Morita et al., 2015) & Parkinson's disease (Unger et al., 2016). Increases in SCFA level, meanwhile, have been linked to autism spectrum disorders (Wang et al., 2012) and chronic psychosocial stress in children (Michels et al., 2017), as well as obesity (Fernandes et al., 2014b; Rahat-Rozenbloom et al., 2014; Schwiertz et al., 2010). Further links have been made between SCFAs and Alzheimer's disease (Zhang et al., 2017) and chronic stress (Maltz et al., 2018) in animal models.

In experiments where SCFAs have been administered to germ-free mice, these compounds work to restore homeostasis in these animals who display abnormal CNS features (including altered dendritic morphology in the hippocampus (Luczynski et al., 2016)). SCFAs have also been shown to alter hippocampal-dependent behaviours including learning and cognition (Arnoldussen et al., 2017; Val-Laillet et al., 2018) and reward-associated behaviours (Byrne et al., 2016; Van De Wouw et al., 2018b). The ability for SCFAs to reach the hippocampus to have a direct effect has yet to be demonstrated, however. When a mixture of SCFAs was given to mice who underwent long-lasting psychosocial stress; anxiety and depressive-like behaviours were reduced while gene expression of the mineralocorticoid receptor was also

decreased in the hippocampus and the hypothalamus (Van De Wouw et al., 2018b). These studies suggest that there may indeed be a link between SCFA production by gut microbiota, the brain, and behaviour; the specific mechanisms underpinning these changes is unclear, and this is what a number of current studies are attempting to address.

One suggested mechanism through which these compounds may exert control over brain and behaviour is via a direct action on neurons and glia, most likely in the hypothalamus where the compounds can most readily enter the brain (Frost et al., 2014a). While it has long been known that Increased fibre metabolism by gastrointestinal bacteria leads to an increase in plasma SCFA levels (Trompette et al., 2014) it was recently shown that peripheral administration of acetate can lead to its detection in the hypothalamus (Frost et al., 2014a). Acetate activity at the hypothalamus has subsequently demonstrated effects on appetite, potentially through the action of SCFAs on monocarboxylate transporters (Frost et al., 2014a; Vijay and Morris, 2014). Once present in the brain, SCFAs can be taken up by these same transporters on neurons and glia (Frost et al., 2014a; Pierre and Pellerin, 2005) and are thought to play an important role in cellular energy metabolism (Rafiki et al., 2003). SCFAs can also modulate intracellular calcium levels in neutrophils, suggesting a role in cellular signalling (Naccache et al., 1988).

In addition to this there is potential for SCFAs to have a direct effect on the brain and exert local action on energy homeostasis, both centrally and peripherally. Related to this is the fact that SCFAs can profoundly influence inflammation, particularly through interaction with the GDPR short-chain fatty acid receptor, GPR43 (Maslowski et al., 2009). Mice administered oral acetate showed a substantial decrease in inflammation, with this effect being absent in GPR43 -/- mice (Maslowski et al., 2009). Butyrate is also known to have many functions, with perhaps the most prominent being histone acetylation through inhibition of histone deacetylase (Davie, 2003) and facilitating the access of DNA repair enzymes (Davie, 2003). Behaviourally, systemic injection of sodium butyrate was shown to elicit an antidepressant effect through transient acetylation of histones in the PFC and hippocampus, combined with alterations in BDNF expression (Wei et al., 2015).

This ability of SCFAs to increase histone acetylation (She et al., 2017) has also been shown to have beneficial effects on hippocampal synaptic signalling (Levenson et al., 2004b). Application of sodium butyrate directly to hippocampal slices can enhance LTP, while injection of sodium butyrate improved performance in a contextual fear conditioning test. Similarly, deficits in neuroinflammation and functional connectivity following high-fat diet

administration in rats could be reversed following butyrate supplementation, again suggesting that these compounds are able to exert positive influences in the brain (Zhai et al., 2019).

Supplementation with acetate, in the form of glyceryl triacetate has also been demonstrated to have an effect on the brain. Rats receiving this compound displayed greater responses to cortical iontophoresis compared to control animals while also displaying altered GluN2B subunit expression and improved cognitive flexibility in an attentional set-shifting task (Gronier et al., 2018). `

Recently a number of studies have emerged which demonstrate the ability of antibiotic administration to significantly alter SCFA production. Antibiotics reduce observed levels of all SCFAs (Guinan et al., 2019; Zhao et al., 2016), with additional links being made between SCFA reduction and an increase in *Candida* infection (Guinan et al., 2019). The ability of SCFAs to impact on physiology is clear, and will continue to act as a cornerstone of future gut-brain-microbiota studies. By probing its mechanisms, the underpinnings of these effects may become clear, paving the way for SCFA supplementation as a potential therapeutic intervention of the future.

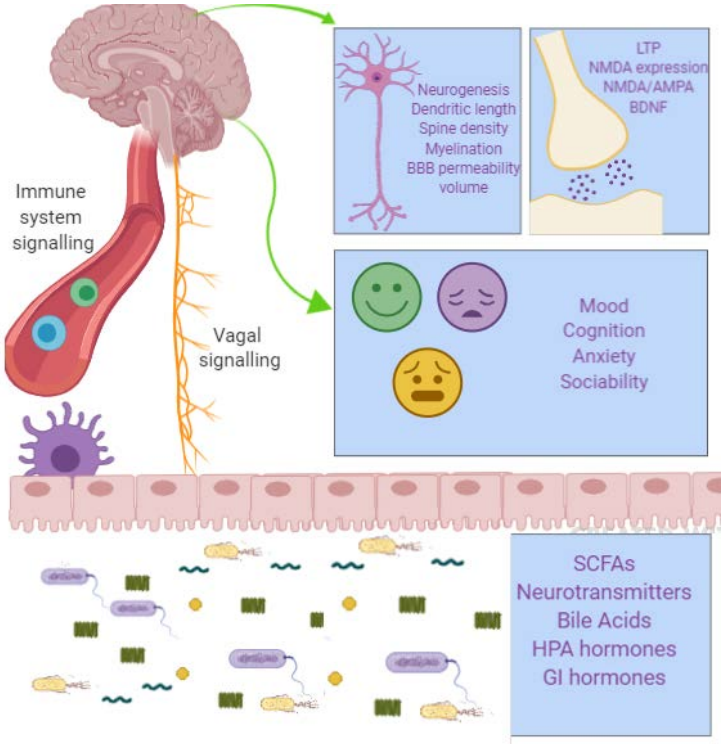


***Figure 11: Microbiota-gut-brain routes of communication and effects on CNS and behaviour**. Communication between the gut and the brain can occur via the vagus nerve, immune system, HPA axis or through metabolites of the microbiota. This can have physical effects on the brain and at the synapse. Effects of gut microbiota modulation have also been observed to impact various aspects of behaviour.*

1.4. Gut microbiota modulation, the brain and behaviour

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While the search for a mechanism underpinning microbiota-gut-brain communication is a relatively recent endeavour, the ability of gut microbiota composition to influence behaviour has been a staple of microbiota-gut-brain axis research from its outset. Alterations in gut microbiota composition have been linked to various neurological and psychiatric disorders including anxiety (Foster and Neufeld, 2013), depression (Foster and Neufeld, 2013), Parkinson's disease (Sampson et al.; Scheperjans et al., 2015), schizophrenia (Dinan et al., 2014) and cognitive decline (Bercik et al., 2011a).

While modulation of gut microbiota composition in human cohorts, particularly through probiotic administration has led to some evidence of their benefit in the treatment of anxiety

(Pirbaglou et al., 2016) and mood disorders (Steenbergen et al., 2015), there are multiple environmental factors, particularly diet, that act as confounding factors, themselves potentially influencing microbiota composition. Furthermore, detailed analysis of the mechanisms underpinning observed effects is limited in clinical trials.

As such, preclinical models, in particular the use of rodents, offer solutions to many of these issues. Variability in diet between groups can be eliminated, for example, and a much broader range of microbiota modulations are possible, as well as allowing more detailed analyses of the changes occurring both centrally and peripherally that may be linked to any observed alterations in behaviour.

Current methods of microbiota manipulation used to determine the influence of bacteria over behaviour include: germ-free animals and antibiotic administration. Additionally, some groups have looked at whether behavioural abnormalities may be reversed through administration of prebiotics, probiotics, or through other methods of recolonization such as faecal microbiota transfer (FMT). The seminal germ free study demonstrating exaggerated stress response and time-dependent reversal by probiotic treatment (Sudo et al., 2004c) became a reference point for many subsequent studies assessing the impact of the microbiota on stress and other psychiatric conditions.

Gut microbiota disruption may also be achieved through administration of a bacteria that disrupts a healthy microbiota, leading to an immune response, altered metabolite production and subsequent effects on behaviour. Introduction of the pathogenic bacteria *Campylobacter Jejuni* resulted in increased anxiety-like behaviour and increased expression of the neuronal activation marker c-fos in areas of the brain associated with anxiety (Goehler et al., 2008). C-fos is an intermediate early gene which has been shown to play a role in learning and memory as well as long-term potentiation (LTP), the processes regarded as the cellular basis of learning-and-memory (Fleischmann et al., 2003). Other modes of microbiota modulation have been more widely studied and will therefore be discussed in greater detail below.

1.4.1. Antibiotics

Administration of antibiotics to animal models, has been used to address a wide range of experimental questions. They can be administered acutely (Bercik et al., 2011a), to mimic a clnical short course, or cocktails may be administered over long periods to effectively eradicate the microbiota (Mohle et al., 2016). Antibiotics can be administered at discrete stages throughout the lifespan that represent key developmental stages including

postnatally (Leclercq et al., 2017b), during early life (O'Mahony et al., 2014) during adolescence (Desbonnet et al., 2015a) or in ageing (van Opstal et al., 2016).

The choice of the specific antibiotic of cocktail used may often appear somewhat arbitrary, though numerous considerations must be taken into account when planning an experiment. A particularly salient factor when studying the microbiota-gut-brain axis is bioavailability following oral administration. Non-absorbable antibiotics such as vancomycin, neomycin and bacitracin deplete the gut microbiota without entering systemic circulation therefore their effects can be said to be solely due to their impact on the microbiota rather than any systemic effects. Some studies control for potential systemic effects using intraperitoneal injection of the administered antibiotics that are given orally, as a control (Tochitani et al., 2016). Conversely, antibiotics such as metronidazole (Jokipii et al., 1977), third generation cephalosporins (Zhang et al., 1989) and minocycline (Yong et al., 2004) are able to enter systemic circulation, and even penetrate the blood-brain barrier (Jokipii et al., 1977) (Zhang et al., 1989) (Yong et al., 2004). Minocycline, for example is known to act as an inhibitor of microglial activation and has been shown to alter synaptic transmission in the hippocampus following intraperitoneal injection (Riazi et al., 2015b). Peripheral administration of ceftriaxone has been shown to modulate synaptic plasticity (Trantham-Davidson et al., 2012) through direct activity at the synapse.

When antibiotics are administered to weaning mothers, a further consideration is whether or not these antibiotics will enter the breast milk of the mother and therefore gut of the offspring in sufficient quantities to alter microbiota composition. While estimates can be made as to the extent to which antibiotics will enter breast milk, the specific concentrations administered to the pup will only be determined following analysis of the milk.

Linked to this is the use of rodent models to shed light on the effects of administration of antibiotics to mothers following cesarean section. Mode of delivery of offspring is the most important risk-factor in the development of maternal postpartum infection (Yokoe et al., 2001). As such, mothers undergoing C-section are recommended to receive prophylactic antibiotic therapy. These may be one of a number of different regimens (Chaim and Burstein, 2003) during which time mothers are recommended to continue breastfeeding (Amir and Academy of Breastfeeding Medicine Protocol, 2014). While each of these antibiotics is considered to be safe for nursing infants, with no adverse effects having been observed (2001; Kaiser et al., 2007) these suggestions are based on acute adverse effects with any long-term impacts not studied to date. This represent an excellent opportunity through

which an animal model may be used to investigate long-term effects of early-life maternal antibiotics on offspring brain and behaviour. The administration of different antibiotics to the mother and measurement of faecal microbiota later in life will also allow the assessment of whether a general disruption of the microbiota may be responsible for the effects or if the presence or absence of specific strains is the cause.

Some work has been undertaken in this arena already, with studies identifying the pre-weaning period as a critical window for alterations in immune response (Russell et al., 2012) and perinatal maternal antibiotic administration has been shown to induce changes in brain and behaviour (Leclercq et al., 2017b). When mothers were administered an antibiotic (penicillin V) from one week prior to delivery until the end of the weaning period, alterations were observed in offspring in anxiety, sociability, social novelty and aggression (Leclercq et al., 2017b). In addition to this, changes were observed centrally in cytokine expression and blood-brain barrier permeability. These changes were associated with alterations in microbiota composition and some were able to be prevented with co-administration of the probiotic Lactobacillus rhamnosus JB-1. (Leclercq et al., 2017b).

Despite the many considerations discussed, antibiotics have been crucial in corroborating the behavioral and biological observations documented in germ-free animals. Indeed, antibiotic administration to laboratory animals has been shown to influence a wide array of behaviours. These include anxiety (Desbonnet et al., 2015b), cognition (Fröhlich et al., 2016), social behaviour (Guida et al., 2018a) and depressive-like behaviour (Guida et al., 2018a) as can be seen in table 1. In addition to these changes in behaviour, changes to the brain in terms of neurogenesis (Möhle et al., 2016), microglial activation (Sampson et al., 2016b), electrophysiology (Guida et al., 2018a) and expression of signalling molecules (serotonin, NPY etc.) (Fröhlich et al., 2016) have been observed. (Table 2).

A final advantage of antibiotics is that they offer a tool to model various clinical scenarios that occur in humans. Administration schedules can model the most prevalent antibiotic regimens that millions are prescribed annually, allowing us to determine the effect that they may be having on the brain and behavior. The flexibility and translational ability of antibiotics make them a hugely valuable tool in the study of the microbiota-gut-brain axis and they will form a key component of future studies in the field. **Table 2** summarizes the current state of knowledge regarding the impact of antibiotics upon brain physiology and behavior.

Table 2: **Studies using antibiotic administration as a method to target the microbiota-gut-brain axis.**

Species/ Strain	Antibiotic Cocktail	Behaviour Assessed/ Other tests.	Age of behavioural assessment	Effects of antibiotic treatment	Ref
Mouse					
NIH Swiss	-Ampicillin (1mg/ml) -Vancomycin (5mg/ml) -Neomycin (10mg/ml) -Metronidazole (10mg/ml) -Amphotericin-B (0.1mg/ml) -In Drinking water -Started at Weaning	-Novel object recognition (NOR) -Light/dark box -Social transmission of food preference -Corticosterone response to restraint stress	7-11 weeks	-Altered gut microbiota composition in adulthood -Reduction in anxiety-related behaviour -Cognitive deficits in novel object discrimination and communication of cued food information -Alteration in the tryptophan/kynurenine metabolic pathway -Significant reduction in hippocampal BDNF, oxytocin, vasopressin expression.	(Desbonnet et al., 2015b)
C57BL/6	-Bacitracin (108mg) -Neomycin (108mg) -Ampicillin (42.3mg) -Meropenem (21.6mg) -Vancomycin (6.48mg) -Dissolved in 4.5ml -Given via gavage 10ml/kg twice daily for 11 days at 8-11 weeks.	-Open Field test (OFT) -Elevated Plus maze (EPM) -Tail suspension test (TST)	8-11 weeks	-Altered gut microbiota composition -Novel object (but not spatial) discrimination was impaired -Brain-region specific changes in expression of relevant signalling molecules (i.e. BDNF, NMDA2B, Serotonin transporter, NPY)	(Fröhlich et al., 2016)

		-Novel object recognition (NOR) -Barnes maze			
C57BL/6J	-Ampicillin & Sulbactam (1.5g/L) -Vancomycin (500mg/ml) -Ciprofloxacin (200ml/ml) -Imipenem & Cilastatin (250mg/ml) -Metronidazole (1g/L) Delivered in drinking water for 7 weeks at 6-8 weeks of age.	-Novel Object Recognition (NOR) -Exercise	13-15 weeks	-Novel object discrimination was impaired -Reduced hippocampal adult neurogenesis -Exercise was shown to improve neurogenesis -These effects are partially mediated by Ly6C^{hi} Monocytes	(Möhle et al., 2016)
C57BJ/6J	-Ampicillin, streptomycin, clindamycin (ASC) (1mg/ml) Delivered in drinking water for two weeks	-Tail suspension test (TST) -Forced swim test (FST) -Rotarod	9-10 weeks	-Depressive-like behavior observed in the FST and TST -Deficit in the ability to discriminate social novelty -Reduced hippocampal BDNF protein levels -Increased hippocampal TrkB protein levels	(Guida et al., 2018a)

	-After 14 days ASC/ control groups were treated via oral gavage with probiotic (Lactobacillus casei) or saline for 7 days	-Muscle strength test -Novel object recognition (NOR) -Y-maze -Hot plate test -3-Chamber social interaction test		-Altered spiking in hippocampal CA3 -Increases in activated microglia/astrocytes in the hippocampus	
C57BL/6J	-Neomycin (5mlml) -Bacitracin (5mg/ml) -Pimaracin (1.25µg/ml) -Delivered in drinking water to dams between E9-E16	-Open field test (OFT) -3-Chamber social interaction test	4-7 weeks	-Altered gut microbiota composition -Reduction in locomotor activity in OFT -No difference in social behaviours between groups -Cross-fostering abolish the behavioural differences at week 4	(Tochitani et al., 2016)

	-IP injection of cocktail used as a control -Administered cocktail to germ-free mice (no observed effects)				
Specific pathogen free BALB/C -Germ-free BALB/C	Non-absorbable antibiotics -Neomycin (5mg/ml) -Bacitracin (5mg/ml) -Pimaracin (1.25µg/ml) -Delivered in drinking water for 7 days -A further group received IP administration of antibiotics	-Light/dark test -Step down test	8-10 weeks	-Altered gut microbiota composition -Anxiolytic-like effect in light/dark box and step-down inhibitory avoidance -Increase in hippocampal BDNF expression -Behavioural changes were independent of inflammatory activity, changes in levels of gastrointestinal neurotransmitters and vagal or sympathetic integrity	(Bercik et al., 2011a)
BALB/C	-Penicillin V (31mg/kg/day) -Delivered in drinking water one week prior to delivery and continued up to weaning (p21)	-Locomotor activity -Elevated plus maze (EPM) -3-Chamber social	6 weeks	-Altered gut microbiota composition in dams and offspring -No effect on locomotor activity -Anxiolytic-like effect observed in the EPM -Increased aggression and reduced social avoidance behaviour	(Leclercq et al., 2017b)

		interaction test		-Increased Avpr1b and cytokine expression in the frontal cortex -Increased tight junction protein levels in the frontal cortex and hippocampus	
APPSWE/PS1ΔE9	-Gentamicin (1mg/ml), -Vancomycin (0.5mg/ml), -Metronidazole (2mg/ml) -Neomycin (0.5mg/ml) -Ampicillin (1mg/ml), -Kanamycin (3mg/ml), -Colistin (6000U/ml) -Cefaperazone (1mg/ml) -Delivered via gavage from postnatal day 14 until day 21 -Mice were then supplemented with ABX-containing drinking water (1/50th gavage concentration) for the duration of lifespan	-None	Mice were culled for immunohistochemistry at 5- 6 months	-Altered gut microbiota composition -Altered inflammatory cytokine composition - Reduction in Aβ plaque deposition - Increased concentration in soluble Aβ levels - Reduction in reactive gliosis surrounding Aβ plaques	(Minter et al., 2016b)

Thy1-α-synuclein (mouse model of Parkinson's disease)	-Ampicillin (1g/L) -Vancomycin (0.5g/L) -Neomycin (0.5g/L) -Gentamycin (100mg/L) -Erythromycin (10mg/L) From 5-6 weeks to 12-13 weeks	-Beam Traversal -Pole descent -Adhesive removal -Hind Limb Clasping reflex sore -Inverted Grid	12-13 weeks	-Antibiotic administration ameliorated locomotor deficits induced by α-synuclein overexpression -Reduction in microglial diameter in the caudate-putamen and substantia nigra	(Sampson et al., 2016b)
Rat					
Sprague Dawley	-Ampicillin (1g/L) -Vancomycin (500mg/L) -Ciprofloxacin (20mg/L) -Imipenem (250mg/L) -Metronidazole (1g/L) -Delivered in drinking water for 13 weeks	- None	-NA	-Altered expression of miRNAs in the amygdala and PFC **Amygdala:** ↓ miR-206-3p & miR-219a-2-3p ↑ miR-369-3p **PFC:** ↓ miR219a-5p	(Hoban et al., 2017)
Sprague Dawley	-Ampicillin (1g/L)	-Open field test (OFT)	17-22 weeks	-Impairment in spatial memory observed in the MWM	(Hoban et al., 2016b)

	-Vancomycin (500mg/L) -Ciprofloxacin HCL (20mg/L) -Imipenem (250mg/L) -Metronidazole (1g/L) -Delivered in drinking water for 6 weeks	-Elevated plus maze (EPM) -Forced-swim test (FST) -Morris water maze (MWM) -Colorectal distension (CRD) -Hot plate		-Increased visceral sensitivity observed in CRD -Depressive-like behaviour observed in the FST -Alterations in CNS serotonergic turnover -Reduction in hippocampal CRHR1 expression -Increase in amygdala BDNF expression	
Sprague Dawley	-Vancomycin in three concentrations (10, 30 100 mg/kg) Also assessed an antibiotic cocktail: -Pimaracin (5mg/kg), bacitracin (100mg/kg), neomycin (100mg/kg). -Delivered via oral gavage for 10 days beginning at P4	-Novel object recognition (NOR) -Open Field test (OFT) -Morris water maze (MWM) -Colorectal distension (CRD)	8-11 weeks	-Neonatal vancomycin significantly altered gut microbiota composition -Increased visceral sensitivity observed in CRD -Behaviour in adulthood was not affected by early-life antibiotic administration -Early-life antibiotic cocktail also had no effect on behaviour	(O'Mahony et al., 2014)

	-Dose of cocktail was halved after 5 days				
Wistar	Dams were fed either control diet, or diet supplemented with 1% SST (SuccinylSulfaThiazole) for one month before breeding and maintained until gestational day 15 (G15)	-Open field test (OFT) -Social interaction test -Marble burying -Elevated Plus maze (EPM) -Prepulse inhibition	6-7 weeks	-Reduction in social interactions -Anxiety-like behaviour observed in the EPM -Altered sensorimotor gating	(Degroote et al., 2016)
Non-rodent species					
Zebrafish *Danio rerio*	Ofloxacin Ciprofloxacin Enrofloxacin Doxycycline Chlortetracycline Oxytetracycline	Social cohesion	3 months of age	-Reduction in social cohesion behaviour -Increase in anxiety-like behaviour observed as a reduction in shoaling -Alterations in the expression of genes associated with locomotion	(Wang et al., 2016)

	Cocktail of all antibiotics with a dose ranging from 6.25, 12.5 and 25 mg/L administered in the water tank. Antibiotic cocktail was administered for 3 months.				
Fruit fly *Drosophila melanogaster*	Tetracycline 50 μg/mL Rifampicin 200 μg/mL Streptomycin 100 μg/mL dissolved in CMY medium individually or together as a cocktail	Multiple choice mating tests	-NA	-Male and female mating preference was abolished	(Sharon et al., 2013)

1.4.2. Mode of delivery

Mode of delivery is another factor that has been shown to modulate the composition of the microbiota, particularly in early life (Dominguez-Bello et al., 2010a). When babies are delivered vaginally, the first bacteria they are exposed to are from their mother's vaginal microbiota. When delivery is via C-section, this initial 'seeding' comes from contact with microbes present in the environment and on the mother's skin (Dominguez-Bello et al., 2010a). This leads to an altered microbiota in the gut as well as other body sites in infancy (Dominguez-Bello et al., 2010a; Lee et al., 2016; Neu and Rushing, 2011). For example, there is known to be a greater abundance of the *Bacteroides* genus in naturally born babies compared to caesarean-born (Dominguez-Bello et al., 2010a). While differences become less pronounced between the two groups over time (Backhed et al., 2015), the fact that a difference exists during a critical window of development may have long-lasting consequences (Borre et al., 2014a).

The rate of caesarean section delivery is rising, evidenced by the fact that between the years 2000 and 2015 the number of procedures performed globally almost doubled (Boerma et al., 2018). While this procedure can have a lifesaving impact when required, it is estimated that medically necessary C-sections occur in 10% to 15% of pregnancies (2015). The rates of elective c-section section surgeries have increased for cultural, cosmetic or other healthcare reasons to as high as 43% in Latin America (Boerma et al., 2018). Accumulating evidence suggests, however, that C-section should only be performed where there is a medical indication (Lumbiganon et al., 2010), and for good reason.

Delivery via c-section has been shown to have consequences on offspring during childhood, with increases in the rates of coeliac disease, (Decker et al., 2010; Marild et al., 2012), hypertension (Li et al., 2013), type 1 diabetes (Cardwell et al., 2008) and obesity (Li et al., 2013) in children delivered via this method. The metabolic consequences of being born via caesarean have also been shown to persist into adulthood (Darmasseelane et al., 2014; Horta et al., 2013). While these studies track the consequences of C-section delivery, they do not offer a mechanism through which the changes are brought about. We suggest that the altered gut microbiota following caesarean-section delivery may play a significant role in the development of such changes.

To date, few links have been made between birth via caesarean section and alterations in behaviour in a clinical cohort (Curran et al., 2016; Leung et al., 2017). While a study of ~2.7 million individuals found a "modest" (~20%) increase in the relative risk of a diagnosis of

autism spectrum disorder this effect was no longer seen when a sub-analysis of sibling controls was made (Curran et al., 2015a; Curran et al., 2015b). The establishment of links between mode of delivery, gut microbiota and behaviour clinically is made difficult by the numerous confounding factors that are present. These include breastfeeding vs formula feeding, antibiotic use, diet, socioeconomic status, and medication use among many others. In order to control for such factors, rodent models of caesarean section has been developed

The rodent c-section model has begun to provide useful information on the effects of mode of delivery on development. While many have recorded the effects on the microbiota, numerous studies have assessed the impact of c-section delivery independent of microbiota composition. The same altered gut microbiota composition and immune system development seen in babies born by c-section are also observed in mice born via this method (Hansen et al., 2014). Transfer of the gut microbiota from C-section mice to germ-free mice led to the same immune alterations, highlighting the importance of gut microbiota composition in immune system development.

Rodent models of caesarean section are also useful tools for determining the effect of mode-of-delivery on development of brain and behaviour. Research has found that discrete brain regions may be more susceptible to mode-of-delivery induced changes, for example (Chiesa et al., 2018). Dendritic arborization in hippocampal pyramidal neurons is underdeveloped at birth in CS mice compared to vaginally born (though this effect is not observed one day later, suggesting that CS may lead to an accelerated neuronal growth after birth) (Chiesa et al., 2018). Additionally, uncoupling protein 2 (UCP2) which regulates neuroprotection and synaptogenesis in the adult brain, is downregulated in the hippocampus of CS born mice compared to naturally born controls, potentially leading to changes in plasticity in later life (Seli and Horvath, 2013; Simon-Areces et al., 2012).

Whole-brain differences between the different modes of delivery have also been observed. C-section delivery has been linked to increased levels of neuronal cell death at birth compared to vaginally delivered controls (Castillo-Ruiz et al., 2018b). This suggests that there may be a protective effect of vaginal delivery, potentially mediated through vasopressin expression in the hypothalamus (Castillo-Ruiz et al., 2018b). When germ-free and conventionally colonized mice with the same mode of delivery were assessed for neuronal cell death some regions exhibited higher rates in GF mice while others were lower, thus indicating that microbiota composition does play a role in this process (Castillo-Ruiz et al., 2018a).

In addition to structural changes in the brain, mode-of delivery has been shown to alter behaviour in mice. Early life behaviour is particularly affected with vocalizations following maternal separation differing between natural born and C-section groups (Castillo-Ruiz et al., 2018b). Preterm C-section delivery in particular modifies communicative behaviours in pups. While these preclinical studies are beginning to shed light on the impact that mode of delivery may exert, such research is still in its infancy and there is significant scope for increasing the body of research in this area over the coming years both in the effects of c-section on behaviour and interventions that may play a role following this method of birth.

In order to overcome the developmental disruptions that occur following C-section birth various strategies have been developed that target the microbiota in this critical stage of early life. A strategy that has been used clinically is vaginal seeding. This method addresses the altered microbiota immediately following birth (Dominguez-Bello et al., 2010a), seeding infants with the microbes that they would have received following vaginal birth (Dominguez-Bello et al., 2016). It involves swabbing the neonate with vaginal fluid in the minutes following delivery, and while a pilot study has demonstrated positive results (Dominguez-Bello et al., 2016), its use has been controversial with both positive (Knight and Gilbert, 2016) and negative (Cunnington et al., 2016) response from experts in the field. The potential impact of the transfer of the vaginal microbiome has also been observed preclinically. When C-section born mice are exposed to the vaginal microbiome of prenatally stressed mice, these pups displayed changes not only in the gut but also in hypothalamic gene expression in adulthood similar to prenatally stressed males (Jašarević et al., 2018).

Other interventions that target the gut microbiota and can be administered to either the mother or the offspring are beginning to be studied and include prebiotic, probiotic and synbiotic administration which will be discussed in greater detail in the following sections. Administration of a prebiotic diet has been shown to reverse many of the immune changes that occur following C-section delivery (Zachariassen et al., 2019). Such interventions, therefore, may provide the cornerstone of microbiota-modulation therapies in the future.

1.4.3. Prebiotics

Targeting the microbiota with prebiotic and probiotic therapies is one of the routes through which it may be possible to exert a positive effect over various aspects of host physiology including brain and behaviour (Dinan et al., 2013a). Indeed the specific use of such interventions to beneficially impact behaviour has led to the coining of the term

'psychobiotics' (Dinan et al., 2013a). The ease of administration and potenital lack of side-effects of these interventions marks them out as a particularly desirable therapeutic approach. Probiotics in particular fit this bill as they may be obtained simply through dietary modulation.

Prebiotics act as a source of energy for bacteria, aiming to encourage the growth of beneficial bacterial strains. They have been defined by the Scientific Association for Probiotics and Prebiotics as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). They may be obtained via dietary fibre, through foods such as asparagus, banana, oats, and wheat. Additionally, they may be administered as dietary supplements such as inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS). Furthermore, human milk oligosaccharides (HMOs), a key component of maternal breast milk, act as a prebiotic (Bode, 2009) highlighting the importance of diet and maternal care during a critical period of development.

Prebiotic administration has been shown to have many beneficial effects on brain and behaviour in animal models. FOS and GOS have been shown to decrease anxiety-like (Burokas et al., 2017; Tarr et al., 2015) and antidepressant-sensitive behaviours (Burokas et al., 2017) while increasing sociability (Burokas et al., 2017). Probiotic treatment has also demonstrated beneficial effects on learning and memory in mice (Vazquez et al., 2015) and rats (Gronier et al., 2018; Vazquez et al., 2016). Further to this, prebiotic supplementation has shown to lead to beneficial effects on neurophysiology through increases in hippocampal BDNF expression (Burokas et al., 2017) and hippocampal dendritic spine density (Waworuntu et al., 2016), decreases in neuronal death (Song et al., 2013) and improvements in long-term potentiation (Oliveros et al., 2016b; Vazquez et al., 2015)

Furthermore, when gut microbiota alterations and increases in inflammatory markers were observed following C-section delivery (Zachariassen et al., 2019), a prebiotic diet was able to re-establish gene expression of intestinal immune markers and iNKT cells (Zachariassen et al., 2019) thereby highlighting a potentially useful treatment option following early-life disruption of gut microbiota composition.

1.4.4. Probiotics

Probiotics refer to species of bacteria which, if ingested in adequate amounts confer health benefits to the host (Butel, 2014). Probiotic administration has been shown to exert exert a

wide range of effects upon host health, with various strains improving metabolism, immunity, endocrine function, and slowing aging (El Aidy et al., 2015; Patterson et al., 2016). In addition to their administration alone, probiotics may be administered in conjunction with prebiotics in the form of 'synbiotics'. Here, the administration of this combination leads to an improved survival of the administered bacterial strain and potentially improved welfare of the host (Gibson and Roberfroid, 1995).

Probiotic administration has been shown to influence aspects of the central nervous system including neuropeptide expression (Dhaliwal et al., 2018b; Lee et al., 2018a), receptor subunit expression (Barrera-Bugueno et al., 2017; Bravo et al., 2011a) neuroinflammation (Chunchai et al., 2018b; Distrutti et al., 2014b) and synaptic transmission (Buffington et al., 2016; Distrutti et al., 2014b).

The use of probiotics as an interventional tool within rodent models has also highlighted the potential for their use to modulate numerous behavioural characteristics. To date, probiotic administration has shown positive effects on anxiety-like behaviour (Bercik et al., 2011a; Bravo et al., 2011a; Moya-Perez et al., 2017), cognition (Lee et al., 2018a; Sun et al., 2016), stress response (Gareau et al., 2011), antidepressant-sensitive behaviour (Bravo et al., 2011a; Desbonnet et al., 2010a; Dhaliwal et al., 2018a) and social behaviour (Buffington et al., 2016).

An additional area in which probiotics and synbiotics have been shown to be beneficial in mice is in the reversal of the effects of microbiota disruptions following antibiotic administration or C-section delivery. Disruptions in *Clostridia* and *Biffidobacteria* species could be reversed following administration of *B. longum* and human milk oligosaccharides (Musilova et al., 2017) as well as increasing anti-inflammatory IL-10 production (Musilova et al., 2017). Disruptions in social behaviour, cytokine expression and blood-brain-barrier integrity following perinatal maternal antibiotic administration could be reversed following probiotic administration (Leclercq et al., 2017c). Furthermore, antibiotic-induced changes in immobility in the tail suspension test, social recognition, BDNF expression and hippocampal electrophysiology have been shown to be reversed following one week of probiotic gavage (Guida et al., 2018b). Deficits in hippocampal neurogenesis and memory retention following antibiotic administration could only be reversed following reconstitution with normal gut bacteria if a probiotic was co-administered (Mohle et al., 2016) again highlighting the benefits of these treatments, either alone or in combination with other microbiota-targeting interventions.

Such positive results explain why the development of commercial probiotics has been at the centre of many recent studies (Bibiloni et al., 2005; Brenner and Chey, 2008) and this trend seems set to increase in scope in the future.

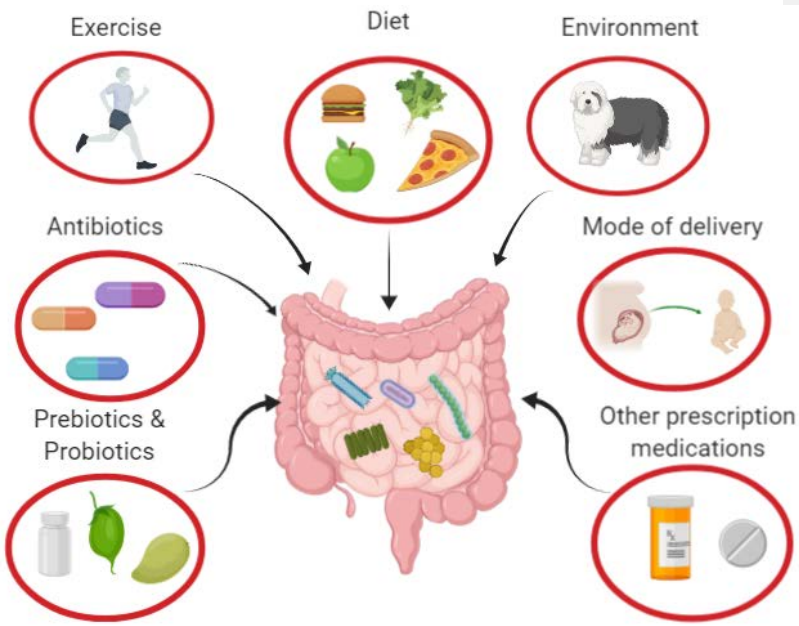

***Figure 12**: **Factors influencing gut microbiota composition.** These are many and varied and include: Prebiotics, probiotics, antibiotics, exercise, diet, environment, mode of delivery and prescription medication.*

1.5: The microbiota gut brain axis through the lifespan

As discussed in section 1.1.1 the microbiota in early life and in old age differs significantly from the relatively stable composition that is present in adulthood (Claesson et al., 2012; Salazar et al., 2014; Yatsunenko et al., 2012). As such it has been suggested that the particular composition of the microbiota may play a more significant role in altering the gut-brain axis during these periods, as well as making them more susceptible targets for microbiota-modulating therapies. (Borre et al., 2014a; Gensollen et al., 2016; Gur et al., 2015). Here we will discuss differences in the microbiota-gut-brain axis throughout the lifespan.

1.5.1. Early Life

Gut microbiota composition in early life is influenced by a small number of factors. These include mode of delivery of birth, antibiotic exposure in early life and breastfeeding vs formula feeding (Rinninella et al., 2019). As mentioned in section 1.4.2, mode of delivery leads to alterations in gut microbiota composition. Dendritic arborization in hippocampal pyramidal neurons is underdeveloped at birth in C-section born mice compared to vaginally born (Chiesa et al., 2018) while whole-brain differences have also been observed. Furthermore, C-section delivery has been linked to increased levels of neuronal cell death at birth compared to vaginally delivered controls (Castillo-Ruiz et al., 2018b).

Behavioural alterations have also been observed in early life between different modes of delivery. Ultrasonic vocalizations differ between C-section and vaginally born animals (Castillo-Ruiz et al., 2018b). Birth via C-section has been linked to increased anxiety-like behaviour, reductions social recognition and a heightened response to stress (Morais, 2018)

Maternal antibiotic administration is often required following C-section delivery in order to prevent infection (Cunningham GF, 2005) and this can lead to exposure of offspring to antibiotics through breast milk (Kaiser et al., 2007). Early-life exposure through perinatal antibiotic administration has been shown to cause alterations in anxiety, sociability, social novelty and aggression in the offspring, as well as changes in blood-brain barrier permeability and cytokine expression in the frontal cortex (Leclercq et al., 2017b). These changes were associated with alterations in microbiota composition and some could be prevented with co-administration of the probiotic *Lactobacillus rhamnosus* JB-1 (Leclercq et al., 2017b). Clinically, antibiotic use in the first year of life has been linked to neurocognitive outcomes later in childhood, with more behavioural difficulties and more symptoms of depression at follow-up (Slykerman et al., 2017).

As mentioned previously, particular sugars found in human breast milk (referred to as human milk oligosaccharides, HMOs) have prebiotic properties (Bode, 2009). While there are a paucity of studies in humans that assess specific effects of these compounds on brain and behaviour, there are a number of studies in rodents that have found them to be beneficial. When mice and rats were administered a diet supplemented with the most abundant HMO, 2´-fucosyllactose beneficial effects were observed in spatial learning, working memory and operant conditioning, as well as leading to improvements in hippocampal LTP and BDNF expression (Vazquez et al., 2015). That a subsequent study found that these effects of 2´-fucosyllactose were dependent on the integrity of the vagus nerve (Vazquez et al., 2016) lands weight to the theory that these effects are due to the activity of the microbiota-gut-brain axis.

1.5.2. Adolescence

Adolescence is a time of vast change throughout the body. As well as the well documented hormonal changes that occur during this period, it is also a significant period for brain development. Alterations occur in brain volume, synaptic pruning and myelination during this period (Blakemore and Choudhury, 2006; Casey et al., 2008; Spear, 2000). Along with these physical alterations it is also a period of significant lifestyle change, meaning that adolescents are particularly vulnerable to mental health problems (Kessler et al., 2005; Lee et al., 2014; Paus et al., 2008).

Clinical studies have found that as adolescents approach adulthood their microbiota becomes more similar to the adult microbiota (Agans et al., 2011). There are few preclinical studies during this period but interesting results have been observed. Differences in microbiota composition between the sexes are only seen following the onset of puberty (Markle et al., 2013) and that probiotic treatment during a period of early-life stress reverses stress-induced changes in the timing of puberty onset in mice (Cowan and Richardson, 2018).

Antibiotic administration during adolescence alters cognition, social behavior, and anxiety-like behaviour in adulthood with central expression of BDNF and oxytocin also observed (Desbonnet et al., 2015a). The research during this window builds on studies assessing the effects of microbiota modulation during early life highlighting the fact that interventions exert a more profound effect when administered in early life compared to similar interventions given during adulthood (Buffington et al., 2016; Diaz Heijtz et al., 2011; Ellekilde et al., 2014; Olszak et al., 2012b; Sudo et al., 2004b). As such targeting these critical periods remains a rich topic to be assessed in future studies.

1.5.3. Ageing

A functional role for the microbiota in ageing is suggested by many clinical and preclinical studies. The fact that germ-free rodents live longer than conventionally colonized controls highlights this key role (Glimstedt, 1959; Gustafsson, 1946). In humans, age-related shifts are linked to various measures of health including cognition, inflammation and depression (Claesson et al., 2011; Claesson et al., 2012).

Another key factor linked to gut microbiota composition is "inflammaging" (Thevaranjan et al., 2017). This is the decline in adaptive immunity and increased proinflammatory status observed in old age (Franceschi et al., 2000). Inflammaging affects the speed of the ageing process and may be linked to the development of age-related diseases (Xia et al., 2016). These include Alzheimer's disease (Giunta et al., 2007) heart disease and type II diabetes (Boren and Gershwin, 2004; Franceschi et al., 2001; Lencel and Magne, 2011). Compositional changes in the microbiota that occur with age have been shown to induce intestinal inflammation (Guigoz et al., 2008) while fecal microbiota transfer from old to young mice was also associated with an Inflammaging phenotype (Fransen et al., 2017), both strengthening the link between gut microbiota composition and inflammaging.

Administration of microbiota-targeting diets to prevent the age-associated decline of beneficial *Bifidobacterium* has been found to have positive effects on gut microbiota composition and associated health. A recent study found that a 14-week long dietary intervention with prebiotics increased both *Bifidobacterium* and *Akkermansia* in middle-aged mice (Boehme et al., 2018; Johnson et al., 2011). Another interesting study in the short-living killifish showed that exchange of microbial communities from young to old via fecal transplantation increased longevity in the older group (Smith et al., 2017) again highlighting the microbiome as a target for modulating therapies throughout the lifespan.

In addition to the physical effects mentioned, there are also effects of ageing on the central nervous system. A number of psychiatric conditions, most notably anxiety, depression and social withdrawal are frequently reported in the elderly (Prenderville et al., 2015). Furthermore, the aforementioned peripheral inflammation, linked to gut microbiota composition, is known to directly affect neuroimmune processes in the central nervous system resulting in impaired cognitive function (Kelly et al., 2016b). In mice, older animals have been shown to exhibit deficits in spatial memory and increases in anxiety-like behaviour compared to younger animals. These changes were also associated with increased gastrointestinal permeability, increased proinflammatory cytokines, and a shift towards a

microbiota profile previously linked to inflammatory disease (Scott et al., 2017a). A stress-induced immune priming has also been observed in middle-aged mice, which can be reversed through prebiotic diet administration alongside reversal of age-related increases in activated microglia (Boehme et al., 2019b).

Each of these studies demonstrate the importance of microbiota composition throughout the lifespan and how various aspects of physiology, immunity and behaviour can be affected by gut bacteria. They also emphasize how targeting the microbiota with specific prebiotics, probiotics and synbiotics at specific points in life can serve as a novel therapeutic avenue in the future.

1.6. Preclinical models and the role of the microbiota in neurological and psychiatric disorders.

The use of rodent models allows researchers to delve deeply into the microbiota-gut-brain axis, to investigate the behavioural and neurochemical aspects of experimental models such as the germ-free mouse in order to probe the mechanisms that link the microbiota to the brain and that underlie any observed behavioural changes. Preclinical models also allow the assessment of a vast array of modulations of the microbiota as mentioned above, including antibiotics, probiotics, FMT and dietary modulation. In each of these there is also the ability to assess numerous aspects of physiology, again with the aim of improving knowledge about the mechanisms underlying the changes in brain and behaviour.

While such experiments have accelerated the field into its current level of expertise there remains a translational gap between preclinical and clinical studies. Some novel techniques have been used to attempt to bridge the two consolidate these two areas of research. For example, when microbiota differences were observed between patients with major depressive disorder and healthy controls (Kelly et al., 2016b; Zheng et al., 2016), a pooled faecal sample from these depressed groups was transferred to a germ-free rodent (Zheng et al., 2016) or a rodent with an antibiotic-depleted microbiota (Kelly et al., 2016b). The behavioural phenotype was also transferred. Another way that preclinical models are aiming to answer questions about aspects of the brain and behaviour is through the use of animal models of psychiatric and neurological disorders. To date examples of conditions that have been studied in this manner include Alzheimer's disease (Minter et al., 2016a), Parkinson's

disease (Sampson et al., 2016b), anxiety (Jørgensen et al., 2014) and epilepsy (Olson et al., 2018).

1.6.1. The Gut microbiota and neurodevelopmental disorders

One key clinical condition that is consistently linked to alterations in gut microbiota composition and in which these links are being investigated both clinically and preclinically is autism spectrum disorder (ASD) (Adams et al., 2011). Indeed probiotics have been suggested as a potential intervention in the condition (Gilbert et al., 2013), though much further research remains to be undertaken in the field.

The aetiology of ASD is not completely known but believed to involve both genetic and environmental factors. Approximately 50% of the disorder is believed to be accounted for via de-novo mutations, short nucleotide polymorphisms and common variants that occur across many incidents of the disorder (Gaugler et al., 2014; Iossifov et al., 2015; Kong et al., 2013). There remains a large scope, therefore for environmental factors to exert significant impact over the behavioural aspects of the disorder. Inflammation is a factor that may play an important role in its development, for example. Post-mortem tissue from ASD patients has shown both increases in activation of astrocytes and microglia, as well as increases in levels of proinflammatory cytokines in the prefrontal cortex (Vargas et al., 2005). Furthermore, genes linked to ASD that encode for immune system features are mutated in the disorder, leading to disruptions in structural and functional connectivity in areas of the brain key for socio-communicative function (Estes and McAllister, 2015; Hsiao, 2013).

In addition to immune dysfunction, gastrointestinal abnormalities are another key feature of ASD (Adams et al., 2011; Williams et al., 2012). Abdominal pain, constipation, diarrhoea and acid reflux are all common symptoms; along with increased intestinal permeability (Emanuele et al., 2010). As both immune function and gastrointestinal issues have been strongly linked to gut microbiota composition (Fung et al., 2017), investigating links between the gut microbiota and ASD was a logical progression. In addition to these physiological features of ASD, gut microbiota composition has also been linked to the behavioural features of the disorder. These include social communications (Buffington et al., 2016), anxiety (Leclercq et al., 2017c), cognitive performance (Frohlich et al., 2016) and repetitive behaviours in rodent models (Desbonnet et al., 2013).

Alterations in gut microbiota composition between ASD and neurotypical individuals have been noted. The genus *Clostridium* have been observed in a greater abundance in ASD individuals (Finegold et al., 2002; Song et al., 2004), furthermore, individuals with ASD have

a decreased *Bacteriodetes/Firmicutes* ratio and an increase in *Lactobacillus and Desulfovibrio* species which correlate with the severity of ASD symptoms (Tomova et al., 2015). Levels of the short-chain fatty acids acetate, propionate and butyrate are reduced in ASD patients (Adams et al., 2011). In spite of these links between ASD and gut microbiota composition there is no consensus on which bacteria may form an 'ASD microbiome'.

The potential for the use of the gut microbiota as a therapeutic tool has gained interest recently. In one such study, a standardized human gut microbiota was transferred to children with ASD (Kang et al., 2013). This treatment led to an improvement in GI function as well as a reduction in behavioural ASD scoring. In spite of this, it not yet known if the gut microbiota itself in autism can impact behavioural or gastrointestinal symptoms of ASD. Furthermore, treatment with antibiotics has also shown to be of benefit in the disorder. When children with ASD were treated with the broad-spectrum antibiotic, vancomycin, it led to a significant improvement in behavioural symptoms (Kang et al., 2017). And while the long-term consequences of prolonged antibiotic administration may preclude it from being a maintenance strategy in the disorder this result, combined with the previous data demonstrating a positive effect of probiotic treatment highlights the critical role that the gut microbiota may play in the behavioural aspects of the disorder.

Identification of specific bacterial taxa and associated bacterial metabolites that can trigger changes in GI physiology and modulate the onset of autistic behaviours is thus an important quest in the search for precision microbiota-based therapeutics in ASD (Gilbert et al., 2013). Preclinical research and the development of well-validated animal models are also crucial in this endeavour (Hsiao et al., 2013b; Vuong and Hsiao, 2017). A recent study found that when gut microbiota was transplanted from humans with ASD to germ-free mice there was an increase in ASD-like behaviours in these mice compared to germ-free mice receiving the microbiota of typically developing controls (Sharon et al., 2019). Furthermore, when the BTBR mouse model of ASD was treated with metabolites that are enhanced in the microbiota of typically developing individuals behavioural abnormalities and neuronal excitability were normalized (see figure below) (Sharon et al., 2019). Targeting the microbiome offers hope as a future area of therapy in ASD.

1.6.2. The BTBR mouse strain and other rodent model of ASD

Preclinical studies of ASD generally rely on well-validated rodent models to gain a more robust understanding of the clinical picture of the condition as well as the mechanisms

underpinning any observed differences. As genetic, molecular, structural, or physiological mechanisms are often unclear in ASD, rodent models of the disease are classified according to the behavioural phenotype. The first class of behaviours being impairments in social and communicative behaviour, the second an excess of repetitive behaviours (Meyza and Blanchard, 2017). `

A number of preclinical models of ASD have been generated, each in a different manner and with different models exhibiting a greater preponderance of a particular subset of symptoms. Most interestingly, each has been linked with disturbances of the gut microbiota that may act as a key component for future treatments of the disorder.

The BTBR T+ Itpr3tf/J mouse strain is one of the most widely used models of ASD. Similar to humans, these animals display robust deficits in social interactions and enhanced engagement in repetitive behaviours (Meyza et al., 2013; Moy et al., 2007). Moreover, the autistic-like behaviour of BTBR mice is largely driven by multiple genetic alterations (Meyza and Blanchard, 2017). While structural alterations are present in numerous brain areas in these animals (Mercier et al., 2012), perhaps the most striking difference is the lack of a corpus callosum (Mercier et al., 2012).

In addition to the vast structural, molecular, and behavioural differences brought about by these genetic alterations, the BTBR model is also sensitive to environmental influences. such as dietary intervention (Ruskin et al., 2013). In a study in which juvenile mice were administered a ketogenic diet these animals showed improvements in sociability, and repetitive behaviour, as well as improvements in seizure-activity in the hippocampus (Ruskin et al., 2013). Since this diet is known to modify gut microbiota composition in children (Lindefeldt et al., 2019) this raises important questions about therapeutic targeting of the gut microbiota through dietary modulation.

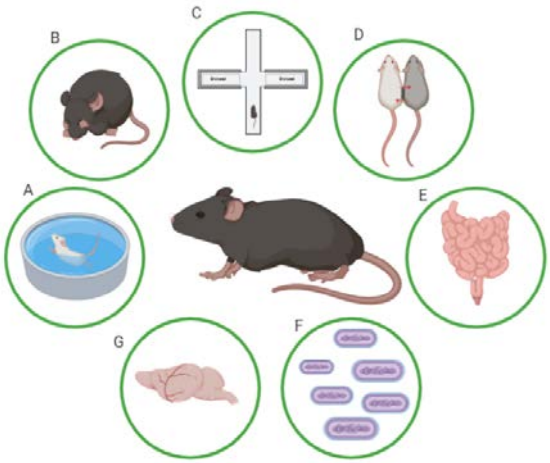

Fig 13: The BTBR mouse model of ASD: *BTBR mice display a number of distinct behavioural and physiological features. (A) Reduced immobility in the forced swim test (B) Increased grooming behaviour (C) Increased anxiety-like behaviour in the elevated plus maze (D) Reduced sociability (E) Altered intestinal physiology (F) Altered gut microbiota composition (G) Altered brain structure, including the lack of a corpus callosum*

BTBR animals have also been shown to exhibit alterations in gastrointestinal physiology that closely mirror the changes observed in a clinical setting (Golubeva et al., 2017). These mice display reduced intestinal motility, indicative of constipation (Golubeva et al., 2017). In addition, they express altered gastrointestinal permeability in both the small and large intestines (Coretti et al., 2017). This altered permeability may play a role in the translocation of bacteria or their metabolites into systemic circulation where there is the potential to exert an inflammatory response (Golubeva et al., 2017). Profound alterations in the microbiota composition characterized by deficits in the relative abundance of *Bifidobacterium* and *Blautia* genera are also observed compared to C57BL/6 mice. The absence of these two bacterial genera was linked to additional deficits in bile acid signaling in these mice, which may contribute towards its observed GI physiology (Golubeva et al., 2017). These animals were shown to differ in gut microbiota composition from C57BL/6 controls with dietary modulation affecting the two strains differently (Klein et al., 2016; Newell et al., 2016)

In addition to the genetic alterations that account for the majority of the differences observed in the BTBR model, environmental factors are the other critical component capable of the generation of a robust ASD-like phenotype. For example in the maternal immune activation model of ASD, in which the pregnant dam is exposed to the viral component and TLR3 agonist, poly I:C, offspring mice developed autistic-like behaviours while also displaying an altered gut

microbiota composition (Hsiao et al., 2013b). Furthermore, *in utero* exposure to the antiepileptogenic valproic acid (VPA) has been shown to result in intestinal inflammation (Rosenfeld, 2015), a disrupted gut microbiota composition assessed via the *Firmicutes:Bacteroidetes* ratio (de Theije et al., 2014b) as well as an increase in the abundance of the species *Desulfovibrio* (Finegold, 2011). This model has also established social deficits, as well as altered turnover of serotonin in the amygdala (de Theije et al., 2014a; de Theije et al., 2014b).. It is not only the composition of the microbiota directly that is believed to be important, but their production of metabolites such as the short-chain fatty acids (Shultz et al., 2015). Treatment of BTBR animals with a SCFA, butyrate has been shown to both lower exacerbated repetitive behaviour and also enhance preference for social novelty (Kratsman et al., 2016) again highlighting the importance of microbial metabolites.

In conclusion, current evidence implicates the microbiota-gut-brain axis as a critical signaling pathway in autism spectrum disorder, where both gastrointestinal function and host behaviour are affected, and the microbiota displays significant potential as a target for future therapeutic interventions.

1.7. Goals and aims of this thesis

The overall goal of this thesis was to investigate the impact of gut-brain axis disruption throughout the lifespan as well as the associated neurobehavioural consequences.

To this end, we employed various methods to disrupt the microbiota in early life. Given the importance of the initial colonization on brain development and behaviour, we utilised a mouse model to assess the long-term consequences of birth by C-section. We also investigated the potential for psychobiotic administration to reverse the behavioural alterations brought about by these disruptions.

We then investigated the additional impact of maternal antibiotic administration in the first week of life on behaviour in these animals. To further probe the effects of early-life maternal antibiotic administration, we compared the effects of administration of a single antibiotic to a cocktail of antibiotics on behaviour in early life and in adulthood. Finally, in order to examine the impact of a life with a disrupted microbiota we assessed behavioural differences in between BTBR mice and C57BL/6 in old age.

In an attempt to address a proposed mechanisms of behavioural alterations we assessed the ability of various aspects of microbiota modulation to impact synaptic plasticity in the brain. This was achieved through the assessment of plasticity-related genes following maternal

antibiotic administration, as well as determining the effects of gut microbiota metabolites on synaptic signalling in the hippocampus.

Aim 1: To investigate whether the mode of delivery of birth can alter microbiota-gut-brain axis function with behavioural, physiological and microbiota alterations in adulthood (chapter 3)

Aim 2: To determine whether disruption of the microbiota in early-life following maternal antibiotic administration can lead to alterations in behaviour throughout the lifespan (chapters 2 and 3)

Aim 3: To assess whether administration of psychobiotics (prebiotics, probiotics & synbiotics) can impact behaviour and microbiota composition in adulthood following early-life microbiota disruption (chapter 3)

Aim 4: To examine the behavioural and immune system differences between a mouse model of ASD and C57BL/6 mice in old age. (chapter 4)

Aim 5: To probe the potential for the microbiota to impact synaptic plasticity through assessing plasticity-related gene expression following early-life antibiotic administration and through measurement of electrophysiological alterations in the hippocampus following perfusion with gut microbiota metabolites (Chapters 2 & 5)

Chapter 2

Maternal Antibiotic Administration During a Critical Developmental Window Has Enduring Neurobehavioural Effects in Offspring Mice

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2.1. Abstract

Rates of perinatal maternal antibiotic use have increased in recent years linked to prophylactic antibiotic use following Caesarean section delivery. This antibiotic use is necessary and beneficial in the short-term; however, long-term consequences on brain and behaviour have not been studied in detail. Here, we endeavoured to determine whether maternal administration of antibiotics during a critical window of development in early life has lasting effects on brain and behaviour in offspring mice. To this end we studied two different antibiotic preparations (single administration of Phenoxymethylpenicillin at 31mg/kg/day; and a cocktail consisting of, ampicillin 1mg/ml; vancomycin 0.5mg/ml; metronidazole 1mg/ml; ciprofloxacin 0.2mg/ml and imipenem 0.25mg/ml). It was observed that early life exposure to maternal antibiotics lead to persistent alterations in anxiety, sociability and cognitive behaviours. These effects in general were greater in animals treated with the broad-spectrum antibiotic cocktail compared to an individual antibiotic with the exception of deficits in social recognition which were more robustly observed in Penicillin V exposed animals. Given the prevalence of maternal antibiotic use, our findings have potentially significant translational relevance, particularly taking into account the implications on infant health during this critical period and into later life.

2.2. Introduction

As the links between gut microbiota composition and behaviour become more established through both clinical and preclinical studies, research has begun to focus on the effects of microbiota disruption during critical windows of development. To date, the impact of antibiotic administration has been assessed throughout the lifespan in animal models including perinatally (Leclercq et al., 2017b), during early life (O'Mahony et al., 2014), adolescence (Desbonnet et al., 2015a), adulthood (Hoban et al., 2016b) and in old age (van Opstal et al., 2016). Of particular interest is the early postnatal period as this is a critical time for both neuronal development (Zoghbi, 2003) and the initial seeding of the microbiota (Mutic et al., 2017). Indeed microbial colonization during this period has been linked to development of the hypothalamic-pituitary-adrenal (HPA) axis, influencing stress response in mice (Sudo et al., 2004b). However, due to the high rates of antibiotic use in the postpartum period in both mother and infant, disturbance to the microbiota in early life is commonplace, which has the potential to significantly impact on brain and behaviour in the offspring.

Maternal antibiotics are administered to prevent postpartum infection in the mother for reasons including trauma sustained during delivery, post-surgical complications following C-section, or physiological changes that may occur during pregnancy (Cunningham GF, 2005). During these treatment periods, mothers are recommended to continue breastfeeding and each of the antibiotics used maternally are considered safe for nursing infants with no reported adverse effects on the neonate being observed during early life (2001; Kaiser et al., 2007). Monitoring of adverse events in the offspring following maternal antibiotic administration is generally confined to the duration of treatment and a short period thereafter, neglecting to account for any potential long-term consequences.

Maternal antibiotic use has however, been linked to the development of asthma in offspring (Stokholm et al., 2014), and this link is present whether antibiotics were administered before or during pregnancy, or in the weeks following birth (Stokholm et al., 2014). Furthermore, the fact that antibiotics administered to the mother can reach breast milk and potentially exert a direct impact on the offspring microbiota must be considered. This potential for disruption to the microbiota during a critical window may have significant impact later in life and these effects require more detailed analysis.

Antibiotic exposure during early-life, however, is not solely as a result of maternal antibiotic administration as antibiotics are also the most common class of drug that is administered to infants directly (Chai et al., 2012), with oral penicillins particularly prevalent (Chai et al., 2012). While antibiotic use among children may be critical to maintain health during this vulnerable period, there are also associated risks. Since it is known that the microbiota can influence host metabolic activity (Tremaroli and Bäckhed, 2012), disruption of its composition during this period can impact the host by either promoting weight gain (Azad et al., 2017) or by stunting growth (Cox and Blaser, 2015b), thereby having lasting effects on body weight throughout the lifespan (Cox and Blaser, 2015b). Links between childhood antibiotic use and development of IBD (Hviid et al., 2011) and asthma (Ahmadizar et al., 2017) have also been made. Antibiotic use in the first year of life has been linked to neurocognitive outcomes later in childhood, with more behavioural difficulties and more symptoms of depression at follow-up (Slykerman et al., 2017).

Antibiotics also serve as a valuable tool to modulate the microbiota in preclinical models that assess behaviour. Compared to other modes of gut microbiota modulation, such as the germ-free mouse, antibiotics offer far greater flexibility over the extent to which they disrupt the microbiota. They can be administered at any stage of the lifespan, either acutely (Bercik et al., 2011a) to mimic a short course as seen clinically, or in cocktails administered over prolonged periods to effectively eradicate the microbiota (Guida et al., 2018a; Mohle et al., 2016).

Animal models of maternal antibiotic administration in the perinatal period have been shown to have long-term effects on offspring metabolic activity (Cox et al., 2014) and immune system development (Russell et al., 2013), with the pre-weaning period having been identified as pivotal for these changes in immune response (Russell et al., 2012). Indeed, a "critical window" has been suggested in immune system development, during which disruption, if it occurs, may lead to predisposition to a number of diseases that are initiated by an abnormal mucosal immune response (Penders et al., 2007)

Furthermore, antibiotic administration during this window has been shown to induce changes in brain and behaviour. When dams were administered penicillin V from one week prior to delivery until the end of the weaning period, alterations were observed in anxiety in the offspring, as well as changes in sociability, social novelty and aggression (Leclercq et al., 2017b). These changes were associated with alterations in microbiota composition and some could be prevented with co-administration of the probiotic *Lactobacillus rhamnosus* JB-1

(Leclercq et al., 2017b). This study in particular highlights the potential for antibiotic administration during this critical window to have long-term effects on brain and behaviour and provides the basis for the selection of the single antibiotic used in our study.

As well as effects on behaviour, administration of antibiotics directly to rodent offspring has shown to have an influence on the brain, including alterations that impact synaptic plasticity in these animals. Aspects of brain physiology including BDNF expression (Fröhlich et al., 2016), NMDA receptor subunit expression (Fröhlich et al., 2016) neurogenesis (Möhle et al., 2016) and electrophysiological recordings (Guida et al., 2018a) have each been shown to be altered following antibiotic administration. Such changes in the brain may provide clues towards a mechanism for the behavioural disruptions observed following antibiotic administration.

The widespread alterations that are observed across different animals, strains and antibiotic cocktails in the above studies demonstrates the powerful effects that antibiotics can have on the brain and on behaviour. Our study aims to shed further light on these findings by assessing changes in offspring behaviour following administration of either a single antibiotic or a cocktail of antibiotics to mothers for one week, beginning one day following the birth of their offspring. The results of these investigations may prove to be clinically relevant to how maternal infections in the perinatal period are treated in the future.

2.3. Materials and Methods

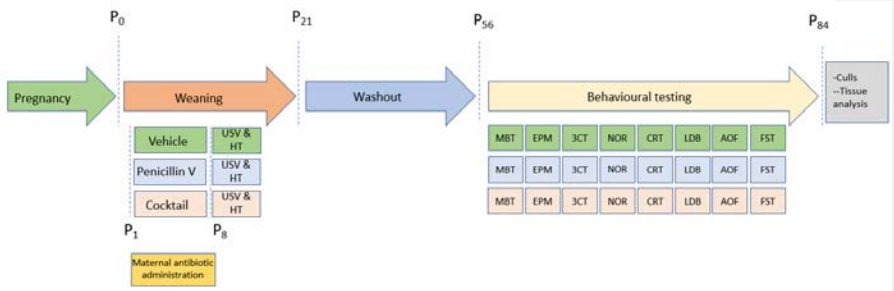


***Fig 1: Study Design**. One day following the birth of the pups weaning mothers were divided into three groups: Vehicle, Penicillin V or antibiotic cocktail, with interventions administered for 7 days in drinking water. Early life behavioural tests were carried out at postnatal day 9 (Ultrasonic Vocalization, USV) and 10 (Homing test, HT). Behavioural tests were then undertaken in beginning at 8 weeks of age following a washout period. Tests were undertaken in the following sequence: Marble burying test (MBT); Elevated Plus Maze (EPM); Three Chamber test (3CT); Novel Object Recognition (NOR); Carmine Red Test (CRT); Light-dark box (LDB); Aversive Open field (AOF) and Forced Swim test (FST). Animals were culled at the end of the experiment and relevant tissues harvested for analysis. N numbers for behavioural tests: Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15*

2.3.1. Animals

All animal experiments were approved by the animal experimentation ethics committee at University College, Cork (UCC), and by the health products regulatory authority (HPRA) of Ireland in accordance with EU directive 2010/63/EU. 8-week-old female and male C57BL/6 were obtained from Envigo laboratories, UK. Breeding began after 2 weeks of acclimatization to the holding room. Animals were kept under a strict 12:12h light-dark cycle and in a temperature controlled room (20 ±1 °C, 55.5% humidity), with food and water supplied *ad libitum.* Male offspring were weaned at P21, and housed in groups of 3-4 mice per cage. Groups consisted of offspring from 14 independent litters. In addition to animals purchased for breeding, 8-week-old C57BL/6 mice were purchased from Envigo laboratories, UK, for use as conspecifics in the three-chamber test of sociability.

2.3.2. Antibiotic Administration

In order to deplete the maternal gut microbiota, dams were administered antibiotics in drinking water for seven days, beginning one day after the birth of the pups (P1). Dams were exposed to either a single antibiotic – Penicillin V, at a dose of 31mg/kg/day (as per (Leclercq et al., 2017b), or a cocktail of antibiotics (consisting of Ampicillin 1mg/ml; Vancomycin

0.5mg/ml; metronidazole 1mg/ml; ciprofloxacin 0.2mg/ml and Imipenem 0.25mg/ml), or water. Antibiotics were dissolved in autoclaved water and changed every two days. Control animals received just autoclaved water, which was also changed every two days. Liquid intake was estimated by measuring bottle weights before replenishment. Maternal weights were taken daily during the antibiotic administration period in order to ensure that excessive weight loss did not occur. Cages were cleaned every second day during treatment (as well as in control groups) in order to prevent the re-establishment of a normal microbiota through coprophagy.

2.3.3. Behavioural testing

2.3.3.1. Isolation-induced ultrasonic vocalisation tests (USV)

Isolation-induced ultrasonic vocalisations (USV) are produced by mouse pups during the first two weeks of life when separated from littermates (Scattoni et al., 2009a). At postnatal day 9 (P9) pups were removed from the home cage and habituated in a room away from their mother and littermates for ten minutes. During the experiment, animals were placed in a clean, plastic container, within a sound-attenuating chamber. Ultrasonic vocalisations were monitored using an ultrasonic-sensitive microphone – a bat detector (US mini-2 bat detector, Summit, Birmingham, USA) tuned into the range of 60-80kHz – suspended above the isolated pup for three minutes, the number and total duration of calls were noted.

2.3.3.2. Homing test

The homing test (HT) evaluates the tendency of pups to recognize the nest of their mother and siblings at P10 and was carried out as described previously (Fiori et al., 2017). The floor of a clean mouse cage was subdivided into three areas by wire mesh dividers, one of which was uniformly covered with wood shavings from the home cage, thus containing familiar odour stimuli. The opposite space was covered with wood shavings from the cage of another litter (born at approximately the same time) - the middle section was covered in clean bedding material. Individual pups were placed in the middle section for one minute, the dividers were then removed, and the pups were allowed to move freely for 2 minutes. Total time spent in each area was noted.

2.3.3.3. Defensive Marble Burying

The defensive marble-burying test (MBT) measures repetitive and anxious behaviours, with a greater number of marbles buried representing increasing levels of anxiety. The test was undertaken as previously described (Golubeva et al., 2017). Cleaned cages were lined with a

5-cm layer of chipped cedar-wood bedding. Twenty glass marbles were arranged in an equidistant 5x4 orientation on top of the bedding. Animals were allowed to habituate to the testing room for thirty minutes prior to testing. During the test phase, each mouse was placed in the test cage and allowed to explore for 30 minutes. At the end of the 30 minutes, animals were returned to their home cage and the number of marbles buried recorded and photographed. Any marble covered greater than two thirds with bedding was considered to be buried.

2.3.3.4. Elevated Plus maze

The elevated plus maze (EPM) is a commonly-used behavioural test to screen for anxiety-like behaviours (Rodgers and Dalvi, 1997). The apparatus is constructed from plexiglass and is arranged into a plus (+) shape with two open and two closed arms (arms are 50cm in length, 5cm wide and closed arms have a 15cm wall surrounding). The apparatus is raised one metre above the ground to increase anxiety in the open arms. The apparatus is separated from the rest of the room using identical white curtains to mitigate for visual clues. The experiment also takes place under red light at defined light intensities. To start the test an animal was placed in the open arms of the apparatus facing one of the open arms and allowed to explore for five minutes. The apparatus was cleaned with 10% ethanol after each subject to prevent olfactory clues from the previous mouse. The test was recorded using a video camera placed directly overhead. Scoring of the test assessed the total number of entries to open and closed arms as well as time spent in each and the number of head dips. Entries to the open and closed arms were defines as when mice placed all four paws on the corresponding arm.

2.3.3.5. Three-chamber test (3CT)

This test for sociability was undertaken in a rectangular box (40cm x 15cm) divided into three chambers (a left and right with a smaller centre chamber. Chambers were separated by partitions with a small semi-circular opening at the bottom, the left and right chambers contained a wire mesh cage. The test consisted of three ten-minute trials as has been described previously (Scott et al., 2017b).

1. **Habituation**: animals were allowed to explore the three chambers for ten minutes with mesh cages in left and right chambers being left empty.

2. **Sociability**: an unfamiliar mouse was placed in one of the mesh cages with an object (plastic rubber duck) placed in the other – again, animals were allowed to explore for ten minutes.

3. Social novelty preference: the object was replaced with a novel animal, while the now familiar animal remains in position – exploration was undertaken for ten minutes.

All animals were age- and sex-matched, with each box cleaned and lined with fresh bedding between trials. For each of the three stages, behaviour was recorded with an overhead camera and interaction times in each chamber were measured.

2.3.3.6. Novel Object Recognition (NOR) test

This NOR test is used to determine hippocampal-dependent memory and takes place during four trials over the course of two days (Lueptow, 2017).

Experiments were performed in dim light (45 lux) in a square box (45cm x 45 cm x 45cm). Large objects were used, a plastic flask filled with blue liquid, and a purple bottle.

The first day consisted of two habituation experiments separated by three hours – in both cases the animals were allowed to explore the arena for ten minutes before being returned to the home cage.

The second day also consisted of two trials, the first being familiarization with the objects, where two identical objects were positioned on adjacent corners approximately 5cm from each wall of the open field, and each animal was introduced for a ten-minute exploration period. Three hours later, the novelty test was performed. One familiar object was replaced with a novel object and each animal was introduced for a ten-minute exploration period. After each test the animal was returned to their home cage after the ten-minute exploration period.

On each day, animals were acclimatized to the testing room for approximately one hour before being placed in the box. Between trials, objects and testing arenas were cleaned with 70% ethanol and rinsed with water before drying. Recordings were made with a camera placed above the apparatus and scoring was undertaken manually from these videos. Object exploration was defined as when the animal's nose came within a 2cm radius of the object.

2.3.3.7 Light-dark box (LDB)

The LDB test acts as a measure of anxiety-like behaviour and exploits the conflict between the tendency of mice to explore novel environments, and their tendency to avoid open, brightly-lit areas (Bourin and Hascoet, 2003). The testing arena is a box consisting of a 'lighted chamber' (45cm x 22cm x 22cm) painted white and illuminated to 1000 lux, and a 'dark chamber' (21cm x 14cm x 21.5cm) inserted into the box, covered, and painted black to avoid

light entry. Animals were moved to the experimental room and allowed to acclimatize for one hour before experimentation. Mice were placed in the 'lighted chamber' facing away from the dark chamber. Mice were allowed to explore for ten minutes before being returned to their home cage. Apparatus was cleaned with 10% ethanol and allowed to dry between experiments. Recordings were made with a camera placed above the apparatus and scoring was undertaken manually, assessing the time spent in each chamber, the number of transitions between chambers, and the number of exploratory rearings made by each animal.

2.3.3.8 Forced swim test (FST)

In this test mice were gently placed in a cylinder containing water (23-25^{o}C) (Temperature monitoring is essential as alterations may impact performance in the test (Jefferys and Funder, 1994)) at a height of 17 cm. Animals were left in the water for 6 minutes with activity being recorded by a camera positioned overhead. Immobility time was scored for the last 4 of the 6 minutes. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery. This has previously been described in our lab (Desbonnet et al., 2013).

2.3.4. Whole Intestinal transit (Carmine red test - CRT)

A test mouse was administered with carmine dye by oral gavage; the latency for the excretion of the first red-coloured faecal pellet was recorded.

2.3.5. Murine HPA axis response

Blood samples were taken to assess the HPA response to a mild acute stress (i.e: FST) in adulthood. Samples were obtained from the tail at 30, 60, 90 and 120 minutes following the forced swim test, and stored at -80^{o}C until analysis. Total corticosterone was measured according to manufacturer's protocol. A corticosterone ELISA kit was used to determine plasma concentrations (Enzo life sciences, Farmingdale). Plasma dilution was 1:50.

2.3.6. RNA isolation, synthesis of cDNA and qPCR analysis

Total RNA was isolated from the hippocampus (HIP) using the GenElute Mammalian Total RNA minprep kit, as per manufacturer's instructions (Sigma-Aldrich). RNA concentration was quantified using the ND-1000 spectrophotometer (Nanodrop). Following RNA extraction equal amounts of RNA were reverse transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, life technologies, Carlsbad, CA). All cDNA was stored at –20^{o}C until time of assay. Gene expression was analysed using SYBR green technology and

gene specific primers on an LC480 Lightcycler II (Roche Scientific). Expression levels were calculated as the average of three technical replicates for each biological sample from all three groups relative to β-actin expression. Fold changes were calculated using the ΔΔCt method (Livak and Schmittgen, 2001). PCR primers were designed using published sequence data obtained from the NCBI database.

BDNF(L): AGTCTCCAGGACAGCAAAGC (20)

BDNF(R): TGCAACCGAAGTATGAAATAACC (23)

GluN2a(L): TACTCCAGCGCTGAACATTG (20)

GluN2a(R): TACTCCAGCGCTGAACATTG (20)

GluN2b(L): TACTCCAGCGCTGAACATTG (20)

GluN2b(R): TACTCCAGCGCTGAACATTG (20)

PSD-95(L): TACTCCAGCGCTGAACATTG (20)

PSD-95(R): TACTCCAGCGCTGAACATTG (20)

CamKII(L): TACTCCAGCGCTGAACATTG (20)

CamKII(R): TACTCCAGCGCTGAACATTG (20)

fos(L): TACTCCAGCGCTGAACATTG (20)

fos(R): TACTCCAGCGCTGAACATTG (20)

2.3.7. Statistical analysis.

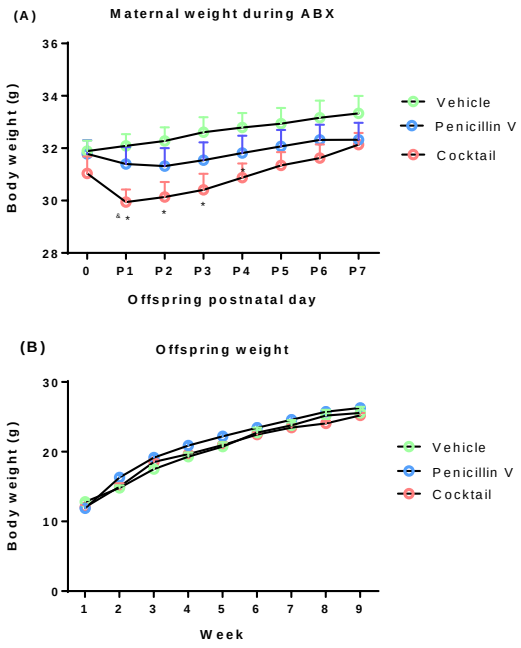
Data distribution was checked by Kolmogorv-Smirnov test and variances were compared using Levene's test. For parametric data, a Paired Student t-test, a One-way ANOVA, Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni post-hoc was applied accordingly to the protocol adopted. For nonparametric data, a Kruskal-Wallis test followed by U-Mann Whitney was used. All statistical analyses were carried out using IBM SPSS Statistics 22.0 for Windows software package. Extreme outliers and technical outliers were excluded when values are *2 x Standard Deviation from the mean***.** F values, P values are presented in the text of the results section.

2.4: Results

2.4.1. The effects of antibiotic administration on maternal body weight

Mothers exposed to the antibiotic cocktail one day following the birth of their pups experienced weight-loss in the days after initiation of treatment. **(Fig. 1A)** (Repeated-measured two-way ANOVA, $F_{(14,84)}$ = 3.516: P = 0.0002 (interaction between treatment and time), $F_{(7,84)}$ = 35.89, P < 0.0001 (time), $F_{(2, 12)}$ = 2.283: P = 0.1445 (treatment)). Post hoc assessment found a significant difference in body weight between vehicle and cocktail groups (P<0.05) at P1-4 only, and between cocktail and Penicilln V group at P1 only (P<0.05) after which no differences were observed between the groups.

Offspring weight was observed throughout the course of the experiment, however no differences were observed **(Fig. 1B)** (repeated measured two-way ANOVA: $F_{(2,37)}$ = 2.128: P = 0.1334 (treatment), $F_{(8,296)}$ = 487.0, P < 0.0001 (time), $F_{(16,296)}$ = 1.720: P = 0.0422 (interaction between treatment and time). Though there is an effect of time on offspring weight, post-hoc analysis does not reveal a difference between the groups at any individual time point.



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The significant interaction however, you just glanced over. A significant interaction usually invites a post-hoc contrast analysis, which is missing??

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***Figure 1: Maternal and offspring weights**. (A) Significant maternal weight loss was observed between antibiotic cocktail group and control from treatment day 1 to treatment day 4. There was also significant weight-loss in the cocktail group compared to the penicillin V group on day 1. (B) No differences were observed in body weight between the offspring groups. Data expressed as mean ± SEM. Repeated measures two-way ANOVA with Bonferroni post-hoc test [Vehicle n=5, Penicillin V. n=5, cocktail n=5]. *p<0.05 between control and cocktail group. &p<0.05 between Penicillin V and cocktail group*

2.4.2. Early-life behaviours are altered following antibiotic administration

As the disruption to the maternal microbiota through the administration of antibiotics in the drinking water occurs during a critical point of development in early life, the effects at this early stage are measured by observing behaviour in the days following cessation of antibiotics to the mothers. It was observed that in early-life (P9) there was no difference in either the total number of vocalisations **(F~~f~~ig. ~~2a~~2A)** ($F_{(2,36)}$=0.9887,P=0.3877) or the total duration of vocalisations **(F~~f~~ig. ~~2b~~2B)** ($F_{(2.36)}$=2.413,P=0.111) between any of the groups. Offspring attachment to maternal bedding was also analysed using a one-way ANOVA, **(Fig. 2C)** ($F_{(2,36)}$=2.287,P=<0.005). A post-hoc analysis revealing that ~~with~~ the time spent in maternal bedding ~~being~~ was significantly reduced in animals whose mother had received a cocktail of antibiotics compared to those whose mothers received vehicle. ~~(Fig. 2c2C) ($F_{(2,36)}$=2.287,P=<0.005). Indicating that these animals may be exhibiting early social recognition and maternal attachment deficits compared to the other groups tested.~~

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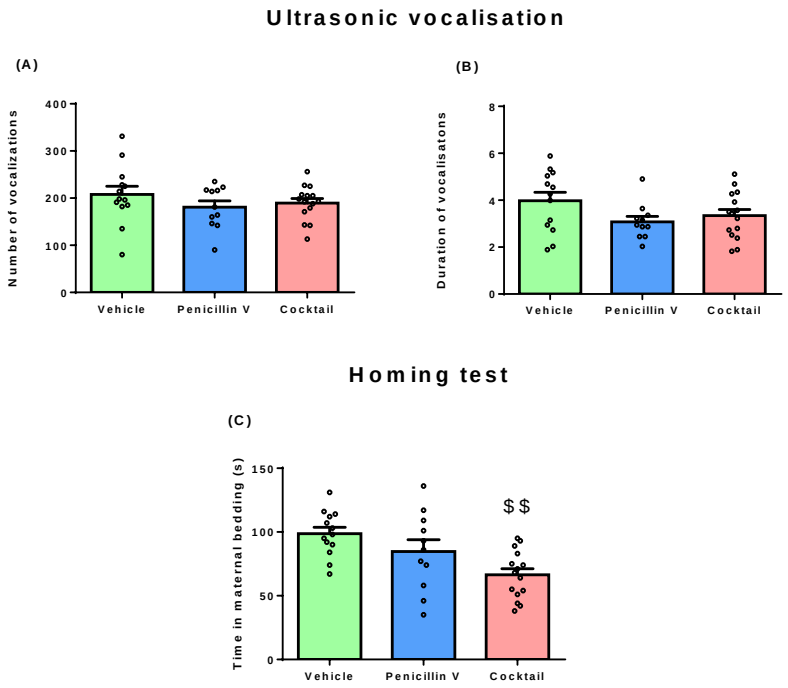

Fig 2: Early-life behavioural alterations following maternal antibiotic administration. *(A) & (B) early-life maternal antibiotic administration had no impact on either total number or duration of vocalisations (C) early-life administration of an antibiotic cocktail to mothers caused a significant reduction in the time spent in maternal bedding arena compared to a group where the mother received only water. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15]. $$<0.01 between vehicle and cocktail groups.*

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2.4.3. Anxiety-like behaviours are altered by early-life maternal antibiotic administration

When mice are exposed to an unfamiliar environment, the increased potential for threat means that there may be an associated increase in anxiety. It is possible to measure this in rodents via a number of behavioural tests. In the marble burying test, increased levels of marble burying correspond to increased anxiety and is regarded as neophobic behaviour (Wolmarans et al., 2016). Here, differences between the groups are seen following a one-way ANOVA analysis ($F_{(2,36)}$=1.136,P<0.05), with a post hoc analysis determining that there was a statistically significant increase in the number of marbles buried in both the Penicillin V (P<0.05) and the antibiotic cocktail group (P<0.05) ~~the offspring of mothers administered~~

~~both low-dose penicillin V, or an antibiotic cocktail buried a significantly greater number of marbles (f(Fig. 3)~~ ~~($F_{(2,36)}$=1.136,P=<0.05).~~

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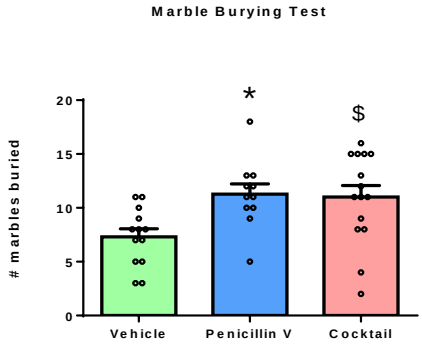

Fig. 3: Maternal antibiotic treatment leads to increased anxiety-like behaviour in the marble-burying test. *Offspring from mothers in both of the treatment groups buried a significantly greater number of marbles than offspring in the control group. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15]. *p<0.05 between vehicle and Penicillin V, $p<0.05 between vehicle and cocktail.*

The elevated plus maze provides an additional measure of anxiety-like behaviour ~~in these experimental animals~~. As rodents generally display an aversion to open and elevated spaces, an increased amount of time in the 'open' arms of the maze represents an anxiolytic-like behaviour (Treit et al., 1993). In this test, differences were observed in the number of entries to the open arms of the maze ~~. The EPM results~~ **(Ffig. 4~~a~~4A)** ~~(~~$F_{(2,29)}$=2.190,P=<0.05); with a post-hoc analysis revealing a statistically significant decrease in the number of entries to the open arm following administration of Penicillin V (P<0.05) as well as the antibiotic cocktail (P<0.05). This corresponds to what was observed in ~~correspond with that seen in~~ the marble burying test **(Ffig. 3).** ~~In some measures of the experiment. As well as measuring durations in each area, t~~he number of entries to the open arms also serves as a measure of anxiolytic-like behaviour (Pellow et al., 1985) and while entries to the open arms are altered by treatment~~, and is significantly reduced in both groups whose mothers received antibiotic treatment **(fig. 4a)** ($F_{(2,29)}$=2.190,P=<0.05); whereas~~, the number of entries to the closed arms remains unaffected **(Ffig. 4~~b~~B)** ($F_{(2,29)}$=0.5219,P=0.402). When the amount of time in the open arms is assessed, no changes are observed following any of the antibiotic treatments **(Ffig. ~~4~~4C~~c~~)** ($F_{(2,29)}$=0.9291,P=0.9152). The number of head dips were similarly unaffected **(Ffig. 4D~~d~~)** ($F_{(2,29)}$=0.166,P=0.4311).

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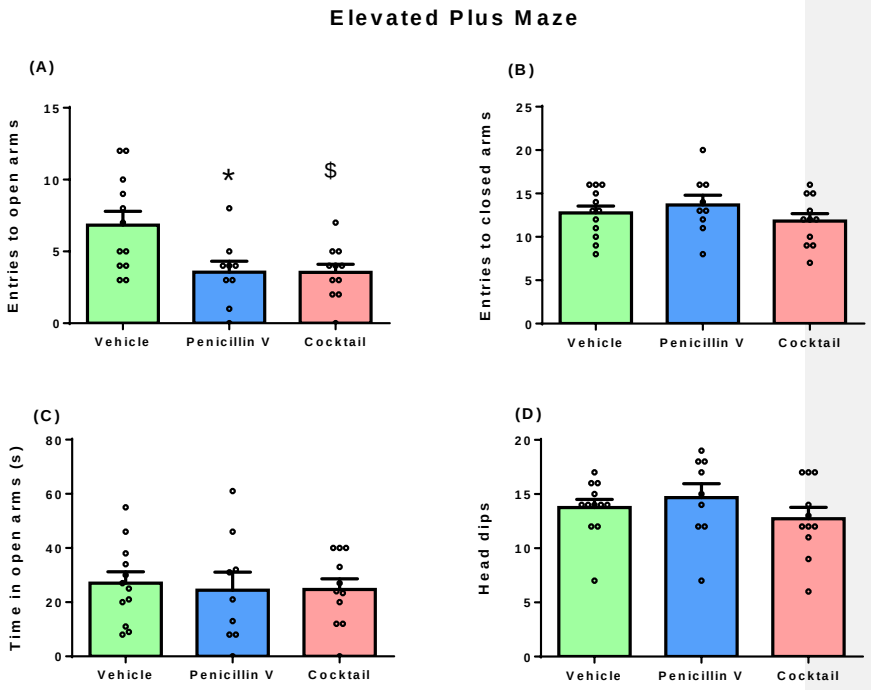

Figure 4: Maternal antibiotic treatment decreased the number of entries into the open arms in the EPM test. *(Aa) the number of entries into the open arms was significantly reduced following treatment with penicillin V or the antibiotic cocktail. No effects of treatment were observed in the number of entries into the closed arms (bB), or in the time spent in the open arms (Cc) or the % time spent in the open arms (dD). Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=12, penicillin V n=9, antibiotic cocktail n=11]. *p<0.05 between vehicle and Penicillin V, $p<0.05 between vehicle and cocktail.*

The light-dark box test acts as another measure for rodent anxiety, taking into account the natural aversion of mice to brightly illuminated areas as well as well as their spontaneous exploratory behaviour in novel environments (Takao and Miyakawa, 2006). Here, time spent in the dark differs between the groups **(Fig 5A)** ($F_{(2,36)}$=15.92, P<0.05), with a post-hoc analysis revealing a significant reduction in the time in the dark in the cocktail-treated group (P<0.01). ~~the offspring of mice treated with an antibiotic cocktail (but not penicillin V) spent significantly less time in the dark than the control group **(fig. 5a)** ($F_{(2,36)}$=0.01592, P<0.05).~~ The number of transitions **(Fifig. 5Bb)** ($F_{(2,36)}$=7.585, P=0.0956), however, and the number of exploratory rearings **(Ffig. 5Cc)** ($F_{(2,36)}$=0.08713, P=0.9167) were unaffected.

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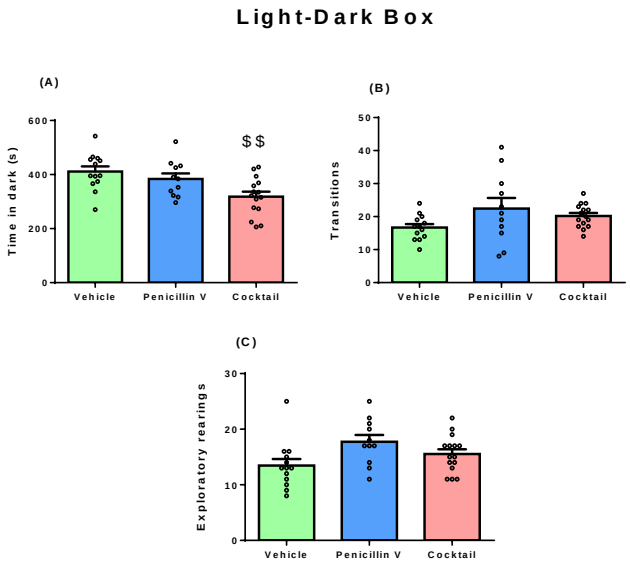

Fig 5: Maternal antibiotic cocktail administration reduces the amount of time spent by offspring in the dark in the LDB. *(A~~a~~) the amount of time spent in the dark is reduced in the offspring of mothers treated with an antibiotic cocktail compared to vehicle-treated animals. No differenced were observed in the number of transitions (B~~b~~) or the number of exploratory rearings (~~c~~C). Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15]. $$<0.01 between vehicle and cocktail groups .*

2.4.4. Antidepressant-sensitive behaviour remains unaffected

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The mouse FST is used as a measure of antidepressant-sensitive behaviour in animals and regularly serves to determine the efficacy of novel antidepressant compounds (Can et al., 2012). It has also been used as a measure of the effect of microbiota manipulation on these behaviours (Guida et al., 2018a; Hoban et al., 2016b). Antibiotic administration has been shown to increase immobility time in the FST in rodent models (Guida et al., 2018a; Hoban et al., 2016b). These effects were not observed in either of the antibiotic treatment groups. **(fig 6)** ($F_{(2,36)}$=0.5641,P=0.2293).

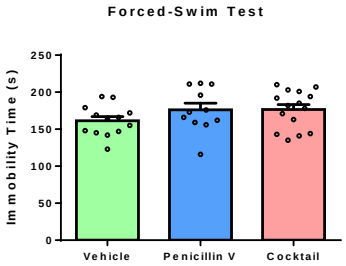

Fig. 6: No effect of maternal administration of penicillin V or an antibiotic cocktail on immobility time in the forced swim test. *Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15].*

2.4.5. Maternal penicillin administration induces deficits in social recognition

The three-chamber test measures various aspects of social behaviour in rodents. Social preference is assessed by giving mice the choice of interacting with either a novel mouse, or an object **(Fig. 7A)**. Here all groups exhibit normal social behaviour. Following a ~~T~~two-way ANOVA statistically-significant differences were observed for factor side (mouse vs object), ($F_{(1,66)}$=262.8, P<0.0001) as well as a statistically significant effect of treatment (~~,~~ $F_{(2,66)}$=7.123, P=0.0016). No statistically significant interaction between treatment and side was observed, however~~,~~ ($F_{(2,66)}$=0.7064, P=0.4971). Post hoc analysis confirms that in each of the groups there was a significantly greater time spent in the side of the mouse vs the side of the object (Vehicle object vs Vehicle mouse: P<0.001, Penicillin V object Vs Penicillin V mouse: P<0.001, Cocktail object vs cocktail mouse: P<0.001). ~~When given the choice, all groups exhibited normal social behaviour, **(fig 7a)** (Two-way ANOVA, F(2,66)=7.123, P=0.0016 (treatment), F(2,66)=0.7064, P=0.4971 (interaction between treatment and mouse/object)). Post hoc tests confirm that they spend more time with the mouse than the object (Vehicle object vs Vehicle mouse: P<0.001, Penicillin V object Vs Penicillin V mouse: P<0.001, Cocktail object vs cocktail mouse: P<0.001).~~

When the test was repeated, with the object being replaced with an unfamiliar mouse, the test ~~can be used to~~ measures social recognition and memory. In this case, different effects are ~~seen following three treatments **(fig**~~observed **(Fig. 7B~~~~b~~~~)**. Once again, a Two-way ANOVA reveals a statistically significant effect of the factor side (~~,~~ $F_{(1,62)}$=41.00, P<0.0001). No effect of factor treatment ($F_{(2,62)}$=0.2414 P=0.7863) was seen though there was an effect of side x treatment ($F_{(2,62)}$=3.661, P=0.0314). This demonstrates that animals in some groups had no preference for novel over familiar mouse. Post-hoc contrast analysis confirmed that vehicle-treated (P<0.001) and cocktail-treated (P<0.01) groups had a preference for the novel over familiar animals, but that this preference was not seen in animals treated with penicillin V.

~~(Two-way ANOVA F (2,62) = 0.2414 P=0.7863 (treatment), F (2,62)=3.661, P=0.0314 (interaction between treatment and mouse/object). Post hoc tests show reveal an greater amount of time spent with the novel mouse in the vehicle group (P<0.001) and the antibiotic cocktail group (P<0.01) but not in the penicillin V group.~~

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But I am a bit confused, because if this is for the effect of side, then the df do not add up. If this is an effect for treatment, that means that veh-pen-cocktail is different, which would match the df, but not the figure.
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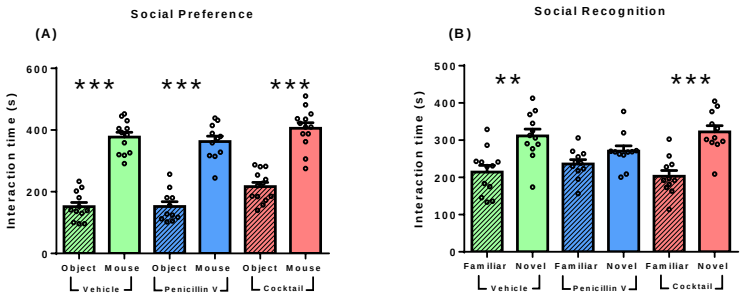

Fig 7: Effect of maternal antibiotic administration on sociability in the three-chamber test. *(~~a~~A) All three groups exhibited a significantly increased preference for a mouse over an object. (~~b~~B) the control and antibiotic cocktail groups exhibited a preference to interact with a novel over a familiar mouse. This preference is not evident in the penicillin V group. Data are expressed as mean ± SEM. Data analysed by means of two-way ANOVA with Bonferroni post-hoc test [Vehicle n=12, penicillin V n=11, antibiotic cocktail n=13]. **p<0.01., ***p<0.001.*

2.4.6. Maternal antibiotic cocktail administration induces cognitive deficits in offspring

The novel object recognition test measures hippocampal-dependent memory in rodents. Harnessing the inherent preference of mice for novelty, this test determines the memory of previously encountered objects in these animals.

When a two-way ANOVA was applied to the results of this test **(Fig. 8A)** a statistically significant effect was seen for the factor object (~~factor~~ $F_{(1,72)}$ = 31.39, P<0.0001), though not for the factor treatment ~~factor~~ ($F_{(2,72)}$=2.087: P=0.1315). A robust interaction between object and treatment was seen, however, ($F_{(2,\ 72)}$=4.141: P=0.0198). ~~Differences were observed between the groups **(fig 8a)** (Two-way ANOVA, F (2, 72)=2.087: P=0.1315 (treatment), F(2, 72)=4.141: P = 0.0198 (interaction between treatment and Novel/familiar object)).~~ Post hoc comparison confirmed that while the vehicle treated group (P<0.05) and the penicillin V treated group (P<0.05) spent a greater time with the novel over the familiar object, this was not the case in the cocktail-treated group. ~~Furthermore,~~

W~~w~~hen the percentage time spent with the novel object is assessed, ~~, there is a significant decrease in the interaction time in the antibiotic cocktail group only~~ **(F~~f~~ig. 8B~~b~~)** ({One Way ANOVA $F_{(2,36)}$=0.4085,P<0.05) differences are seen between the groups. With a post-hoc analysis revealing a significant decrease in the time % interaction with the novel object in the cocktail-treated group, P<0.05.

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Factor B: object F-N (2 levels);
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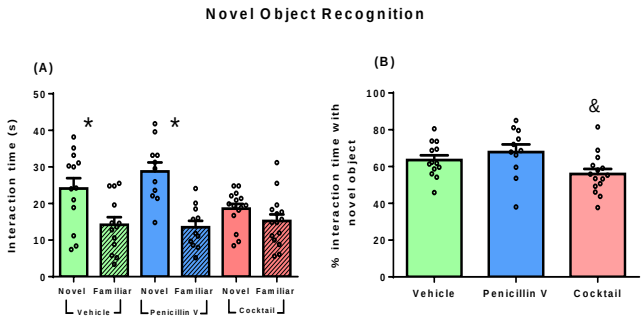

Fig 8: Maternal administration of an antibiotic cocktail affects cognition in the novel object recognition test. *(~~A~~a) Both the vehicle group and the penicillin V treated group spend significantly more time investigating the novel object than the familiar object, this preference is lost following maternal administration of an antibiotic cocktail. Data are expressed as mean ± SEM. Data analysed by means of two-way ANOVA with Bonferroni post-hoc test (~~B~~b) this effect is retained when the % time spent with the novel object is measured. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15]. *p<0.05 between novel and familiar groups, &p<0.05 between penicillin V and cocktail groups.*

2.4.7. Plasma corticosterone levels are unaffected

Plasma corticosterone acts as a measure of HPA axis activation following an acute stressor. Increased levels of circulating corticosterone following a stressor indicate that there is an abnormal response to the event. Here, no differences were observed in HPA axis response between any of the groups **(fig. 9.)** (Repeated-measures Two-way ANOVA: $F_{(2, 28)} = 1.182$: P = 0.3217 (Treatment), $F_{(4, 112)} = 1139$, P < 0.0001, (time); $F_{(8, 112)} = 1.724$: P = 0.1005 (interaction between treatment and time)

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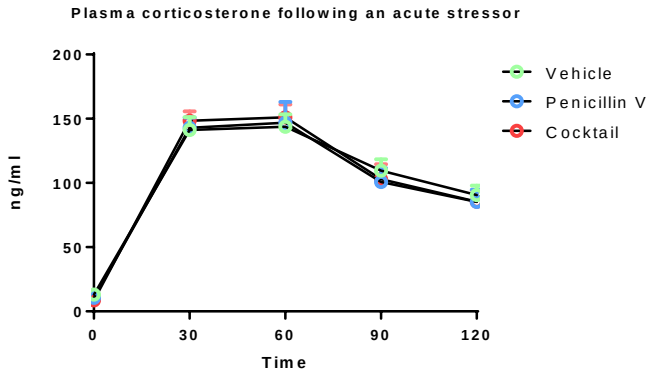

Fig. 9: No differences were observed in plasma corticosterone between groups following an acute stressor. *Both a two-way ANOVA for all groups, as well as a one-way ANOVA and Tuckey post-hoc test for each time point were carried out. No differences were observed between groups. [Vehicle n=11, Penicillin V. n=10, cocktail n=10].*

2.4.8. Physiological alterations

As well as behavioural assessments, physiological differences were measured both during the experiment, as well as following the completion of behavioural assessments. Intestinal transit was assessed using the inert dye carmine red **(Ffig. 10Aa)** ($F_{(2,33)}$ =0.6576,P=0.0903). Previous studies have shown that this measure can be affected by gut microbiota composition (Kashyap et al., 2013); however, we did not observe any differences in transit time in antibiotic treated animals. In addition to this, fat masses **(Ffig. 10Bb)** ($F_{(2,36)}$=0.7018,P=0.0784) **(Ffig. 10Cc)** ($F_{(2,36)}$=0.4632,P=0.2240) **(Ffig. 10Dd)** ($F_{(2,36)}$=0.8245,P=0.0761) **(Ffig. 10Ee)** ($F_{(2,36)}$=1.755,P=0.3141) and cecum weight **(fiFig. 10Ff)** ($F_{(2,36)}$=2.106,P=0.1160) as well as spleen weight ($F_{(2,36)}$=2.637,P<0.05) were assessed when the animals were culled. Following post-hoc analysis, the The only difference that was

observed between the groups was spleen weight, which was significantly increased (P<0.05) in the group treated with an antibiotic cocktail (**Ffig. 10Gg**). ~~($F_{(2,36)}$=2.637,P<0.05)~~

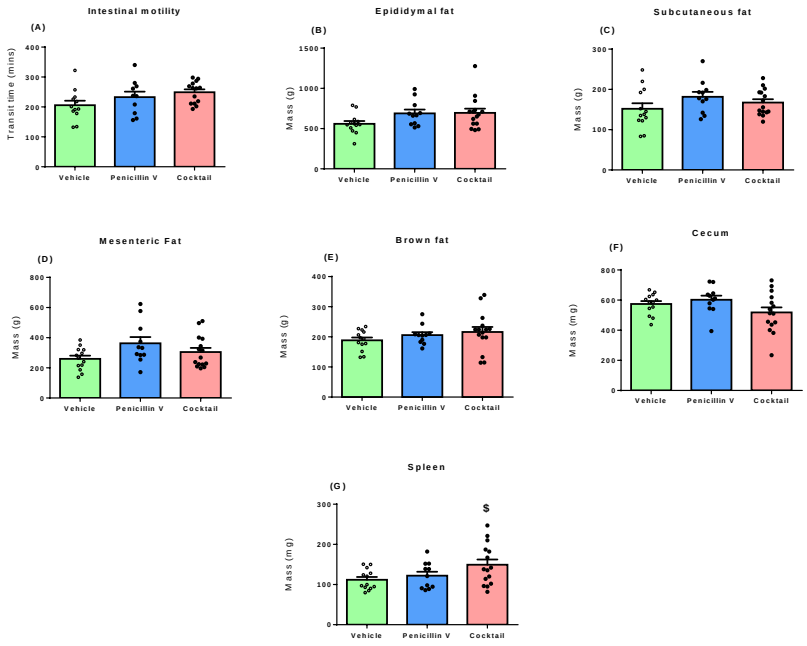

Fig. 10. Intestinal motility and tissue weights following maternal antibiotic administration. *(Aa) intestinal motility was unaltered in both treatment groups compared to control. Tissue weights following the animal culls were also generally unaffected for (bB) epididymal fat, (cC) subcutaneous fat, (dD) mesenteric fat, (eD) cecum weight, (fF) brown fat. (gG) spleen weight was significantly increased following maternal administration of an antibiotic cocktail. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Bonferroni post-hoc test [Vehicle n=13, penicillin V n=11, antibiotic cocktail n=15]. $p<0.05 between vehicle and cocktail groups.*

2.4.9. Hippocampal PCR analysis

In order to determine whether any of the treatment groups were able to affect the expression of plasticity related genes in the hippocampus, expression of a number of these genes was measured in the hippocampus. Following a One-Way ANOVA differences were seen in BDNF expression ($F_{(2,21)}$=1.577,P=0.0393). Post-hoc analysis revealed a~~A~~ reduction in hippocampal expression of *BDNF* was ~~observed~~ in the penicillin V group compared to

control (P<0.05) **(Ffig. 11Aa)** ~~(F(2,21)=1.577,P=0.0393)~~**.** None of the other genes were affected, however, including *GluN2A* **(Ffig. 11Bb)** ($F_{(2,22)}$=0.01173,P=0.9883)**,** *GluN2B* **(Ffig. 11Cc)** ($F_{(2,22)}$=0.04879,P=0.9525)**,** *PSD-95* **(Ffig. 11D)** ($F_{(2,21)}$=0.6620,P=0.5262)**,** *CamKii* **(Fifig. 11D)** ($F_{(2,21)}$=0.4272,P=0.6579)**,** *fos* **(Ffig. 11E)** ($F_{(2,22)}$=0.9823,P=0.3903)**.**

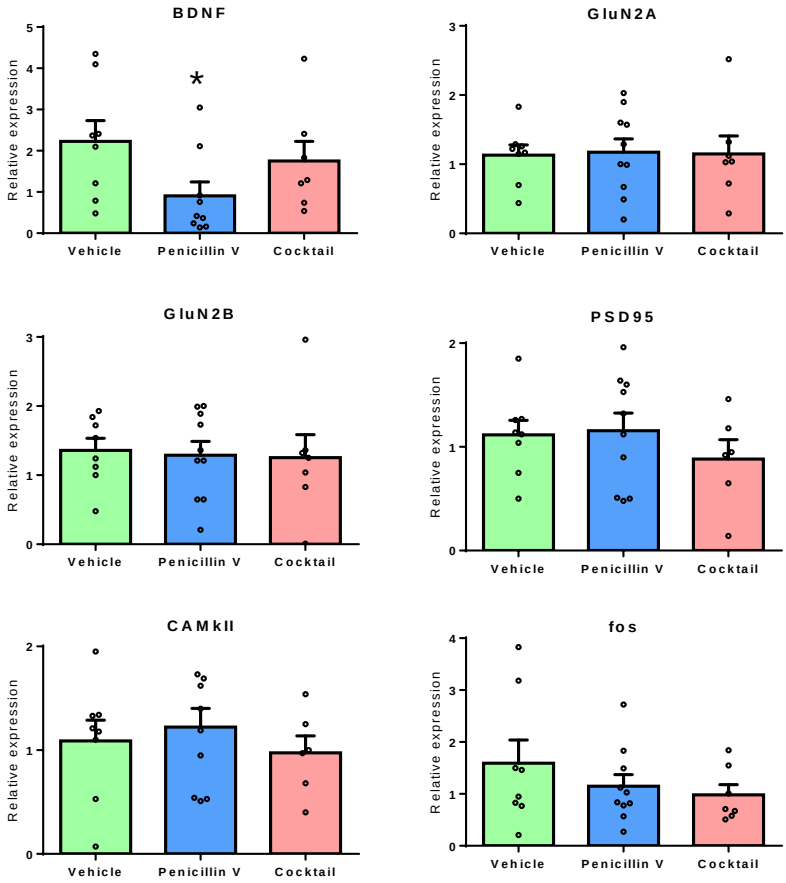

Fig. 11. Hippocampal mRNA expression of synaptic plasticity genes. *(Aa) BDNF (B) GluN2A (C) GluN2B (D) PSD95 (E) CamKII (F) fos. Data are expressed as mean ± SEM. Data analysed by means of one-way ANOVA with Fishers LSD. [vehicle n=8, penicillin V n=10, antibiotic cocktail n=7]. *p<0.05 between vehicle and Penicillin V groups.*

2.5. Discussion

The increase in maternal antibiotic use in the days and weeks following delivery, primarily driven by an increase in rates of caesarean section (Chaim and Burstein, 2003) has led to a source of gut microbiota disruption that, until this point has been relatively understudied. There are clinical reports linking maternal antibiotic use with an increase in the prevalence of asthma later in life (Stokholm et al., 2014), as well as showing that direct antibiotic administration in early life is linked to metabolic disorders (Stokholm et al., 2014), IBD (Hviid et al., 2011), asthma (Ahmadizar et al., 2017) and negative neurocognitive outcomes later in childhood (Slykerman et al., 2017). Furthermore, preclinical studies have linked perinatal maternal antibiotic administration and effects on behaviour later in life (Leclercq et al., 2017b).

Here, we assessed the effects of administration of either a single antibiotic (a low-dose penicillin) or a cocktail of five antibiotics (designed to ablate a large portion of the existing gut microbiota community) nursing dams. This allowed us to compare a clinically relevant dose of an antibiotic to a much more comprehensive disruption of gut microbiota composition. Effects of maternal antibiotic administration on behaviour were observed both in early life as well as in adulthood, with the antibiotic cocktail generally leading to more pronounced behavioural effects . Post-mortem hippocampal mRNA expression of genes related to behaviour and synaptic plasticity was also undertaken, maternal penicillin exposure in early life leading to a significant reduction in hippocampal BDNF in adulthood.

As it has been shown that pre-weaning exposure of pups to antibiotics via the mother leads to metabolic (Cox et al., 2014), immune (Russell et al., 2013) and behavioural changes (Leclercq et al., 2017b) we administered our antibiotic interventions to dams from the day following birth of the pups (P1). Ingested penicillin V is found in the breast milk (Anderson, 1977) and as such is able to exert direct effects over the microbiota composition of the offspring while they are nursing. Our results will determine if there are any effects of antibiotic treatment that are differentially affected following administration only during the weaning period.

Previous studies using perinatal antibiotic administration have shown an alteration of the gut microbiota composition (Cox and Blaser, 2015b; Cox et al., 2014; Leclercq et al., 2017b) that they suggest as the mechanism behind behavioural and physiological alterations. The risk of penicillin V exerting a direct effect on the brain is very low due to its negligible penetration of the blood-brain barrier (Andrew and Miltefosine, 2010) as well as its rapid

renal clearance (Andrew and Miltefosine, 2010) therefore accumulation in the serum should not occur. Within the cocktail group, direct effects of metronidazole on the brain are possible. It crosses the blood-brain barrier (Jokipii et al., 1977) and it is known to enter the breast milk (Passmore et al., 1988). In order to control for this, in subsequent studies it may be worthwhile to administer an intraperitoneal injection of antibiotics as a control, as has been undertaken in other antibiotic studies (Tochitani et al., 2016). In our study, however, behavioural testing took place a minimum of seven weeks following the completion of the course of antibiotics, therefore any residual toxic effects of the drugs are regarded as highly unlikely.

An additional factor that may lead to alterations in behaviour in adulthood is maternal behaviour and care during the weaning period. While maternal care is a factor known to alter behaviour later in life (Liu et al., 2000) is was not monitored in our study. Care was taken to determine that no abnormalities were observed in nests, and that no cannibalism occurred. These factors were not altered between groups, suggesting that maternal antibiotic administration did not affect maternal care, a finding consistent with previous studies (Leclercq et al., 2017b; Sudo et al., 2004b). The weight of the mothers in the antibiotic cocktail group decreased in the early days following the introduction of the antibiotic cocktail to their drinking water, compared to the other two treatment groups. This observed weight-loss may be due to the aversive taste of metronidazole. Previous studies have shown that this antibiotic, in particular, is associated with reduced fluid consumption (Reikvam et al., 2011) and additional weight loss compared to other antibiotics (Reikvam et al., 2011). Furthermore, bacterial depletion in the gut is known to cause diarrhoea, which is associated with weight loss (McFarland, 2007). By the end of the antibiotic intervention the maternal bodyweight had recovered such that there were no differences between treatment groups and controls.

The initial behavioural tests took place in the days following cessation of maternal antibiotics to determine whether exposure to antibiotics up to this point caused altered behavioural development. Ultrasonic vocalizations are used to assess social development in early life, with different characteristics of the calls being determinants of various social paradigms (Grimsley et al., 2011). Previous research in which microbiota from high-fat-diet-fed mice was transplanted to mothers prior to breeding and the USV characteristics of the offspring measured found that these mice vocalized less upon maternal separation than pups from control dams (Bruce-Keller et al., 2017). In our study however, no differences were observed either in the duration, or number of vocalizations. This may be due to the fact that in the

previous study dysbiosis was present in the mothers prior to breeding, and in our study, dysbiosis only occurred in the days following the birth of the pups.

The second test of early-life behaviour undertaken analysed attachment to maternal bedding at P10. The premise of this test is that recognition of maternal odours is vital for mother-offspring pairing through facilitating the establishment of social behaviours. Mouse pups at P10 have demonstrated the ability to recognize pertinent social stimuli and move towards their mother's nest when removed from it (Macri et al., 2010). We observed the animals whose mothers received an antibiotic cocktail spent less time in the maternal bedding than the control offspring. This pairing between mother and offspring is important for the development of social behaviours (Brennan and Kendrick, 2006). Interestingly, it is the penicillin group which displayed a disruption in social preference in the three chamber test in adulthood. And, although these two tests measure different aspects of sociability, it may be that the differential effects on the microbiota in early life have different effects on development of sociability. Another factor that may play a role is the alteration in odour cues in the bedding of cocktail-treated mothers. Such scents involved in recognition have been linked to the presence of fermentative bacteria (Shropshire and Bordenstein, 2016). As such, ablation of the microbiota has the ability to modulate the levels of these bacteria and therefore the intensity of the odour cues.

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When behavioural tests were carried out in adulthood, numerous differences were observed between the antibiotic-treated groups and the control groups in which the mothers received water only. Anxiety-related behaviour was particularly affected. Both groups in which the dams received antibiotics in the drinking water displayed increased levels of anxiety-related behaviour in the MBT, demonstrated by an increase in the number of marbles buried. Similar results were observed in the EPM, where both groups had significantly fewer entries to the open arms of the maze. Previous antibiotic studies have produced variable results in tests of anxiety. In rats, perinatal antibiotic administration to the dam led to increased anxiety-like behaviour in the EPM (Degroote et al., 2016) with an antibiotic study in zebrafish also producing an increase in anxiety-like behaviour (Wang et al., 2016). Antibiotic studies in mice however, have reported reductions in anxiety-like behaviour (Desbonnet et al., 2015a; Leclercq et al., 2017b). In a further study in which antibiotic treatment caused anxiolytic behaviour (Bercik et al., 2011a), when testing was repeated following a two-week washout period, anxiety-like behaviour returned to normal. The changes seen in anxiety levels a number of weeks following the cessation of antibiotics, as in our study suggests that early-life bacterial colonization is pivotal in the establishment of these behaviours. Similarly, in

germ-free mice where alterations in anxiety are observed, this behaviour was normalised when mice were colonised by bacteria after weaning and subsequently assessed in adulthood , suggesting adolescence as an additional critical period of microbiota modulation of brain circuitry linked to anxiety, such as the amygdala (Clarke et al., 2013). These results suggest the existence of multiple windows during which the microbiota my impact on normal neuronal development.

In the light-dark test, we observed that the antibiotic cocktail group spent significantly less time in the dark area than the control, indicating an anxiolytic effect of antibiotic cocktail. This finding is more in line with what was observed in other studies assessing the impact of antibiotic administration on anxiety (Desbonnet et al., 2015a; Leclercq et al., 2017b). It does not correspond, however, with what we observed in the other tests of anxiety. This may be explained by the fact that each of the tests for anxiety utilized measure different aspects of anxiety-related behaviour (Miyakawa et al., 2003). The LDB test is a measure of bright-space anxiety while the EPM, which is carried out under red light, is more of a measure of open-space anxiety (Takao and Miyakawa, 2006).

In the FST there were no observed differences in the immobility time between any of the groups. This differs from previous studies in which antibiotic administration led to an increased immobility time in the test, and an increase in antidepressant-sensitive behaviours (Guida et al., 2018a; Hoban et al., 2016b). In each of these cases, there was a direct administration of the antibiotics to the study animals, potentially leading to a more significant disruption of the microbiota. A greater susceptibility to change during adulthood suggests that microbiota-based interventions may be of greater benefit in depressive-like conditions than disorders of anxiety in adults.

The link between microbiota and social behaviour is gaining much attention across species (Sherwin et al., 2019). Indeed, the absence of a microbiota from birth corresponds with impaired sociability and social memory in adulthood (Desbonnet et al., 2014), in germ-free mice. Moreover, recolonization of germ free mice with microbiota post-weaning was sufficient to reverse such effects. In our current study, maternal antibiotic treatment did not induce any alteration in the amount of time the animals spent interacting with a mouse over an inanimate object, with all groups exhibiting a significant preference for the mouse. When presented with a novel and familiar mouse, however, the penicillin-treated group did not display a preference between either mouse, indicating an impairment in social recognition mirroring the germ free recolonized situation. The fact that the single antibiotic had this

effect but the cocktail did not is of note. While this corresponds with a recent paper in which perinatal administration of penicillin V caused impairments in social novelty but not sociability (Leclercq et al., 2017b) , this paper only assessed the effects of a single antibiotic compared to control. Previous studies in which antibiotic cocktails were administered has led to conflicting results, with both a lack of effect on social behaviour (Tochitani et al., 2016) as well as disruptions in the ability of mice to distinguish social novelty (Guida et al., 2018b) being observed. Overall, these results highlight the complexity of social behaviour. It may be that the specific bacteria targeted by penicillin V may exert a greater impact on brain areas regulating social development; however, this would require a more detailed temporal analysis of microbiota composition throughout early life in subsequent studies.

Cognitive deficits are one of the most commonly observed behavioural deficits following antibiotic administration in rodents (Desbonnet et al., 2015a; Fröhlich et al., 2016; Guida et al., 2018a; Möhle et al., 2016). In our study, we also found that while the control and single antibiotic groups spent significantly more time exploring the novel object than the familiar, this was not the case in the antibiotic cocktail group. Furthermore, when the percentage time spent with the novel object was assessed, the cocktail group performed worse than the single antibiotic group. Again, this finding potentially links the level of cognitive disruption to the extent of microbiota disruption. Further studies should aim to probe some of the mechanisms underpinning these changes through assessing alterations in protein expression; for example, cognitive disruption following antibiotics has been linked to altered BDNF (Fröhlich et al., 2016) and microglial expression in the hippocampus (Guida et al., 2018a).

When physiological measurements between the two groups were assessed, few differences were observed. No differences in fat weights between groups were observed, indicating that the metabolic alterations seen in other instances of perinatal antibiotic administration (Cox et al., 2014) were not present in our study. Additionally, intestinal motility, which has been shown to be altered following antibiotic administration due to the impact on enteric nervous system signalling (Delungahawatta et al., 2017a) is unaffected in our study. No differences were observed in plasma corticosterone expression in response to a stressor, corresponding with the results of previous antibiotic research (Desbonnet et al., 2015a; O'Mahony et al., 2014) The spleen weight of the offspring of cocktail-treated offspring was increased compared to those whose mother received vehicle. As this is an aspect that has been shown to be altered in other rodent antibiotic models (Kennedy et al., 2018), it may be a factor worthy of investigation in future rodent antibiotic studies.

Post-mortem analysis of hippocampal tissue found that the offspring of the penicillin V treated mothers had a significant reduction in hippocampal BDNF compared to vehicle. This results is perhaps unsurprising as this effect has previously been observed following antibiotic treatment (Desbonnet et al., 2015a; Fröhlich et al., 2016; Guida et al., 2018b). What is noteworthy, however is that the same effect was not observed in the antibiotic cocktail group. Again, this may be linked to the extent of antibiotic transmission from dam to pup and therefore the antibiotic exposure. This is an aspect of the study that would require further scrutiny in future antibiotic investigations. When other plasticity-related genes were assessed no differences were observed between the groups. Interestingly, in one of the studies in which hippocampal BDNF was shown to be reduced by antibiotic treatment (Fröhlich et al., 2016) no effect of treatment was seen on the expression of GRIN2B, mirroring our results. It is clear that plasticity related genes can be differentially affected by microbiota modulation and further investigation of these differences may be key to understanding observed behavioural changes.

It seems clear therefore that maternal administration of antibiotics in the early postnatal period has the ability to alter behaviour both in early-life, as well as in adulthood. Based on our data it appears that more significant disruption of the microbiota (through administration of a cocktail of antibiotics, rather than a single antibiotic) leads to more pronounced effects on behaviour. This study brings a degree of novelty to the field as it compares the administration of two different modes of bacterial knockdown under identical conditions and, while many of the effects are mirrored between the groups, there are also a number of differences. This highlights a key issue in the field to date. The enormous variation among preclinical studies in the specific antibiotics that are administered, the route of administration, the age and duration of administration, and the age at which behavioural tests are undertaken. Each one of these factors is pivotal to the effects on brain and behaviour. As such, while we may agree that disruption of the microbiota through antibiotic administration has the ability to modulate brain and behaviour, future studies should place a much greater emphasis on how the other factors may influence outcomes.

The results of this study also raise a number of interesting questions that may be assessed in subsequent or follow-up studies in the future. Firstly, alteration of when antibiotic administration occurs would allow the assessment of their impact during different developmental stages. For example, would maternal antibiotic administration during gestation as well as after the birth of the pups exacerbate the effects in the pups? What would be the long-term behavioural impacts of multiple, shorter courses of antibiotics? This

scenario would mimic more closely the observed clinical situation. A further addition to this study would be to determine whether the addition of a microbiota-modulating treatment could serve to reduce the level of behavioural alterations. Previous studies have found that the addition of probiotics to antibiotic-treated animals can reverse behavioural (Leclercq et al., 2017b) as well as inflammatory, biochemical, and electrophysiological alterations (Guida et al., 2018a). This treatment would also be of great practical use to mothers offering a safe method to prevent any long-term consequences of this treatment.

These data provide compelling information regarding the use of antibiotics in early-life. While it is not simple to extrapolate data obtained in rodent models to humans, these data support the notion that there are potential long-term detrimental consequences of maternal antibiotic use in the perinatal period. The effects observed in the antibiotic cocktail group tend to be more severe, and this is a dose that is unlikely to ever be used in a clinical setting. The use of a single, low-dose penicillin does have significant negative effects on anxiety and social behaviour in later life These results in combination with those observed in the antibiotic-treated group suggest that disruption of the microbiota during a critical period in early can have long-term consequences on neurodevelopment and the prevalence of psychiatric disorders later in life.

Chapter 3

Enduring Effects of Caesarean-Section Birth on Behaviour in Mice. A Target for Psychobiotic Intervention?

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3.1. Abstract

Mode of birth delivery is known to be a critical factor in the initial seeding of the microbiota, influencing its composition in early life. Birth via Caesarean section has been linked to alterations in metabolism and immune system development. More recently, the ability of mode of delivery to influence brain and behaviour in later life has become an important topic of research. Here we aimed to determine whether birth via caesarean section can influence behaviour in adulthood in a mouse model, finding significant disruptions in anxiety-like, cognitive, social, and antidepressant sensitive behaviours. We then assessed whether additional microbiota disruption through maternal antibiotic administration, as is undertaken clinically following Caesarean section delivery, can modulate these effects. Deficits in anxiety-like, social, and antidepressant behaviours were observed following this addition. Finally, the ability of microbiota modulating prebiotic, probiotic and synbiotic treatments to improve behaviour were assessed in each of the groups with many beneficial effects observed, particularly in antidepressant sensitive behaviours and stress response. Given the increased rates of caesarean section delivery globally, these results are encouraging for the potential for microbiota-modulating therapy to positively impact the potential enduring effects.

3.2. Introduction

Caesarean section (C-section) is a lifesaving procedure in perinatal medicine. However, the rates of C-section are increasing dramatically (Boerma et al., 2018) and the long-term consequences of this are only beginning to be explored (Dominguez-Bello et al., 2010a). Indeed with the relative risk early-life development of coeliac disease (Decker et al., 2010; Marild et al., 2012), hypertension (Li et al., 2013), type 1 diabetes (Cardwell et al., 2008) and obesity (Li et al., 2013) increased following this mode of delivery, it is a topic that demands further research. Particularly given that in many cases, these effects have been shown to persist into adulthood (Darmasseelane et al., 2014; Horta et al., 2013).

An altered gut-microbiota composition following C-section delivery has been suggested as a causative factor for many of these effects. While the notion of a sterile *in-utero* environment is a topic currently being debated (McDonald and McCoy, 2019), there is no doubt that mode of delivery remains one of the most important single factors for initial colonization (Dominguez-Bello et al., 2010a) with a recent study showing that C-section leads to a disrupted transmission of maternal *Bacteroides* strains as well as increased pathogen colonization (Shao et al., 2019).

When babies are delivered vaginally, the first bacteria they are exposed to are largely from their mother's vaginal microbiota. When a baby is delivered by C-section this initial 'seeding' comes from contact with microbes present in the environment and on the mother's skin. This leads to an altered microbiota in the gut as well as other body sites in infancy (Dominguez-Bello et al., 2010a; Lee et al., 2016; Neu and Rushing, 2011). While differences become less pronounced between the two groups over time (Backhed et al., 2015; Koletzko et al., 2018; McCann et al., 2018), the fact that a difference exists during this critical window of development may have long-lasting consequences (Borre et al., 2014a).

Altered gut microbiota composition and immune system development seen in babies born by C-section have also been observed in mice born via this route (Hansen et al., 2014; Morais, 2018) It is possible, however, to probe the underlying mechanisms in much greater detail in the rodent model. The fact that gut microbiota transfer from C-section to germ-free (GF) mice could also transfer the associated immune alteration highlights the importance of gut microbiota composition during this early stage (Zachariassen et al., 2019).

Rodent models of C-section also allow assessment of mode of delivery on the development of brain and behaviour (Chiesa et al., 2018; Morais, 2018). Dendritic arborization in

hippocampal pyramidal neurons is underdeveloped at birth in C-section born mice compared to vaginally born (Chiesa et al., 2018) while whole-brain differences have also been observed. Furthermore, C-section delivery has been linked to increased levels of neuronal cell death at birth compared to vaginally delivered controls (Castillo-Ruiz et al., 2018b).

In addition to structural changes in the brain, mode-of delivery has been shown to alter behaviour in mice. Early-life behaviour is particularly affected with vocalizations following maternal separation differing between vaginally born and C-section groups (Castillo-Ruiz et al., 2018b). We have previously shown that birth via C-section can induce deficits in early life behaviour, increase anxiety-like behaviour, reduce social recognition and lead to a heightened response to stress (Morais, 2018).

Clinical guidelines suggest that prophylactic antibiotics should be administered to all women undergoing C-section (2018) in order to prevent infection at the site of incision (Kankuri et al., 2003). While this antibiotic administration is essential in the short-term and is regarded as safe to the infant, equally important are the potential long-term implications such as the effect on the developing immune system, and, of interest to our study, potential effects on central nervous system development and behavioural impacts in later-life.

Maternal use of antibiotics in the perinatal period has been associated with increased development of childhood diseases that may persist into adulthood. These include asthma (Stensballe et al., 2013), wheezing, allergy (Kummeling et al., 2007) and metabolic disturbances (Cox et al., 2014). Study of perinatal antibiotic administration has also been undertaken in relation to behavioural and central nervous system development. Maternal administration of phenoxymethylpenicillin has been shown to have effects on gut microbiota composition, brain cytokines and behaviour in offspring adulthood (Leclercq et al., 2017c). As such, this was the administration protocol chosen in our study to determine whether C-section induced changes can be modulated by maternal antibiotic administration and indeed if these can be further augmented with a synbiotic combination.

To date, research into the protective effects of prebiotic and probiotic treatment in an altered mode of delivery is scarce. In humans, synbiotic treatment has been shown to restore a disrupted microbiota in babies born by C-section, while also increasing the production of anti-inflammatory cytokines (Musilova et al., 2017) with similar effects seen on microbiota and inflammation seen following a probiotic diet (Zachariassen et al., 2019). Furthermore, antibiotic-induced changes in immobility in the tail suspension test, social recognition, BDNF expression and hippocampal electrophysiology in mice could be reversed following one week

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of probiotic gavage (Guida et al., 2018b). Hippocampal neurogenesis and memory retention have been shown to be reduced in mice following antibiotic administration (Mohle et al., 2016). Reconstitution with normal gut bacteria (SPF) did not fully restore these factors unless a probiotic was co-administered (Mohle et al., 2016).Thus the potential for beneficial effects of prebiotic and probiotics can be seen both following birth via C-section or after antibiotic administration.

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Our study aims to shed further light on microbiota-targeted treatments by assessing their potential protective effect both individually and combined as a synbiotic in a murine C-section model. We will then aim to determine whether further perturbation of the microbiota during a critical window in early life by antibiotic administration can exacerbate any behavioural alterations and what potential benefit synbiotic administration may have in this scenario. The comparisons of each of these groups along with the addition of microbiota modulating treatments brings an aspect of novelty to the study of the gut-brain axis, while also mirroring the clinical scenario in many cases and therefore having a large degree of translational relevance.

3.3. Materials and Methods

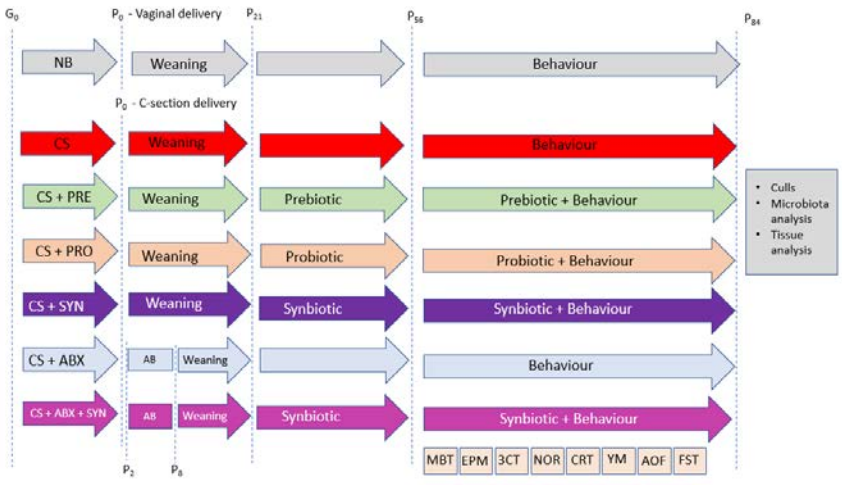

***Fig 1**: Study design. Mice were born either vaginally (NB) or via C-section (CS). Following birth the C-section animals were further divided into vehicle or antibiotic-treatment groups. The vehicle treated dams received water during weaning and following weaning at P21 offspring were maintained on regular chow (CS), regular chow with*

Probiotic gavage (CS+PRO) Prebiotic diet (CS+PRE), or Prebiotic gavage and prebiotic diet (CS+SYN). In the antibiotic-treatment groups, nursing dams received antibiotics for seven days (P1-P8) following the birth of the pups. Following weaning the maternal antibiotic treated pups either received standard chow (CS+ABX) or prebiotic diet and probiotic gavage (CA+S+ABX+SYN). A behavioural battery was undertaken at eight weeks of age. This battery consisted of the marble burying test (MBT), elevated plus maze (EPM), three chamber test (3CT), novel object recognition test (NOR), the carmine red test (CRT), spontaneous alternation behaviour in the Y-maze (YM), the aversive open field test (AOF) and the forced-swim test (FST). All treatments were maintained from weaning until the end of the experiment.

3.3.1. Animals

All animal experiments were approved by the Animal Experimentation Ethics Committee in University college Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland in accordance with EU directive 2010/63/EU. 8-week-old female and male NIH Swiss mice were obtained from Envigo Laboratories, UK. Breeding began after 2 weeks of acclimatization to the animal holding room. Animals were kept under a strict 12:12-h dark-light cycle and temperature (20 ±1 ^{o}C,55.5% humidity), with food and water supplied *ad libitum.* Male offspring were weaned at P21, and group housed in 3-4 mice per cage. Groups consisted of offspring from 28 independent litters. In addition to animals purchased for breeding, 10-week old NIH swiss mice were purchased from Harlan laboratories, UK, for use as conspecifics in the three-chamber test of sociability.

3.3.2. C-section surgery

Mice were time-mated and the presence of a gestational plug was marked as gestational day 0.5 (G 0.5). Males were removed from the cage and pregnant females were not disturbed apart from cage cleaning. At full-term (G20.5) female mice were euthanized by cervical dislocation. To reduce the risk of bacterial contamination abdominal skin was sprayed with 70% ethyl alcohol prior to retraction. Incision of the abdomen was made and the uterus removed and placed on sterile gauze (on top of a heating pad set at 30 degrees, in order to prevent hypothermia of the foetus in the uterus). Pups were removed through gentle pressure with a sterile swab and the umbilical cord was cut. A sterile cotton bud was used to tear the amniotic membrane and massage the pup until spontaneous breathing was noted. Pups were then transferred to a foster dam that had given birth on the same day. Additional pregnant females were allowed to deliver spontaneously, and the litters were used as full-term vaginal delivery controls.

3.3.3. Antibiotic administration

In order to deplete the maternal gut microbiota, dams were administered antibiotic in drinking water. Phenoxymethylpenicillin (penicillin V) was administered at a dose of 31mg/kg/day (Leclercq et al., 2017c). Average daily water consumption was calculated, and the body weight of each animal was then used to determine the amount of antibiotic required. Antibiotic was dissolved in autoclaved water and changed every two days. Control animals received autoclaved water without any antibiotics which was also changed every two days. Bottle weights were taken after replenishment. Animal weights were taken every day during the period of antibiotic administration to ensure that excessive weight loss did not occur. Cages were cleaned every second day in order to reduce the risk of re-establishment of a normal microbiota during the treatment period.

3.3.4. Probiotic & prebiotic (synbiotic) administration

Male offspring were weaned at postnatal day 21 (P21) and assigned either to the prebiotic, probiotic, synbiotic or control groups. Probiotic and synbiotic animals received a daily oral gavage of 200ul of freeze dried Biffidobacteria (*B. breve, B. longum & B. biffidum*) resuspended in sterile water. Control animals received a daily gavage of 200µl sterile water in order to control for the stress of gavage. The prebiotic element of the prebiotic and synbiotic groups was an arabinoxylan,wheat-based prebiotic (Naxus). This was administered ad libitum to these groups via the diet. Control and prebiotic only groups received standard chow. Pre-and probiotics were administered from weaning (P21) through the duration of behavioural tests until euthanasia.

3.3.5. Behavioural testing:

3.3.5.1 Defensive marble burying

The defensive marble-burying test measures repetitive and anxious behaviours, with a greater number of marbles buried representing increasing levels of anxiety. The test was undertaken as previously described (Golubeva et al., 2017). Briefly, cleaned cages were lined with a 5-cm layer of chipped cedar-wood bedding. Twenty glass marbles were arranged in an equidistant orientation in a 5x4 orientation on top of the bedding. Animals were allowed to habituate to the testing room for thirty minutes prior to testing. During the test phase, each mouse was placed in the test cage and allowed to explore for 30 minutes. At the end of the 30 minutes, animals were returned to their home cage and number of marbles buried

recorded and photographed. Any marble covered with greater than two thirds in bedding was considered to be buried.

3.3.5.2. Elevated Plus maze

The elevated plus maze (EPM) is a commonly-used behavioural test to screen for anxiety-like behaviours (Rodgers and Dalvi, 1997). The apparatus is constructed from plexiglass and is arranged into a plus (+) shape with two open and two closed arms (arms are 50cm in length, 5cm wide and closed arms have a 15cm wall surrounding). The apparatus is raised one metre above the ground to increase anxiety in the open arms. The apparatus is separated from the rest of the room using identical white curtains to mitigate for visual clues. The experiment also takes place under red light at defined light intensities. To start the test an animal is placed in the 'hub' at the centre of the apparatus facing one of the open arms and was allowed to explore for five minutes. The apparatus was cleaned with 10% ethanol after each subject to prevent olfactory clues from the previous mouse. The test is recorded using a video camera placed directly overhead. Scoring of the test assessed the total number of entries to open and closed arms as well as time spent in each. Entries to the open and closed arms were considered when mice place all four paws on the arm.

3.3.5.3. Three-chamber test

This test for sociability is undertaken in a rectangular box divided into three chambers (a left and right with a smaller centre chamber. Chambers (15cmx18cm) are separated by partitions with a small semi-circular opening at the bottom, the left and right chambers contained a wire mesh cage. The test consists of three ten-minute trials as has been described previously (Scott et al., 2017b).

1. **Habituation**: animals can explore the three chambers for ten minutes with mesh cages in left and right chambers being left empty.

2. **Sociability**: an unfamiliar mouse is placed in one of the mesh cages with an object (plastic rubber duck) placed in the other – again, animals are allowed to explore for ten minutes.

3. **Social novelty preference:** the object is replaced with a novel animal, while the now familiar animal remains in position – exploration is undertaken for ten minutes.

All animals were age- and sex-matched, with each box cleaned and lined with fresh bedding between trials. For each of the three stages, behaviour was recorded with an overhead camera and interaction times in each chamber were measured.

3.3.5.4. Novel object recognition test

The novel object recognition test is a commonly used test to assess hippocampal-dependent memory and takes place over three trials on three consecutive days as per previous studies assessing the ability of the gut microbiota to impact learning and memory (Desbonnet et al., 2015a).

Day 1: Habituation – animals are habituated to a square open-field box (Perspex sides and base: 32.5cm x 42.7cm) in a dimly lit room by individually placing the mice to the apparatus for ten minute habituation periods.

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Day 2: Two identical objects are positioned on adjacent corners approximately 5cm from each wall of the open field, and each animal was introduced for a ten-minute exploration period. Animals were then placed directly back into their home cages.

Day 3: After a 24-hour inter-trial-interval, one familiar object was replaced with a novel object and each animal was introduced for a ten-minute exploration period.

On each day, animals are acclimatized to the testing room for approximately one hour before being placed in the box. Between trials, objects and testing arenas are cleaned with 70% ethanol and rinsed with water before thorough drying.

Recordings were made with a camera placed above the apparatus and scoring was undertaken manually from these videos. Object exploration was defined as when the animal's nose comes within a 2cm radius of the object.

3.3.5.5. Y-maze spontaneous alteration behaviour

The Y-maze test of spontaneous alteration is a test of the willingness of a mouse to explore a new environment. With rodents typically preferring to visit a new arm of the maze than return to one that was previously visited. It acts as a test of hippocampal-dependent spatial memory. The apparatus consists of three 10cm x 10cm black plexiglass boxes arranged at 120^{o} angles to one another around a central hub. Here the test was carried out as previously described (Senechal et al., 2007). Animals were placed in the central hub and allowed to explore the apparatus for 5 minutes. Animals were allowed to acclimatize to the

experimental room for one hour before testing and the test was carried out under dim lighting. Following the test, animals were immediately returned to their home cage and apparatus cleaned with 70% ethanol. Recordings were made with a video camera placed directly above the apparatus. Movement between arms was noted manually. Alternation behaviour was defined as consecutive entries (i.e. all four paws) into all three arms without repeated entries and was expressed as a percentage of the total arm entries (Sarter et al., 1988).

3.3.5.6. Aversive open field test

The open field (OF) test is used to assess locomotor activity and response to a novel stressful environment. The test was undertaken as previously described (Seibenhener and Wooten, 2015). Mice were placed at the centre of an open field arena (Perspex box with white base, 30cm x 30cm x 20cm) and allowed to freely explore the arena for ten minutes. The distance moved and the velocity of movement in the open field were recorded using the ethovision videotracking system (Noldus information technology). Using this technology, a centre zone and outer zone were demarcated. The time spent in each zone and the frequency of entry into each zone was also noted for each mouse. Mice were place back into their home cage prior to testing. The arena was cleaned with 70% ethanol and allowed to dry prior to the next test.

3.3.5.7. Forced Swim test

In this test mice were gently place in a cylinder containing water (23-25°C) at a height of 17 cm as previously described (Desbonnet et al., 2013). Animals were left in the water for 6 minutes with activity being recorded by a camera positioned overhead. Immobility time was scored for the last 4 of the 6 minutes. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery.

3.3.6. In-vivo Intestinal motility (carmine red test)

Mice were singly housed and habituated to new cages for three hours for acclimatization. Following acclimatization, mice received 200uL oral gavage of Carmine (C1022; Sigma Aldrich) suspended in 0.5% carboximethylcellulose (CMC) sodium salt (Sigma, St Louis, MO, USA). Time of the first red coloured bolus is recorded.

3.3.7. Intestinal Permeability

Intestinal tissue samples were mounted into the Ussing chambers. Short-circuit current (I_{sc}) was recorded in a zero voltage clamp mode; transepithelial electrical resistance (TEER) was

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measured by discharging a 2 mV pulse. 4 kDa FITC-dextran was added to the mucosal chamber at a final concentration of 2.5 mg/mL; 200 µL samples were collected from the serosal chamber every 30 min for the following 3 h.

3.3.8. Murine HPA axis response

Blood samples were taken to assess the HPA response to a mild acute stress (FST) in adulthood, as per Robertson et. Al (Robertson et al., 2017). Total corticosterone was measured according to manufacturer's protocol. Corticosterone ELISA kit (Enzo life sciences, Farmingdale). Plasma dilution was 1:50.

3.3.9. Statistical analysis of behavioural and physiological data

Data distribution was checked by Kolmogorv-Smirnov test and variances were compared using Levene's test. For parametric data, a Paired Student t-test, a One-way ANOVA, Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni post-hoc was applied accordingly to the protocol adopted. For nonparametric data, a Kruskal-Wallis test followed by U-Mann Whitney was used. All statistical analyses were carried out using IBM SPSS Statistics 22.0 for Windows software package. Extreme outliers and technical outliers were excluded when values are *2 x Standard Deviation from the mean*. Differences for microbiota composition and diversity were calculated using the package in R and corrected for multiple comparisons using the Benjamni-Hochberg method**.** F values, P values are presented in the text of the results section.

3.3.10. Gut microbiota analysis

DNA was extracted using the Qiagen QIAmp Fast DNA Stool Mini Kit coupled with an initial bead-beating step. The V3-V4 hypervariable region of the 16S rRNA gene was amplified and prepared for sequencing as outlined in the Illumina 16S Metagenomic Sequencing Library Protocol. Samples were sequenced at Clinical-Microbiomics (Copenhagen, Denmark) on the Illumina MiSeq platform using a 2 × 300 bp kit. Reads were assembled, processed and analysed by the laboratory bioinformatician using the R package. N numbers for microbiota analysis are lower than in the behavioural section due to some samples not meeting criteria for analysis.

3.3.11. Bioinformatic and statistical analysis of microbiota data

DADA2 was used to denoise and call ASVs from the 300 bp paired-end read, taxonomy was assigned using the SILVA SSUREf database version 132. ASVs unknown on a genus level were

excluded, as well as ASVs present in two or fewer samples. Statistical microbiome analysis was carried out in R (version 3.6.1) with Rstudio (version 1.2.1335). The ALDEx2 library (Fernandes et al., 2014a) was used to compute the centred log-ratio transformed values of the remaining taxa. For principal component analysis, a pairwise implementation of the adonis() PERMANOVA function in the vegan library (Oksanen et al., 2017) followed by the Bonferroni-Holm correction was used to test for difference in β-diversity in terms of Aitchison distance. Differential abundance was assessed using a pairwise implementation of the aldex.test() function, followed by Benjamini-Hochberg correction. In these cases, a *q*-value < 0.1 was considered significant. α-diversity was computed using the iNEXT library (Hsieh et al., 2016).

3.4. Results.

3.4.1Physiological changes

3..41.1: Body weight

While birth via caesarean section has been shown to affect metabolic processes in both early-life as well as in adulthood (Cox et al., 2014), no differences in body weight were observed between any of the groups when body weight was measured weekly until adulthood (Fig. 1). (Repeated measures Two way ANOVA $F_{(6, 87)} = 1.906$; $P = 0.0888$ (Treatment), $F_{(48, 696)} = 0.9805$; $P = 0.5126$ (Interaction between treatment and time).

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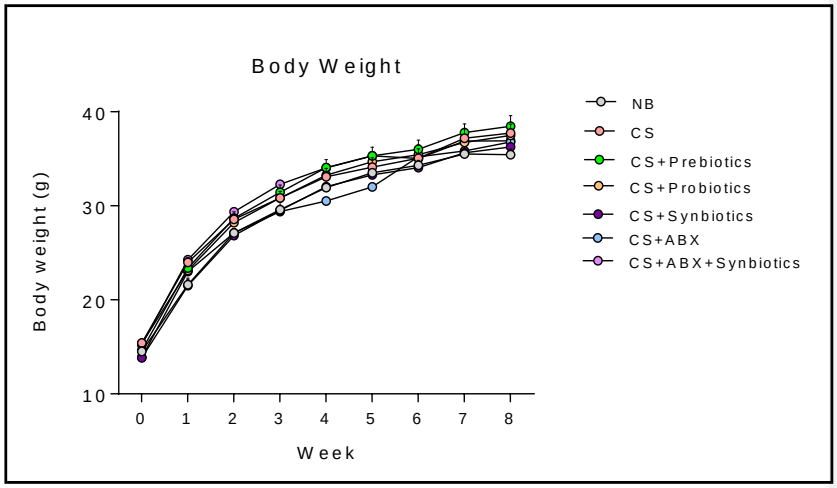

Figure 1: Offspring body weight through the course of the experiment. *A repeated measures two-way ANOVA and Tuckey post-hoc test for each time point did not reveal any alterations in body weight between the groups through the duration of the experiment. [Natural born n=22, C-section n=16, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=10, CS + antibiotics + synbiotics n=9]*

3.4.1.2: Intestinal Motility & colonic permeability

Understanding that an altered gut microbiota may impact on normal function of the gut we assessed gastrointestinal function by looking at both intestinal motility and permeability in C-section born animals. Using carmine red as an in-vivo marker of motility, it was observed that neither mode-of-delivery, nor subsequent microbiota modulations affected gastrointestinal transit **(Fig. 2-A)** (One-way ANOVA. $F_{(6,93)}=0.9882$,P=0.0667).

In addition to this, intestinal permeability was measured. The presence of fluorescein isothiocyanate (FITC) in the plasma of mice reflects the level of paracellular permeability in

the small intestine. No alterations were observed in ileal **(Fig. 2B)** (Repeated-measures two-way ANOVA. $F_{(6,49)} = 1.064$, $P = 0.3968$ (Treatment); $F_{(24, 196)} = 0.9016$: $P = 0.5998$ (interaction between treatment and time).

Similar results were observed in colonic permeability **(Fig. 2C)** (Repeated-measures two-way ANOVA. $F_{(6,49)} = 2.134$, $P = 0.7296$ (Treatment); $F_{(24,196)} = 1.045$: $P = 0.8908$ (interaction between treatment and time).

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Commented [ZV14]: I can see why you use an overall ANOVA to compare across all the groups. There is probably much that speaks in favour of doing this (since you have only one control group), BUT you might miss out on real effects. For example, CS decreases ileum perm compared to NB, but not in colon. Pre-Pro-Syn increase permeability in ileum (and colon??) etc.

Commented [RO15R14]: Good point.

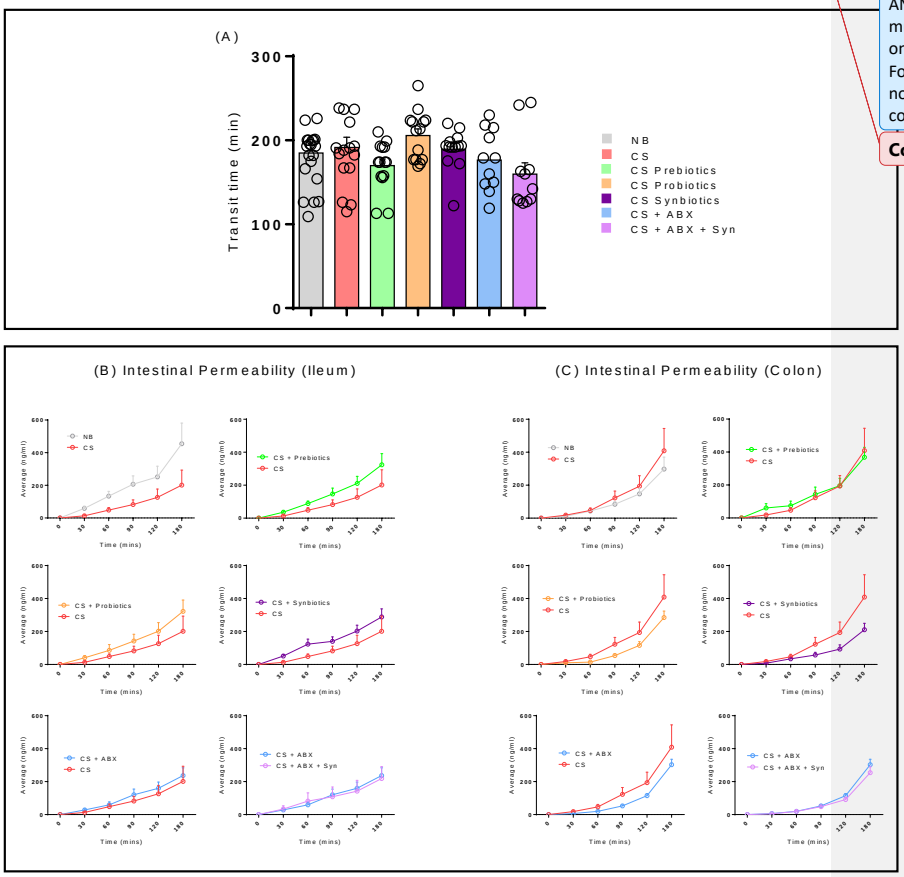

Figure 2: Evaluation of intestinal motility and permeability. *(A) No differences were observed in intestinal motility between any of the groups. Data expressed as mean ± SEM. (One-way ANOVA followed by bonferroni post-hoc test) [Natural born n=22, C-section n=16, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=13, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11] (B-C) C-section birth, nor microbiota modulating treatment made any difference to ileal or colonic permeability. Data expressed as mean ± SEM. Repeated-*

measures Two way ANOVA with Bonferroni post-hoc test. [Natural born n=11, C-section n=8, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10]

3.4.2. Behavioural results

3.4.2.1. Anxiety-like Behaviour

Three tests of anxiety-like behaviours were undertaken to assess different aspects of this phenotype. In the elevated plus maze, birth via caesarean section led to a reduction in the percentage time spent by animals in the open arms of the apparatus, indicative of increased anxiety-like behaviour **(Fig. 3A)** ($F_{(36)}$=2.935,P<0.05). Furthermore, there was an additional effect of C-section delivery on % time in the open arms **(Fig. 3C)** ($F_{(2,35)}$=4.315,P<0.05) with a post-hoc test revealing a significant decrease in the amount of time spent in the open arms in CS+ABX (P<0.05), though no difference was seen when synbiotic was added (CS+ABX+SYN). ~~when animals born via caesarean section were administered antibiotics in early-life this effect was exacerbated **(Fig. 3B)** ($F_{(2,35)}$=0.5195,P<0.05).~~ None of the microbiota-modulating treatments were able to affect anxiety-like behaviour, however **(Fig. 3BC)** ($F_{(3,51)}$=0.7567,P=0.2266).

In the other tests for anxiety that were undertaken, no differences were observed as a result of mode of delivery as seen in the marble burying test **(Fig. 3D)** ($F_{(33)}$=1.264,P=0.2150) **(Fig. 3E)** ($F_{(2,31)}$=0.1220,P=0.2923) **(Fig. 3F)** ($F_{(3,52)}$=1.224,P=0.2823) and the aversive open field test **(Fig. 3G)** ($F_{(33)}$=1.135,P=0.2646) **(Fig. 3H)** ($F_{(2,34)}$=0.1107,P=0.3928), **(Fig. 3I)** ($F_{(3,52)}$=0.6949,P=0.3614).

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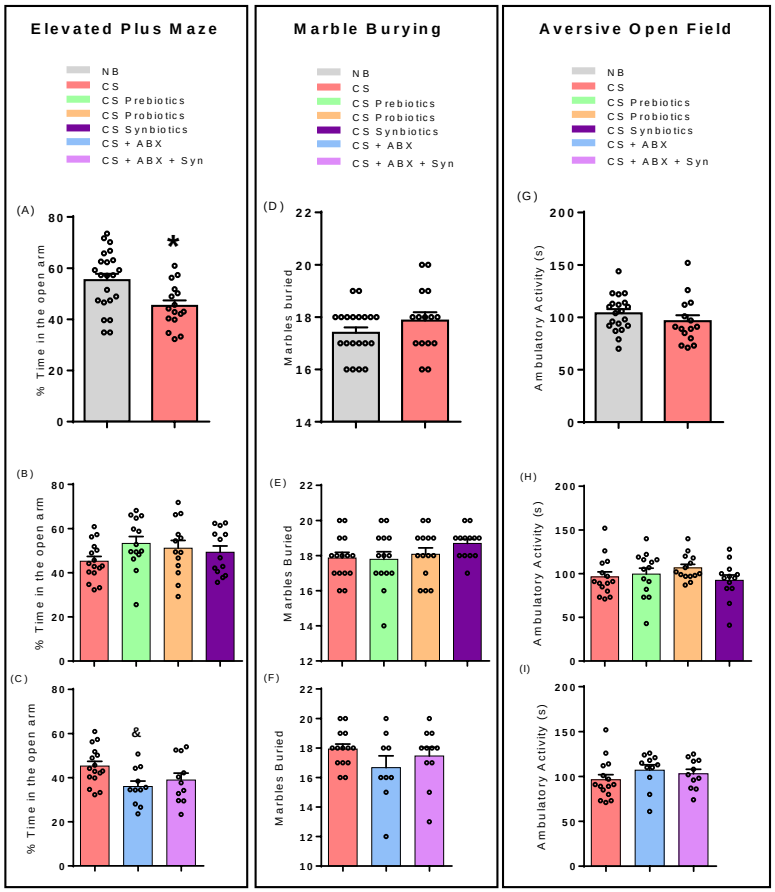

Figure 3: Anxiety-like behaviours. *(A~~e~~) Birth via C-section results in a significant reduction in the % time spent in the open arms of the elevated plus maze. (B) Early-life maternal antibiotics significantly enhance this effect, (C) however, none of the microbiota-modulating treatments had any further effect. (D) No differences were observed in the marble burying test following C-section birth. (E/F) Antibiotic or other treatments did not lead to any further effects (G-I) No differences in ambulatory activity were observed in the open field test. Data are expressed as mean ± SEM. NB vs CS data are analysed by means of a student's T-test with other groups analysed using a one-way ANOVA with Bonferroni post-hoc test [Natural born n=22, C-section n=16, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=14, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11] *p<0.05 between CS an NB, &p<0.05 between CS and CS+ABX.*

3.4.2.2. Cognitive behaviours

In the novel object recognition test, assessing non-spatial object memory, dependent on the hippocampus (Cohen and Stackman, 2015), birth via C-section led to time investigating a novel object than their naturally-born counterparts **(Fig. 4A)** ($F_{(35)}$=2.084,P<0.05). Treatments to enhance or disrupt the microbiota did not alter the time spent investigating the novel object **(Fig. 4B)** ($F_{(2,33)}$=1.245,P=0.6577) **(Fig. 4C)** ($F_{(3,52)}$=0.7424,P=0.1082).

In the Y-maze test for spontaneous alternation, which assesses spatial working and reference memory (Kraeuter et al., 2019) and again depends on hippocampal function (Kraeuter et al., 2019), there was no observed effect of either C-section delivery, or any of the other microbiota modulating treatments given **(Fig. 4D)** ($F_{(34)}$=0.7944,P=0.4325) **(Fig. 4E)** ($F_{(2,34)}$=0.02904,P=0.5012) **(Fig. 4F)** ($F_{(3,53)}$=0.6328,P=0.5552).

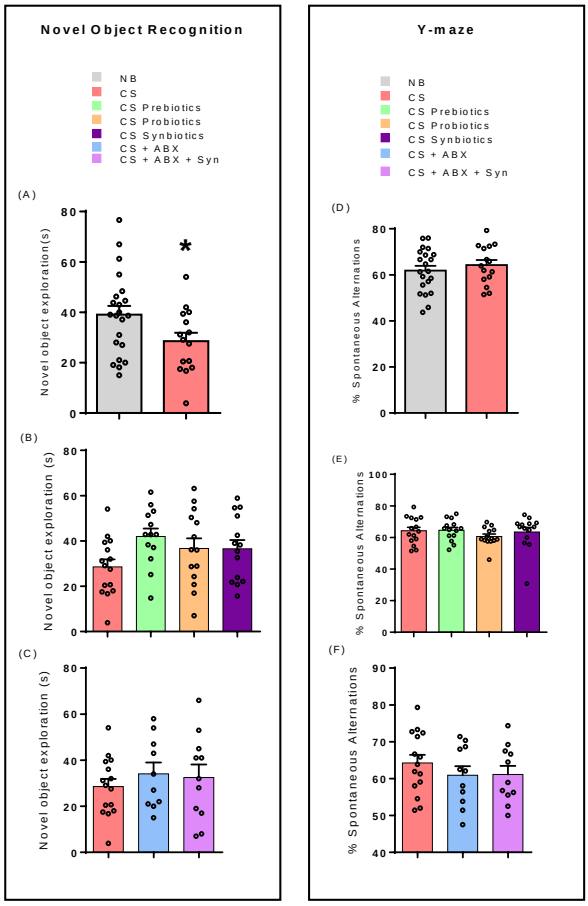

Figure 4: Learning and Memory behaviours *(A) birth via c-section leads to a significant reduction in the amount of time interacting with a novel object in the novel object recognition test. (B) Interaction time is not altered by the addition of early-life antibiotics (C) No effects are observed in any of the treatment groups in c-section animals. (D-F) No differences were observed in spontaneous alteration in the Y-maze test either due to mode-of-delivery, or microbiota modulation. Data are expressed as mean ± SEM. NB vs CS data are analysed by means of a student's T-test with other groups analysed using a one-way ANOVA with Bonferroni post-hoc test [Natural born n=22, C-section n=15, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=14, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11] *p<0.05 between NB and CS groups.*

3.4.2.3. Social behaviour

The three-chamber test for social behaviour is undertaken in two parts each measuring a different aspect of sociability. In the first, there was a strong preference for interaction with the mouse over the object indicating a 'normal' sociability in each of the groups **(Fig. 5A)**. Two way ANOVA indicated a statistically significant effect for factor side (mouse-object) (Two-way ANOVA. $F_{(1, 188)} = 697.1$, P<0.0001 (mouse/object)) with no differences between groups , ($F_{(6,-188)} = 0.7522$, P = 0.6084), and a statistically significant interaction (treatment), ($F_{(6,-188)} = 2.417$, P=0.0284) (interaction between treatment & mouse/object). Post-hoc analysis revealed that there was a statistically significant preference for mouse over object in every group (P<0.001)

In the second part of the experiment animals are permitted to interact either with the familiar mouse from the previous part, or another, novel mouse. **(Fig. 5B)**. (Two-way ANOVA. F (6, 149) = 1.556, P = 0.1639 (treatment), F (1, 149) = 46.79, P < 0.0001 (novel/familiar), F (6, 149) = 3.734, P = 0.0017 (interaction between treatment and novel/familiar). animals are permitted to interact either with the familiar mouse from the previous part, or another, novel mouse. Mice being social animals generally display a preference for interaction with the novel mouseIn this setting, mice usually show more affinity towards novel animalsnovelty and explore the less familiar animal. Two-wWay ANOVA indicated a statistically significant effect for factor side ($F_{(1,-149)} = 46.79$, P<0.0001 (novel/familiar)), no effect for factor treatment (Two-way ANOVA. $F_{(6,-149)} = 1.556$, P=0.1639), but a robust interaction of side x treatment (treatment), F (1, 149) = 46.79, P < 0.0001 (novel/familiar), ($F_{(6,-149)} = 3.734$, P=0.0017) (interaction between treatment and novel/familiar), indicating that animals in some groups had no preference for the novel over familiar animals. Post-hoc contrast analysis confirmed that .In our experiment this preference for novelty was observed in the naturally-born animals, displayed a preference for social novelty (p<0,05) but was absent inwhile animals born via c-section did not, suggesting a deficit in social novelty **(Fig. 5B).** Furthermore, treating c-section animals with either prebiotics (CS+prebiotics) or synbiotics (CS+synbiotics) reversed this social novelty deficit. Post-hoc analysis reveals significantly greater time spent with novel over familiar in the natural born (P<0.05), CS+prebiotic (P<0.05) and CS+synbiotic (P<0.05)) This social recognition can be restored following treatment with the prebiotic or the symbiotic mixture. In contrast, Wwhen c-section animals were treated with an antibiotic, however, they do not exhibit a preference

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for social novelty, ~~nor is this~~and a preference is not restored following synbiotic treatment. These results suggest an exacerbation of the negative effect of caesarean section on social behaviour with early-life antibiotic treatment.

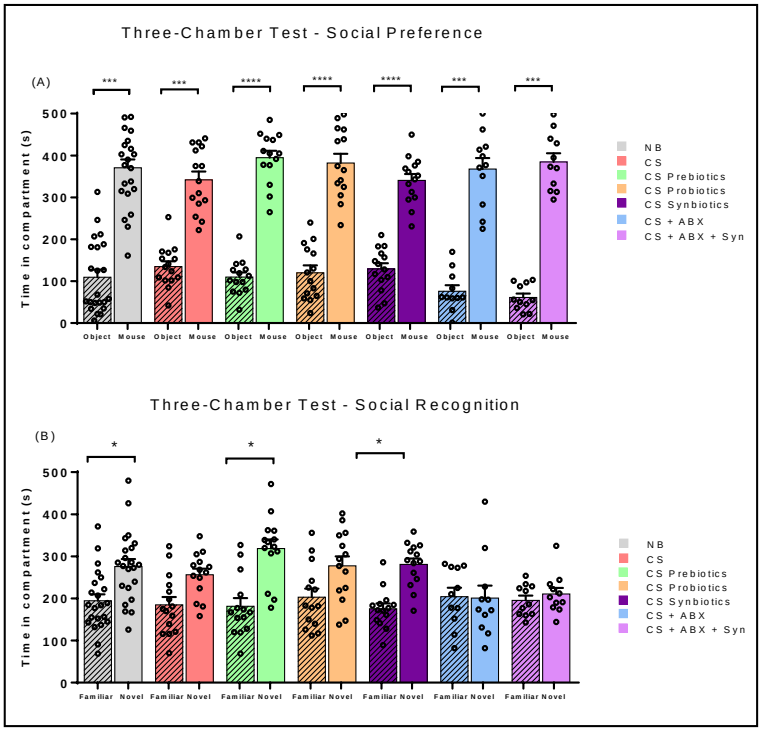

Fig 5: Tests of sociability. *(A) All groups exhibited normal social interaction, with each group showing a preference for a mouse over an object. (B) In a test for social novelty, naturally-delivered animals had a preference for a novel over a familiar mouse, this preference was not present following c-section. Treatment of C-section animals with prebiotics or synbiotics restored the social preference. Early-life antibiotic treatment exacerbates the effect of C-section on sociability as there is no preference for social novelty, nor is it returned following symbiotic treatment. Data analysed by means of two-way ANOVA with Bonferroni post-hoc test [Natural born n=22, C-section n=15, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=14, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11]*p<0.05, ***p<0.001*

3.4.2.4. Antidepressant-sensitive behaviours.

The forced swim test acts as a measure of antidepressant-sensitive behaviour. In our case it is being used to determine the potential impact of mode-of-delivery on these behaviours, any exacerbation following early-life antibiotics, as well as the potential for recovery with microbiota-modulating treatments. It was observed that birth via caesarean section led to a significant increase in immobility time **(Ffig. 6A)** ($t_{(34)}$=2.995,P<0.005). The addition of early-life antibiotic treatment to c-section delivered animals, led to differences between groups ~~to an additional increase in immobility time (Ffig. 6CB)~~ ($F_{(2,34)}$=36.95~~0.7918~~,P<0.001). A post-hoc analysis revealed that there was a statistically significant increase in immobility following the addition of antibiotic treatment (P<0.001) and that the addition of synbiotics to this group reduces immobility time compared to both the CS group (P<0.01) and the CS+ABX group (P<0.001).

~~When a symbiotic mixture was administered to antibiotic-treated animals born via this route there was a significant reduction in immobility time **(fig. 6B)**.~~ When assessing C-section born animals who did not receive antibiotics, again differences are observed **(fig. 6B)** ($F_{(3,53)}$=4.118,P<0.05). A post-hoc analysis reveals that synbiotics have a beneficial effect on immobility, leading to a significant reduction in immobility time compared to the C-section group (P<0.05) ~~**(fig. 6C)**, ($F_{(3,53)}$=0.8239,P<0.05).~~

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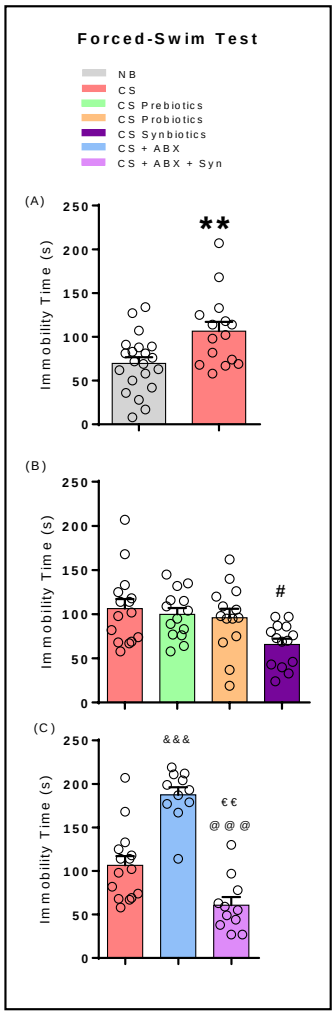

Figure 6: Antidepressant-sensitive behaviour in the forced-swim test. *(A) Birth via C-section leads to a significant increase in immobility time. (B) The addition of early-life antibiotics significantly exacerbated this effect, causing an increase in the amount of time spent immobile in the FST, with symbiotic treatment providing a significant improvement. (C) Again, synbiotic treatment had a beneficial effect in c-section born mice who did not receive antibiotics. Data are expressed as mean ± SEM. NB vs CS data are analysed by means of a student's T-test with other groups analysed using a one-way ANOVA with Bonferroni post-hoc test [Natural born n=22, C-section n=15, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=14, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11] **p<0.01 between NB and CS, #p<0.05 between CS and CS+Syn,, &&&p<0.001 between CS and CS+ABX, @@@p<0.001 between CS+ABX and CS+ABX+Syn, €€p<0.01 between CS and CS+ABX+Syn.*

3.4.3 Stress response: Plasma corticosterone

When plasma corticosterone was measured following an acute stressor, alterations were observed **(fig. 7A)** (Repeated-measures two-way ANOVA. $F_{(6, 88)} = 2.869$, $P = 0.0134$ (treatment), $F_{(4, 352)} = 422.9$, $P < 0.0001$ (time), $F_{(24, 352)} = 2.099$, $P = 0.0022$ (interaction between treatment and time). Post-hoc analysis revealed significantly increased plasma corticosterone concentrations in the CS+ABX group compared to the CS group ($P<0.05$) as well as significantly lower level of plasma corticosterone in the CS+ABX+SYN group compared to the CS+AB group ($P<0.01$). Post-hoc analysis did not reveal differences at other time points, however.

When the 30 minute time-point was analysed in isolation, the same results were seen. There was no effect of C-section at this time point **(Fig. 7B)**~~(One-way ANOVA. t~~$F_{(34)}=0.09862, P=0.9220$). No differences were observed, in CS animals when microbiota modulating treatments were administered ($F_{(3,48)}=0.5872, P=0.4909$) **(Fig. 7C)**. When CS animals are administered antibiotics differences were observed ($F_{(2,33)}=1.347, P<0.05$). Post-hoc analysis revealing that antibiotics increased plasma corticosterone at 30 minutes ($P<0.05$) and that this is significantly reduced with additional synbiotic treatment ($P<0.01$) **(F~~ig. 7D)**.

~~, there was, however a significant increase observed in the C-section animals which received antibiotics, again being reversed following synbiotic administration **(Fig. 7C)**($F_{(2,33)}=1.347, P<0.05$) though none of the treatment groups had an impact on c-section born animals who did not receive this additional perturbation **(fig. 7D)** ($F_{(3,48)}=0.5872, P=0.4909$)~~

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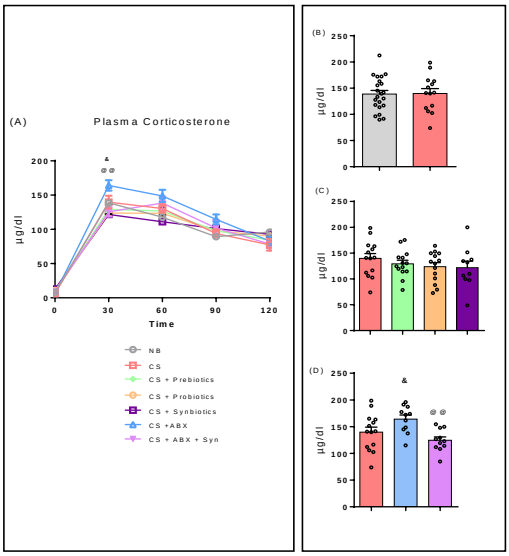

Figure 7: Plasma corticosterone measurement. *(A) Plasma corticosterone concentrations over time for each group. Data are expressed as mean ± SEM. NB vs CS data are analysed by means of a repeated-measures two-way ANOVA with Bonferroni post-hoc test (B-C) Plasma corticosterone concentration at the 30 minute post-stressor timepoint. (B) No difference in plasma corticosterone between C-section and naturally-born animals at T30. Data expressed as mean ± SEM, data analysed by means of a student's T-test. (C) Early-life antibiotic treatment increases plasma corticosterone concentration in C-section born animals at T30. This is not seen in animals who were treated with a synbiotic combination. (D) Microbiota-modulating treatments had no effect on plasma concetration at T30 in C-section born mice. Data are expressed as mean ± SEM. NB vs CS data are analysed by means of a one-way ANOVA with Bonferroni post-hoc test [Natural born n=22, C-section n=14, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=11, CS + antibiotics + synbiotics n=11] &p<0.05 between CS and CS+ABX, @@p<0.01 between CS+ABX and CS+ABX+Syn.*

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3.4.4. Gut microbiota analysis

Gut microbiota analysis was undertaken to determine whether mode-of-delivery or any of the interventions were able to alter the composition of the microbiota. Various measures of alpha and beta diversity were assessed as demonstrated in the following data. No differences in species richness were seen between the different modes of birth in the Chao1 analysis **(fig 8. A)** (U=105.4,P=0.1097). In c-section born animals, there was a decrease in richness following prebiotic treatment. **(fig 8. B)** (U=10.90,P<0.05)**.** Treatment with synbiotic led to a significant increase in richness in the antibiotic-treated groups **(fig 8. C)** (U=11.20,P<0.01.).

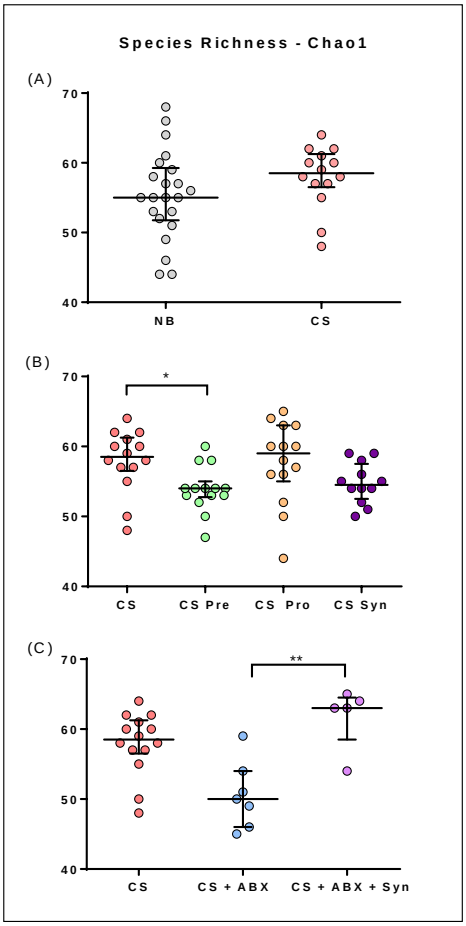

Figure 8: Chao1 species richness measurement. *(A) No differences in species richness were observed between the different modes of delivery (B) Prebiotic treatment in c-section born mice led to a decrease in species richness, with neither of the other two treatments having an effect. (C) Synbiotic treatment led to a significant increase in species richness in antibiotic-treated c-section mice Data are expressed as median with IQR. NB vs CS data are analysed by means of a Mann-Whitney test with other groups analysed by a Kruskall-Wallis test followed by a pairwise Mann-whitney test. [Natural born n=22, C-section n=14, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=7, CS + antibiotics + synbiotics n=5]*p<0.05, **p<0.01.*

Alpha-diversity was assessed using two measures, the Simpson index and the Shannon index. Again, using this measure, no difference was observed based on mode-of-delivery. **(fig 9. A)** (U=123.5,P=0.4377) Synbiotic treatment led to a significant increase in alpha diversity in animals born via C-section. **(fig 9. B)** (U=7.502,P<0.05). Within the antibiotic-treated groups, treatment with synbiotic led to a significant increase in richness **(fig 9. C)** (U=7.637,P<0.05.).

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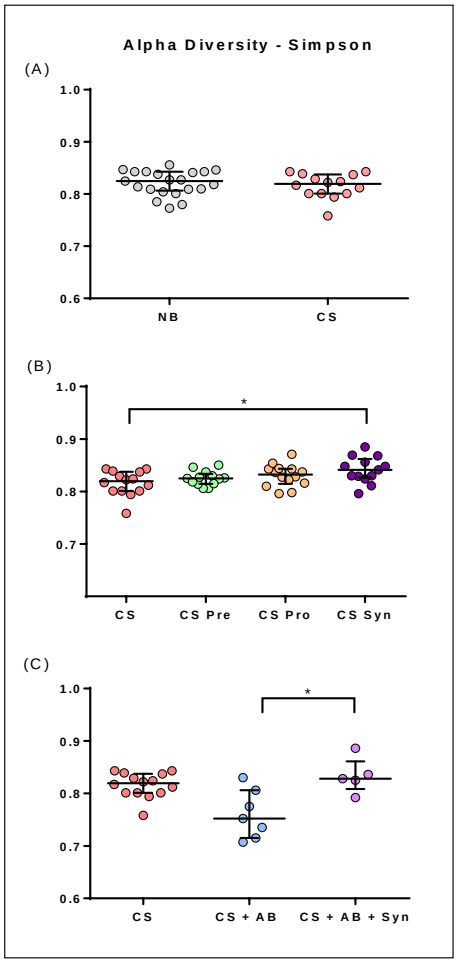

Figure 9: Simpson index measurement. *(A) No differences in Simpson index were observed between the different modes of delivery (B) Synbiotic treatment led to a significant increase in Simpson Index in C-section born animals. (C) Synbiotic treatment led to a significant increase in Simpson index in antibiotic-treated c-section mice Data are expressed as median with IQR. NB vs CS data are analysed by means of a Mann-Whitney test with other groups analysed by a Kruskall-Wallis test followed by a pairwise Mann-whitney test. [Natural born n=22, C-section n=14, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=7, CS + antibiotics + synbiotics n=5]*p<0.05, **p<0.01*

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Shannon index was also assessed. Once again, no difference was observed based on mode-of-delivery. **(fig 10. A)** (U=124.5,P=0.4588). None of the treatment groups led to a difference in c-section born animals. **(fig 10. B)** (U=3.326,P=0.3440). In c-section-born animals, antibiotic treatment led to a reduction in diversity, however this was increased following synbiotic treatment. **(fig 10. C)** (U=14.21,P<0.001.)

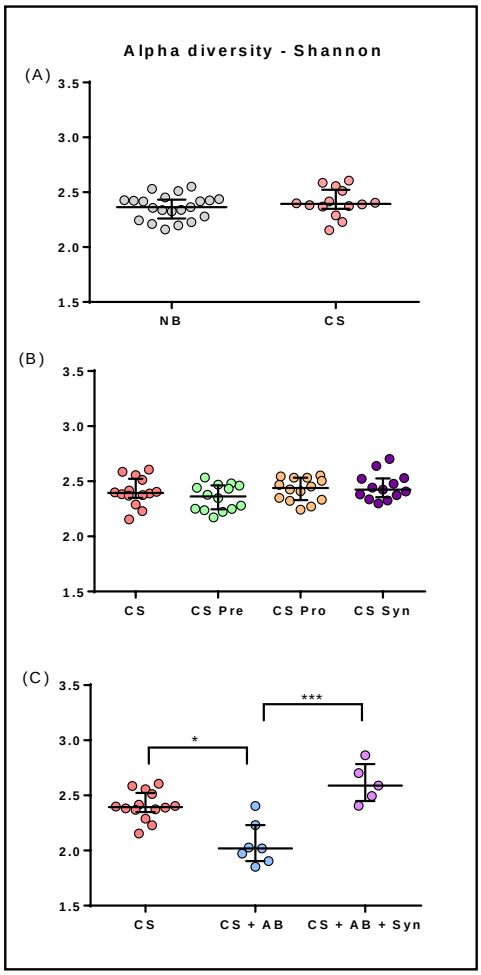

Figure 10: Shannon index measurement. *(A) No differences in Shannon index were observed between the different modes of delivery (B) No effects of pre-,pro- or synbiotic treatment were observed in c-section born mice. (C) Antibiotic treatment led to a significant decrease in Shannon index in c-section born animals. This was significantly increased following synbiotic treatment Data are expressed as median with IQR. NB vs CS data are analysed by means of a Mann-Whitney test with other groups analysed by a Kruskall-Wallis test followed by a*

*pairwise Mann-whitney test. [Natural born n=22, C-section n=14, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=7, CS + antibiotics + synbiotics n=5]*p<0.05, **p<0.01.*

Beta diversity measures to assess compositional differences between groups were also undertaken. No compositional differences were observed between the two modes of delivery **(Fig. 11 A)** (PERMANOVA P>0.05)**.** Within C-section born mice the addition of both prebiotics and synbiotics led to significant compositional differences. Furthermore significant compositional differences were observed between the C-section groups that received probiotic and the two other intervention groups (CS+Prebiotic and CS+synbiotic). **(Fig. 11 B)** (PERMANOVA: P<0.01)**.** There was a significant compositional difference between the C-section group and the C-section group that were administered antibiotics **(Fig. 11 C)** (PERMANOVA: P<0.01), however, synbiotic addition did not cause any further differences.

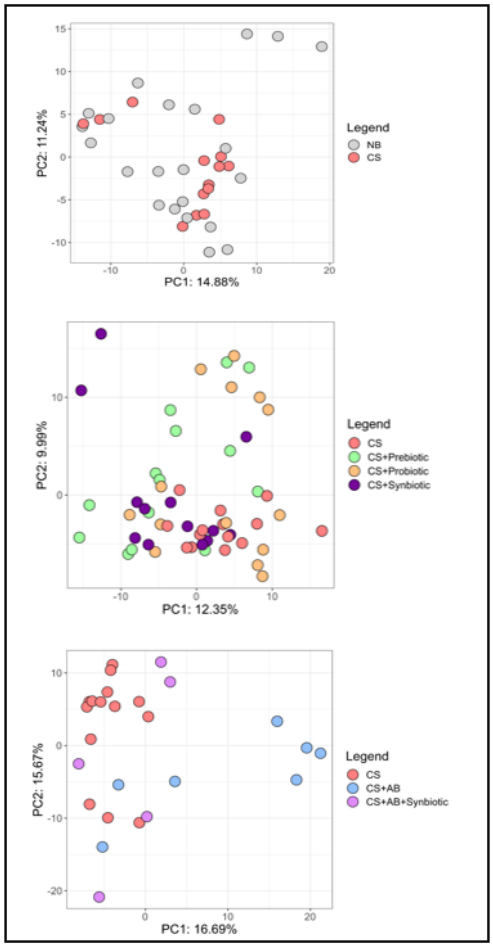

Figure 11: Beta-diversity measurement. *(A) No differences in Beta diversity were observed between the different modes of delivery (B) Both Prebiotic and Synbiotic treatment led to significant compositional differences in CS animals while there were also significant differences between the CS+Probiotic group and the CS+Prebiotic and the CS+synbiotic groups (P<0.01). (C) There were significant differences in composition between the CS and CS+AB groups (P<0.1), with the addition of a synbiotic not leading to any further differences. All statistics were pairwise PERMANOVA followed by Bonferroni correction. [Natural born n=22, C-section n=14, CS + prebiotics n=14, CS + probiotics n=14, CS + synbiotics n=10, CS + antibiotics n=7, CS + antibiotics + synbiotics n=5}*

3.5. Discussion

Mode of delivery is a pivotal factor in determining the initial gut microbiota composition of infants (Brestoff and Artis, 2013; Makino et al., 2013). Disturbances in the makeup of the microbiota during this critical window of development have been implicated in long-term detrimental health effects as well as an increased susceptibility to immune and metabolic disorders (Brestoff and Artis, 2013; Decker et al., 2010; Li et al., 2013; Marild et al., 2012). With links made between c-section birth and alterations in behaviour in early life (Castillo-Ruiz et al., 2018b) there is a great value in determining whether these changes may persist to adulthood, as well as the potential for microbiota modulation to have beneficial effects in infants born through this method.

Here we build on previous data showing that mode of delivery of birth impacts behaviour in adulthood (Morais et al., 2018). Specifically we show that anxiety-like, social, and cognitive behaviours are affected by mode of delivery. Furthermore, the addition of maternal antibiotics in early life was found to exacerbate anxiety-like behaviour, social deficits and antidepressant-sensitive behaviour. The addition of microbiota-modulating treatments (prebiotics, probiotics and synbiotics) was able to rescue some of these deficits while also leading to changes in various measures of microbiota diversity.

Anxiety-like behaviours were assessed using various behavioural paradigms. When we assessed anxiety in the elevated-plus maze it was observed that animals born via C-section spent a lower percentage of time in the open arms of the maze, indicating a greater level of anxiety (Rodgers and Dalvi, 1997). Interestingly, when C-section animals were additionally exposed to early-life antibiotics, there was an exacerbation of the effect, with these animals spending a significantly lower percentage of time in the open arms. This factor is of significant translational interest given that the use of antibiotics for the prophylaxis of post-surgical infection following C-section delivery is regarded as standard treatment (Smaill and Grivell, 2014). None of the microbiota modulating treatments had any effect in the EPM. The results of this test are of interest as disruption of the microbiota has been shown in many cases to reduce levels of anxiety, particularly in germ-free animals (Luo et al., 2018; Neufeld et al., 2011b). An anxiogenic effect of perinatal antibiotic administration has also been seen in the EPM in rats (Degroote et al., 2016). The fact that changes were seen following c-section and exacerbated following antibiotics may suggest that the changes in the microbiota during this critical window of development may lead to subtle changes in brain areas linked to anxiety

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such as the amygdala, with antibiotic administration previously being shown to alter RNA expression in this region (Hoban et al., 2017). While none of the psychobiotic treatments were able to restore behaviour to baseline levels, previous studies have found probiotic treatment beneficial in the reversal of anxiety-behaviour in the EPM (Li et al., 2018a; Luo et al., 2014). This may be linked to the timing of each of these modulations. Pre-weaning bacterial colonization has been shown to be pivotal in the establishment of anxiety-related behaviours (Leclercq et al., 2017c). Furthermore, in germ-free animals in which alterations in anxiety have been observed, behaviour is normalized when these mice are colonized by bacteria after weaning and subsequently assessed in adulthood, suggesting that early-life may be a critical window for microbiota modulation of the brain circuitry affecting anxiety (Clarke et al., 2013). In the other tests of anxiety, microbiota modulation was less impactful. No differences were observed between any of the groups in either the marble-burying test or the open-field test. These differences in results between the tests may be explained by the fact that the different assessments measure various aspects of anxiety behaviour, dependent on different neuronal circuits (Miyakawa et al., 2003).

Two tests of cognition were performed to determine whether caesarean section delivery has an impact on learning and memory in adulthood. The novel object recognition test is a measure of hippocampal dependent memory (Cohen and Stackman, 2015) which is of note as it is the brain area in which dendritic arborization is affected in early-life following c-section delivery (Chiesa et al., 2018). Furthermore, the mitochondrial uncoupling protein, UCP2 which regulates neuroprotection and synaptogenesis in the adult brain, is downregulated in the hippocampus of CS born mice compared to naturally born controls (Seli and Horvath, 2013; Simon-Areces et al., 2012). This may be a factor that is at play in our study as we see that animals born via caesarean section spend significantly less time interacting with a novel object than vaginally-born controls. Cognitive alterations have also been observed following antibiotic administration. Multiple studies have shown that antibiotic administration in rodents leads to cognitive deficits (Desbonnet et al., 2015a; Frohlich et al., 2016; Mohle et al., 2016). Furthermore, these were also linked to deficits in hippocampal BDNF (Desbonnet et al., 2015a), NMDA receptor subunit expression (Frohlich et al., 2016) and hippocampal neurogenesis (Mohle et al., 2016). This would lead us to the hypothesis that antibiotic administration in early life would further disrupt behaviour in the novel object recognition test, however, this was not the case. Nor did probiotic treatment have any beneficial impact in these tests. Treatment with two strains of *lactobacillus* have

shown the ability to rescue some decreases in the novel object recognition test along with *c-fos* expression in the CA1 of the hippocampus (Jeong et al., 2015).

Spontaneous alternation in the Y-maze acts as a measure of working memory (Sarter et al., 1988; Senechal et al., 2007). While this test has not to our knowledge been undertaken in behavioural tests in c-section or antibiotic-treated rodents, it has been assessed following probiotic treatment. A probiotic mixture of two *lactobacillus* strains has shown to reverse age-dependent reductions in spontaneous alternations (Jeong et al., 2015). It was hypothesised that probiotic treatment would exert a similarly beneficial impact in our study, there were, however no deficits following C-section or antibiotic treatment that could serve as a target for reversal. This may, as in the tests for anxiety be a factor of the age at which the microbiota disruptions occur in our study. The subtle differences between the results of the behavioural tests may be due to the development of distinct regions of the hippocampus at various stages in life.

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Strong links have been made between gut microbiota composition and social behaviour (Sherwin et al., 2019). Links between c-section delivery and sociability, however, vary. While a study of ~2.7 million individuals found a modest (~20%) increase in the relative risk of a diagnosis of autism spectrum disorder this effect was no longer seen when a sub-analysis of sibling controls was made (Curran et al., 2015a). The results of the social aspect of our study, were therefore of great interest. In a test for social preference we observed that each of the groups in the study spent significantly more time with a mouse than with an object, indicating normal sociability across the board. No differences were observed in time spent with either mouse or object, or the percentage time spent with each in any of the groups. When the object was replaced with an unfamiliar mouse the test was repeated, this time to test for recognition of social novelty by the animals. More striking results were observed. There was an effect of mode of delivery on social recognition. Animals born vaginally spent significantly more time with the novel mouse than with the familiar counterpart. This is not the case in animals born via caesarean section which do not express a significant preference for either side. Interestingly, this social recognition ability was restored in both the prebiotic and the synbiotic groups, indicating an ability of the prebiotic component in particular to exert beneficial effects on social behaviour. While the caesarean section + antibiotic group, much like the c-section alone group expressed no preference between the two animals (in fact this was the only group to spend more time with the familiar over the novel group) synbiotic treatment was unable to reverse the behaviour. This suggests that early-life antibiotic treatment may indeed exacerbate the extent to which caesarean section delivery

impacts on social recognition. That social preference is unaffected and social recognition appears to be influenced by these modulations is of great interest. Social recognition is seen as a higher-level behaviour than the basic test for social behaviour, having been described as the equivalent of the 'tendency to initiate social contact with a novel individual rather than somebody who is known' (Moy et al., 2004b).

While prebiotic administration has been shown to have beneficial effects in stress and anxiety reduction (Burokas et al., 2017; Savignac et al., 2016; Tarr et al., 2015), their effect on sociability has not been widely studied, making this a novel finding of interest. Probiotic strains of bacteria can improve social behaviour in maternal high fat diet mice displaying a disrupted microbiota (Buffington et al., 2016), though we did not see any benefits of probiotic administration. This finding suggests that the prebiotic component of the synbiotic may be leading to beneficial effects on microbiota composition that encourage recovery from the deficits induced by disturbances in early life.

That there is an additional disruptive impact of early-life antibiotic treatment is of interest translationally. A similar finding, that antibiotic treatment impacted social recognition, but not social preference was reported in a recent study (Guida et al., 2018b). Interestingly, however, they observed that daily gavage of probiotic was able to reverse this effect. That was not the case in our study. This may be due to the developmental window during which the exacerbation to microbiota disruption is occurring. While their antibiotic administration takes place during adulthood, ours is during the first week of life, a key developmental window for sociability (Yoshizaki et al., 2017).

Perhaps the most striking result observed in this study is the effect on antidepressant-sensitive behaviours in the forced swim test. Birth via C-section is sufficient to cause an increase in the immobility time in this test, which is significantly exacerbated in those animals who received early life maternal antibiotics, suggesting an increase in depressive-like behaviours. This is consistent with previous behavioural studies in both mice (Guida et al., 2018b) and rats (Hoban et al., 2016a) in which antibiotics cause an increase in the immobility time in the FST. In a mouse study of antibiotic administration, probiotic administration was able to reverse these changes (Guida et al., 2018b). This somewhat mirrors what we see in our study, where we observe that synbiotic supplementation can reverse the negative effects of this antibiotic administration. While there are no studies to date assessing the effects of prebiotics following antibiotic administration, studies have shown prebiotic treatment to significantly reduce immobility time in the FST (Burokas et al., 2017). Further

to this, we also observed that synbiotics lead to a significant reduction in immobility time when administered to C-section treated animals, though prebiotics and probiotics individually did not exert a protective effect. While previous studies have shown benefits of oral probiotics in the forced swim test of stressed mice (Li et al., 2018a) we did not observe effects of probiotics alone. However when combined with a prebiotic diet significant improvements were observed. What is clear from these findings is that modulation of the gut microbiota during early life can lead to significant alterations in depressive-like behaviour, particularly when exacerbated through antibiotic administration. Synbiotic treatment may be a viable treatment strategy to potentially overcome these problems.

We assessed the response to a stressor through measurement of plasma corticosterone levels at consecutive timepoints. Mode-of-delivery had no influence on this stress response. Similarly, none of the treatments in the c-section born animals led to a change in response. Interestingly however, the addition of early-life maternal antibiotics led to a significant increase in plasma corticosterone at the 30 minute timepoint after the stressor, and this was reversed by synbiotic treatment. This result mirrors what has been seen in stress response in GF animals (Sudo et al., 2004a). GF mice have an exaggerated response to stress that could be reversed following colonization with faeces from specific pathogen free (SPF) mice. Interestingly these reversal effects were only present during a critical developmental window. That the microbiota modulations in our study fall within this window highlight the importance of monitoring microbiota composition throughout the lifespan. Probiotic administration during this window also normalized corticosterone response (Sudo et al., 2004a), while prebiotic administration in adults reduced plasma corticosterone response in mice (Burokas et al., 2017) highlighting the relevance of pre- and probiotics in modulating stress response. The additive effect of these factors may explain the observation that synbiotics were shown to reverse the increase in plasma corticosterone in the antibiotic-treated group.

Other physiological measures made during adulthood displayed few differences between the groups in our study. For example, humans born via C-section are more likely to be obese than their vaginally-delivered counterparts (Horta et al., 2013) however we do not find any effects of mode-of delivery on weight gain. Interestingly, previous studies in a mouse model of c-section also found limited effects of altered microbiota on body weight in male mice but did find increased obesity in female mice (Martinez et al., 2017).

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Administration of antibiotics has been consistently linked to weight gain in later life (Cox and Blaser, 2015a; Cox et al., 2014). Since Penicillin V is known to be found in breast milk following treatment (Anderson, 1977) it can exert a direct effect on the gut microbiota of the offspring. It is perhaps a surprise that this treatment does not lead to any changes in body weight. Furthermore, gastrointestinal motility has been shown to be affected by gut microbiota composition (Quigley, 2011), and direct exposure of the gut to various antibiotics ex-vivo has led to differing effects on motility (Delungahawatta et al., 2017b). While acute exposure was seen to alter motility in our study, the long recovery time following cessation of treatment may explain the lack of difference in gut motility observed between our groups. In addition, antibiotic treatment has been shown to increase gastrointestinal permeability (Feng et al., 2019) when measured shortly after cessation of treatment. Again, the early age of exposure and long recovery time in our study may explain the lack of observed effects. Furthermore, the novelty of the model being used means that a defined effect expected following c-section delivery has yet to be established.

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Analysis of microbiota data in adulthood revealed that the treatments impacted gut microbiota. Species richness was measured through the Chao1 index, giving an estimate of how many different genera can be expected within each sample. We observed no difference in species richness between the two modes of delivery. This is perhaps not surprising, given that variability in microbiota composition is shown to be reduced from childhood to adulthood (Odamaki et al., 2016). It would be interesting to observe in future studies how the microbiota composition changes over the lifespan following c-section delivery. In c-section born mice whose mothers received antibiotics, there was a significant increase in richness with the addition of probiotics. In C-section born mice, prebiotic treatment actually led to a decrease in species richness. This factor has previously been shown to be reduced in antibiotic-treated animals which displayed altered anxiety and cognitive behaviours (Desbonnet et al., 2015a), while also being altered following exposure to a social stressor (Bailey et al., 2011).

The Simpson and Shannon indexes serve as a measure of alpha-diversity, with both giving an estimate of evenness and diversity within the samples. In the Simpson index, synbiotic treatment again had the most profound effect, with both synbiotic treated groups having a significantly greater level of richness compared to the equivalent group not receiving the intervention. In the Shannon measure of diversity, antibiotic treatment led to a significant reduction in diversity in C-section born animals. The fact that these alterations persist many weeks following the cessation of antibiotic administration emphasises the profound effect

that such treatments in early life can have on the microbiota. Synbiotic treatment led to a significant increase in diversity compared to the antibiotic group who did not receive this intervention. Again, similar changes in diversity were observed following antibiotic administration at weaning that corresponds to behavioural alterations similar to those observed here (Desbonnet et al., 2015a). Interestingly, alpha diversity has been seen to be increased in aged mice (Scott et al., 2017b) that display similar behavioural disruptions to those seen our c-section born mice. These conflicting results emphasise the difficulty of corresponding measures of diversity directly to behaviour.

At the end of the experiment we also investigated microbiota composition beta-diversity, the measure of how different two or more microbiomes are to each other, and we see as expected there are no differences between the C-section and naturally-born animals in adulthood. Interestingly, antibiotic treatment of C-section born mice led to an enduring difference in microbiota composition, though, the addition of synbiotic treatment did not cause a difference from either of the other groups. Again, the enduring nature of these changes many weeks after the cessation of treatment is noteworthy. In the c-section born animals, both prebiotic and synbiotic treatment led to a significant difference in beta-diversity compared to untreated animals.

Our data adds to the existing literature (Morais, 2018) suggesting that birth via c-section has the potential to impact behaviour in adulthood. Furthermore, the addition of an early-life antibiotic exacerbated the effects of c-section in anxiety, depressive and social behaviour. This highlights the sensitivity of the developing brain during this critical window and how the extent of microbiota disruption may be related to the extent of behavioural disruption, also suggesting that the microbiota changes during this critical window are subtle and different brain areas are differentially affected. Microbiota modulating treatments show moderate success in reversal of behavioural disruptions. While it should be noted that none of the treatments 'improve' behaviour compared to control animals, significant reversals are made in cases where behaviour is disrupted. This is particularly the case with disruptions in sociability and depressive-like behaviour.

Overall, these findings suggest that birth via caesarean section may lead to behavioural consequences later in life. And while this may be due in part to an altered microbiota during this critical window of development this mode of birth is also known to induce other physiological changes including changes in stress and immune priming that occur during the

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birthing process (Lagercrantz and Slotkin, 1986). Translationally, the addition of early-life antibiotics is of particular interest as it is common protocol in mothers who give birth via this method (Smaill and Grivell, 2014) and we show that it can exacerbate alterations in social and depressive like behaviour as well as physiological response to stress. Moreover, microbiota-modulating treatments may offer the potential to reverse some of the behavioural alterations induced by c-section. Human intervention studies are now warranted.

Chapter 4

Differential Effects of Ageing on Behaviour and Immunity in a Mouse Model of Autism Spectrum Disorder.

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4.1 Abstract

Autism spectrum disorder (ASD) is the most prevalent neurodevelopmental condition globally. In spite of its ubiquity, the vast majority of research into the disorder remains focused on childhood, with studies in adulthood and old age relatively rare. To this end, we explored the differences in behaviour and immune function in an aged BTBR T+ Itpr3tf/J mouse model of the disease compared to a similarly aged C57bl/6 control. We show that while many of the alterations in behaviour that are observed in young animals are maintained (repetitive behaviours, antidepressant-sensitive behaviours, social deficits & cognition) there are more nuanced effects in terms of anxiety in older animals of the BTBR strain compared to C57bl/6 controls. Furthermore, BTBR animals also exhibit an activated T-cell system. As such, these results represent confirmation that ASD-associated behavioural deficits are maintained in ageing, and that that there may be need for differential interventional approaches to counter these impairments, potentially through targeting the immune system.

4.2. Introduction

Autism spectrum disorder (ASD) is a life-long developmental disability characterized by social impairments, communication deficits, restricted interests and repetitive behaviours, as well as cognitive deficits, each of which are heterogeneously expressed throughout the disorder (Association, 2000). Estimates suggest that one in 132 individuals are affected by ASD (Baxter et al., 2015). The aetiology of ASD is not completely known but believed to involve both genetic and environmental factors. Genetic factors influencing development of the disorder include de-novo mutations, short nucleotide polymorphisms and common genetic variations that occur across its many incidents (Gaugler et al., 2014; Iossifov et al., 2015; Kong et al., 2013). One aspect that has been suggested to play a role in the development of ASD is the immune system (Meltzer and Van de Water, 2017). Prenatal immune activation has been shown to lead to lead to an increase in autism-like symptoms both clinically (Zerbo et al., 2015) and in animals (Morais et al., 2018; Weber-Stadlbauer et al., 2017). Postnatally, immune dysregulation and inflammation have been correlated with prevalence of ASD (Theoharides et al., 2016). Post-mortem tissue from ASD patients has shown both increases in activation of astrocytes and microglia, as well as increases in levels of proinflammatory cytokines in the prefrontal cortex (Vargas et al., 2005). Furthermore, genes linked to ASD that encode for immune system features are mutated in the disorder, leading to disruptions in structural and functional connectivity in areas of the brain key for socio-communicative function (Estes and McAllister, 2015; Hsiao, 2013).

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While almost all aspects of ASD have been studied in great detail, an area that has not been the focus of much attention is the impact of the disorder in older individuals and in later life. With an ageing population, increasing numbers of ASD individuals are reaching old age, provision of care for this population has been neglected and is only beginning to be addressed in a few small areas in Europe and the USA (Mukaetova-Ladinska et al., 2012). Of particular concern is the period during which individuals with ASD transition from parental care to a time during which this may no longer be possible, and the effects on cognition that may come with such a change (Mukaetova-Ladinska et al., 2012). While neuroimaging, pharmacological and pathological studies abound in individuals with ASD, to date they have been confined to children and adolescents (with a handful making assessments in middle age). As such, the impact that of but critical age-related physiological changes in the condition has been largely unstudied.

The united nations defines 'older persons' as those over 60 years of age, corresponding to an age of approximately 20 months in a rodent model (Prenderville et al., 2015). Behavioural alterations, as well as the prevalence of neurogenerative disorders are also known to be increased in an ageing population (Derenne and Baron, 2002). The underlying mechanisms including immune and inflammatory disturbances as well as metabolic alterations are only beginning to be understood and are explaining the age-related increases in the prevalence of metabolic and neurodegenerative disorders (Lopez-Otin et al., 2013). As changes in metabolism and cognitive alterations abound in individuals with ASD there is a very strong case to be made to increase the knowledge base of the impact of ageing on these individuals.

In order to probe the links between ageing, ASD, and immune function, preclinical models of the disorder are required to perform the necessary behavioural and immune analyses. The BTBR T+ Itpr3tf/J (subsequently referred to as 'BTBR') mouse strain is one of the most widely used models of ASD. Similar to humans, these animals display robust deficits in social interactions (Bove et al., 2018) and enhanced engagement in repetitive behaviours (Meyza et al., 2013; Moy et al., 2007). Moreover, the autistic-like behaviour of BTBR mice is largely driven by multiple genetic alterations (Meyza and Blanchard, 2017). These include disruptions in an enzyme regulating the metabolism of the glutamate agonist kynurenic acid, leading to alterations in synaptic signalling (McTighe et al., 2013). Expression of the plasticity-related protein, BDNF in the hippocampus is also downregulated compared to C57BL/6 controls (Daimon et al., 2015) in addition to alterations in serotonin (Gould et al., 2011) and cannibanoid (Gould et al., 2014) receptors. Structural alterations are present in numerous brain areas in these animals (Mercier et al., 2012), with perhaps the most striking difference being the lack of a corpus callosum (Wahlsten et al., 2003).

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The BTBR model is also sensitive to environmental influences, such as dietary interventions (Ruskin et al., 2013; Wu et al., 2017). Juvenile mice administered a ketogenic diet showed improvements in sociability, repetitive behaviour, and social behaviour (Ruskin et al., 2013). Since this diet is known to modify gut microbiota composition in children (Lindefeldt et al., 2019) this highlights the potential importance of gut microbiota composition in expression of behavioural symptoms in this model(Kraneveld et al., 2016), with the influence of the microbiota over behaviour linked to neuroimmune interactions (Foster, 2016).

To date, the behavioural and immune disruptions of the BTBR model have not been assessed in ageing. Thus, we aim to assess both of these factors and determine whether there is a greater level of 'inflammaging' (the heightened proinflammatory status and the decline in

adaptive immunity progressively seen in older age (Franceschi et al., 2000)) compared to an age-matched C57 control group.

4.3: Materials and methods

4.3.1. Animals

All animal experiments were approved by the Animal Experimentation Ethics Committee in University college Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland in accordance with EU directive 2010/63/EU. 8-week-old male BTBR and C57BL/6 mice were obtained from Harlan Laboratories, UK and housed in the animal unit until experiments were carried out at 19-21 months of age. Animals were kept under a strict 12:12-h dark-light cycle and temperature (20 ±1 ^{o}C,55.5% humidity), with food and water supplied *ad libitum.* Mice were group housed in 3-4 mice per cage.

4.3.2. Behavioural testing

4.3.2.1. Defensive marble burying

Defensive marble burying was performed as previously described (Golubeva et al., 2017). Briefly, this test measures repetitive and anxious behaviours, with a greater number of marbles buried representing increasing levels of anxiety. Cleaned cages were lined with a 5-cm layer of chipped cedar-wood bedding. Twenty glass marbles were arranged in an equidistant manner in a 5x4 orientation on top of the bedding. Animals were allowed to habituate to the testing room for thirty minutes prior to testing. During the test phase, each mouse was placed in the test cage and allowed to explore for 30 minutes. At the end of the 30 minutes, animals were returned to their home cage and the number of marbles buried recorded and photographed. Any marble greater than two thirds covered in bedding was considered to be buried.

4.3.2.2. Elevated Plus Maze

The elevated plus maze (EPM) is a commonly-used behavioural test to screen for anxiety-like behaviours (Rodgers and Dalvi, 1997). The apparatus is constructed from plexiglass and is arranged into a plus (+) shape with two open and two closed arms (arms are 50cm in length, 5cm wide and closed arms have a 15cm wall surrounding). The apparatus is raised one metre above the ground to increase anxiety in the open arms. The apparatus is separated from the rest of the room using identical white curtains to mitigate for visual clues. The experiment also takes place under red light at defined light intensities. To start the test an animal is placed in the 'hub' at the centre of the maze facing one of the open arms and allowed to

explore for five minutes. The apparatus is cleaned with 10% ethanol after each subject to prevent olfactory clues from the previous mouse. The test is recorded using a video camera placed directly overhead. Scoring of the test assessed the total number of entries to open and closed arms as well as time spent in each. Entries to the open and closed arms were considered when mice place all four paws on the arm.

4.3.2.3. Three-chamber test

This test for sociability was performed as previously described (Scott et al., 2017b). The test is undertaken in a rectangular box divided into three chambers (left, right, and with a smaller centre chamber. Chambers are separated by partitions with a small semi-circular opening at the bottom, and the left and right chambers contained a wire mesh cage. The test consists of three ten-minute trials performed consecutively.

1. **Habituation**: animals can explore the three chambers for ten minutes with mesh cages in left and right chambers being left empty.

2. **Sociability**: an unfamiliar mouse is placed in one of the mesh cages with an object (plastic rubber duck) placed in the other – again, animals are allowed to explore for ten minutes.

3. **Social novelty preference:** the object is replaced with a novel animal, while the now familiar animal remains in position – exploration is undertaken for ten minutes.

All conspecific mice were age- and sex-matched, with each box cleaned and lined with fresh bedding between trials. For each of the three stages, behaviour was recorded with an overhead camera and interaction times in each chamber were measured.

4.3.2.4. Forced-Swim test

The forced-swim test serves as a measure of antidepressant-sensitive behaviours. The test was performed as previously described (Desbonnet et al., 2013). In this test mice were gently place in a cylinder containing water (23-25^{o}C) at a height of 17 cm. Animals were left in the water for 6 minutes with activity being recorded by a camera positioned overhead. Immobility time was scored for the last 4 of the 6 minutes. Following removal from the cylinder, animals were dried gently and placed in a separate cage for recovery.

4.3.2.5. Open Field Test

Animals are moved to the experimental room and allowed to acclimatize for one hour before behavioural analysis. Following this, animals are placed individually in the centre of an open field box (Perspex sides and base: 32.5cm x 42.7cm) and their spontaneous activity was

recorded for five minutes using a camera placed overhead. Animals are returned to their home cage following the experiment, apparatus is cleaned with 10% ethanol and allowed to dry between experiments. Videos were analysed using the Ethovision (Noldus, USA) software. Total distance travelled, ambulatory activity, and time spent in the centre, were all measured and analysed. This test has previously been described (Scott et al., 2017b)

4.3.2.6. Grooming Test

The description of this test for repetitive behaviour has previously been described within our lab (Golubeva et al., 2017). Briefly, animals were moved to the experimental room and allowed to acclimatize for one hour before behavioural analysis. Following this, animals are placed individually into clear Perspex cylinders (10cm diameter and 20 cm high) with a thin layer of bedding in order to reduce neophobia but prevent digging, a potentially competing behaviour. Animals remained in the cylinders for ten minutes and were recorded with a camera placed horizontally level with the cylinders. Grooming time was scored manually by experimenters from watching video files.

4.3.2.7. Novel object recognition test

The novel object recognition test is a commonly used trial to assess hippocampal-dependent memory as described previously (Lueptow, 2017) and takes place over three trials on three consecutive days.

Day 1: Habituation – animals are habituated to a square open-field box (Perspex sides and base: 325cm x 42.7cm) in a dimly lit room by individually placing the mice to the apparatus for ten minute habituation periods. This portion of the experiment also served as the basis for the generation of 'open field test' results.

Day 2: Two identical objects are positioned on adjacent corners approximately 5cm from each wall of the open field, and each animal was introduced for a ten-minute exploration period. Animals were then placed directly back into their home cages.

Day 3: After a 24-hour inter-trial-interval, one familiar object was replaced with a novel object and each animal was introduced for a ten-minute exploration period.

On each day, animals are acclimatized to the testing room for approximately one hour before being placed in the box. Between trials, objects and testing arenas are cleaned with 70% ethanol and rinsed with water before thorough drying.

Recordings were made with a camera placed above the apparatus and scoring was undertaken manually from these videos. Object exploration was defined as when the animal's nose comes within a 2cm radius of the object.

4.3.3 Other physiological and post-mortem analyses

4.3.3.1. In-vivo Intestinal motility (carmine red test)

Mice were singly housed and habituated to new cages for three hours for acclimatization. Following acclimatization, mice received 200uL oral gavage of Carmine (C1022; Sigma Aldrich) suspended in 0.5% carboximethylcellulose (CMC) sodium salt (Sigma, St Louis, MO, USA). Time of the first red coloured bolus is recorded as previously performed (Golubeva et al., 2017)

4.3.3.2. Tissue Collection for Flow Cytometry

Trunk blood was collected in 3 mL EDTA-containing tubes (Greiner bio-one, 454086) and 100µl was put in a separate Eppendorf for flow cytometry. Both tubes were centrifuged for 10 min at 3,500 g at 4°C. The remaining cell pellet of the Eppendorf containing 100 µl blood was stored on ice and subsequently used for flow cytometry. Mesenteric lymph nodes (MLNs) were extracted, fat tissue was removed and stored in RPMI-1640 medium with L-glutamine and sodium bicarbonate (R8758, Sigma), supplemented with 10% FBS (F7524l, Sigma) and 1% Pen/strep (P4333, Sigma) on ice for subsequent flow cytometry.

4.3.4. Flow Cytometry

Blood and MLNs collected when animals were sacrificed were processed on the same day for flow cytometry similar to previously described (Boehme et al., 2019a; Gururajan et al., 2019). Blood was resuspended in 10 mL home-made red blood cell lysis buffer (15.5 mM NH_4Cl, 1.2 mM $NaHCO_3$, 0.01 mM tetrasodium EDTA diluted in deionised water) for 3 minutes. Blood samples were subsequently centrifuged (1500 g, 5 minutes), split into 2 aliquots and resuspended in 45 µl staining buffer (autoMACS Rinsing Solution (Miltenyi, 130-091-222) supplemented with MACS BSA stock solution (Miltenyi, 130-091-376)) for the staining procedure. MLNs were poured over 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 and 1% Pen/strep), centrifuged and $2x10^6$ cells were resuspended in 90 µl staining buffer and split into two aliquots for the staining procedure. For the staining procedure, 5 µl of FcR blocking

reagent (Miltenyi, 130-092-575) was added to each sample. Samples were subsequently incubated with a mix of antibodies (Blood and MLNs aliquot 1; 1 µl CD4-FITC (ThermoFisher, 11-0042-82) and 1 µl CD25-PerCP-Cyanine5.5 (ThermoFisher, 45-0251-80); MLNs aliquot 2; 1 µl CD4-FITC (ThermoFisher, 11-0042-82) and 5 µl CD8a-PerCP-Vio700 (Miltenyi, 130-102-468); MLNs aliquot 3; 2 µl CD11c-PE (Miltenyi, 130-110-838) and 5 µl MHC-II-APC (Miltenyi, 130-102-139)) and incubated for 30 minutes on ice. Blood aliquot 1 was subsequently fixed in 4% PFA for 30 minutes on ice, whilst Blood aliquot 2 and MLNs underwent intracellular staining using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher, 00-5523-00), according to the manufacturers' instructions, using antibodies for intracellular staining (2 µl FoxP3-APC (ThermoFisher, 17-5773-82) and 5 µl Helios-PE (ThermoFisher, 12-9883-42)). Fixed samples were resuspended in staining buffer and analysed the subsequent day on the BD FACSCalibur flow cytometry machine. Data were analysed using FlowJo (version 10). Cell populations were selected as following: T helper cell: CD4+, Cytotoxic T cell: CD8a+, Treg cells: CD4+, CD25+, FoxP3+; Dendritic cells; MHC-II+, CD11c+. The investigated cell populations were normalised to PBMC levels. Gating strategies are depicted in **Supplementary Figures 1, 2 and 3**.

4.3.5 Statistical analysis

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Data distribution was checked by Kolmogorv-Smirnov test and variances were compared using Levene's test. For parametric data, a Paired Student t-test, a One-way ANOVA, Two-way ANOVA and two-way repeated measures ANOVA followed by Bonferroni post-hoc was applied accordingly to the protocol adopted. All statistical analyses were carried out using IBM SPSS Statistics 22.0 for Windows software package. Extreme outliers and technical outliers were excluded when values are *2 x Standard Deviation from the mean***.** F values, P values are presented in the text of the results section.

4.4 Results

4.4.1. Behavioural results

4.4.1.1. Anxiety-like and repetitive behaviours.

As a model of ASD, BTBR mice have demonstrated a robustly anxious phenotype as well as clear repetitive behaviour in various behavioural tests when assessed in early adulthood. Here we show that grooming behaviour remains significantly increased in ageing **(Ffig. 1Bb)** ($T_{(20)}$=13.0 P<0.001)). However, in the marble burying test **(Ffig. 1Aa)** ($T_{(20)}$=0.1137, P=0.9106) no observed difference in anxiety-like behaviour between the BTBR and C57 animals was observed. In a similar vein, in the elevated plus maze **(Ffigs. 1Cc-1Ee)** the time spent in the open arms **(Ffig. 1Dd)** ((($T_{(20)}$=0.191%, P=0.8501) and number of entries to the open arms **(Fiffig. 1Ee)**($T_{(20)}$=1.162,P=0.2590) were not found to be different between groups. The amount of time spent in the closed arm **(Ffig. 1C)** ($T_{(20)}$=2.267,P<0.05) was however, significantly reduced in the BTBR group.

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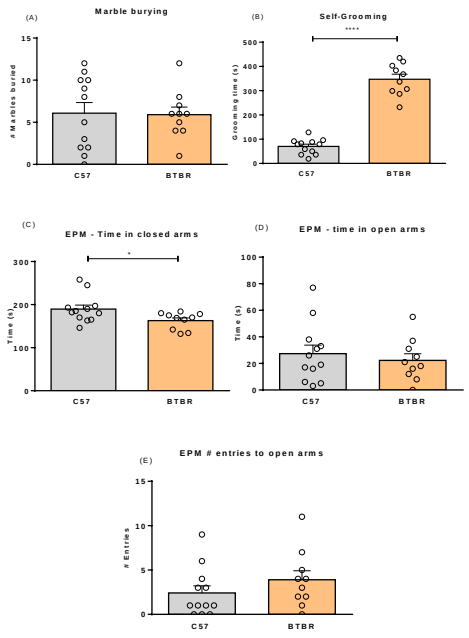

Fig 1: Aged BTBR and C57 mice display variable levels of anxiety across a range of behavioural tests. *(A) marble burying – no differences found in the number of marbles buried between the two strains. (B) grooming – BTBR mice spend significantly more time self-grooming than C57 (C-E) in the elevated plus maze BTBR mice spend less time in the closed arms of the maze, though there are no differences in the time spent in open arms, or the number of entries to the open arms. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. *p<0.05, ****p<0.0001.*

The open-field test is widely used in rodent models and it provides information regarding various aspects of emotionality in rodents (Seibenhener and Wooten, 2015). In our test it was used as a measure of locomotor activity **(Ff~~f~~ig. 2A~~a~~)** and anxiety **(Ff~~f~~ig. 2B~~b~~)**. Aged BTBR animals display greater locomotion than their C57 counterparts **(Ff~~f~~ig. 2A~~a~~)**($T_{(20)}$=9.745,P<0.0001), however, the time spent in the centre of the arena is similar between the groups **(Ff~~f~~ig. 2B~~b~~)**($T_{(20)}$=0.0478,P=0.9726), indicating no difference in anxiety-like behaviour.

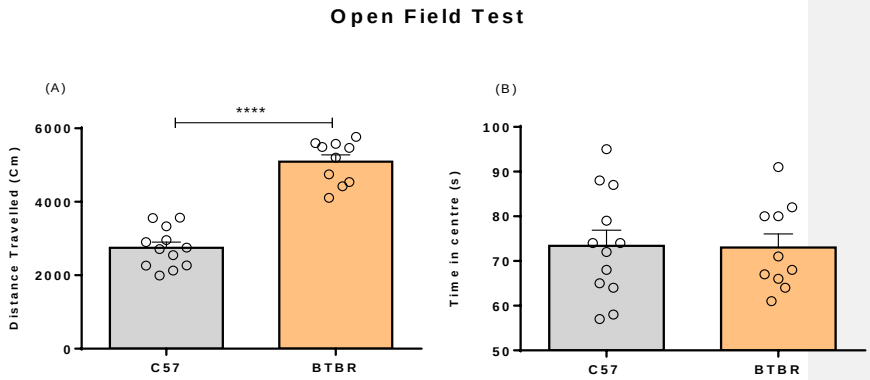

Fig 2: Aged BTBR mice display increased locomotor activity in the open field test. *(A~~a~~) Aged BTBR animals travel a significantly greater distance in during the duration of the test than age-matched C57 controls. (B~~b~~) There is no difference in the time spent in the centre of the arena between the groups. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. *p<0.05.*

4.4.1.2. Antidepressant-sensitive behaviour

The forced-swim test is used as a measure of antidepressant-sensitive behaviour (Yankelevitch-Yahav et al., 2015). Aged BTBR mice had a reduced immobility time in the test, indicating a reduction in depressive-like behaviour **(Fig. 3~~2~~)** ($T_{(20)}$=3.67, P<0.005).

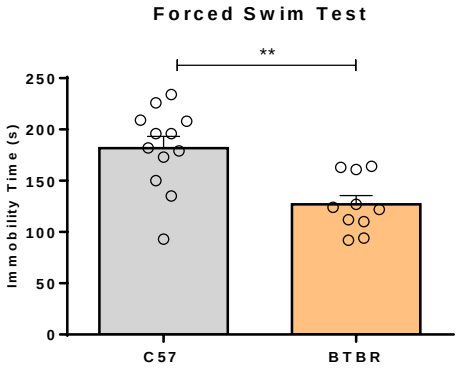

Fig 3: Aged BTBR mice display less antidepressant-sensitive behaviour in the forced-swim test. *BTBR mice spend significantly less time immobile in the FST than aged-matched C57 controls. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. **p<0.005.*

4.4.1.3. Three-chamber test

The ASD-like phenotype of BTBR mice means that a decreased level of sociability would be expected in both subsets of this experiment. Indeed, aged BTBR mice displayed a significantly reduced preference for the novel mouse than the aged-matched C57 control animals **(Fig. 4Aa)** (~~F~~t~~T~~$_{(20)}$=4.496,P<0.001). A similar result was observed in the second part of the experiment in which the mice were given the choice between interacting with a novel and a familiar mouse **(Fig. 4Cc)** (~~F~~t$_{(20)}$=2.664,P<0.05). The BTBR mice displayed a reduced preference for the novel mouse compared to the age-matched C57 control.

When the interaction times were analysed via two way ANOVA **(Fig. 4B)**. There was a significant effect of factor object $F_{(1,36)}$=119.8, P<0.0001. No effect was see of factor strain $F_{(1,36)}$=1.294, P=0.2628, or interaction between object and strain $F_{(1,36)}$=12.00, P=0.0014. Post-hoc analysis revealed that both C57 (P<0.0001) and BTBR (P<0.0005) mice exhibited a significant preference for mouse over object.

Two-way ANOVA analysis was also applied to interaction times in the social recognition test **(Fig. 4D)**. A statistically significant effect was observed for factor novel/familiar $F_{(1,32)}$=10.38, P=0.0029, though no effect of factor strain was observed $F_{(1,32)}$=1.184, P=0.2847, nor was an interaction between (novel/ familiar) and strain observed $F_{(1,32)}$=0.7872, P=0.3816. A post-hoc analysis revealed a preference for a novel over familiar mouse was observed in C57 mice only (P<0.005).

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~~it was found that both C57 ($F_{(22)}$=12.09,P<0.0001) and BTBR ($F_{(14)}$=14.475,P<0.0005) exhibited a significant preference for a mouse over an object **(Fig. 4b)**. However, while a preference for a novel over familiar mouse was observed in C57 mice ($F_{(18)}$=3.162,P<0.005), this was not seen in the BTBR animals ($Ft_{(14)}$=1.518,P=0.1512) **(Fig. 4d)**.~~

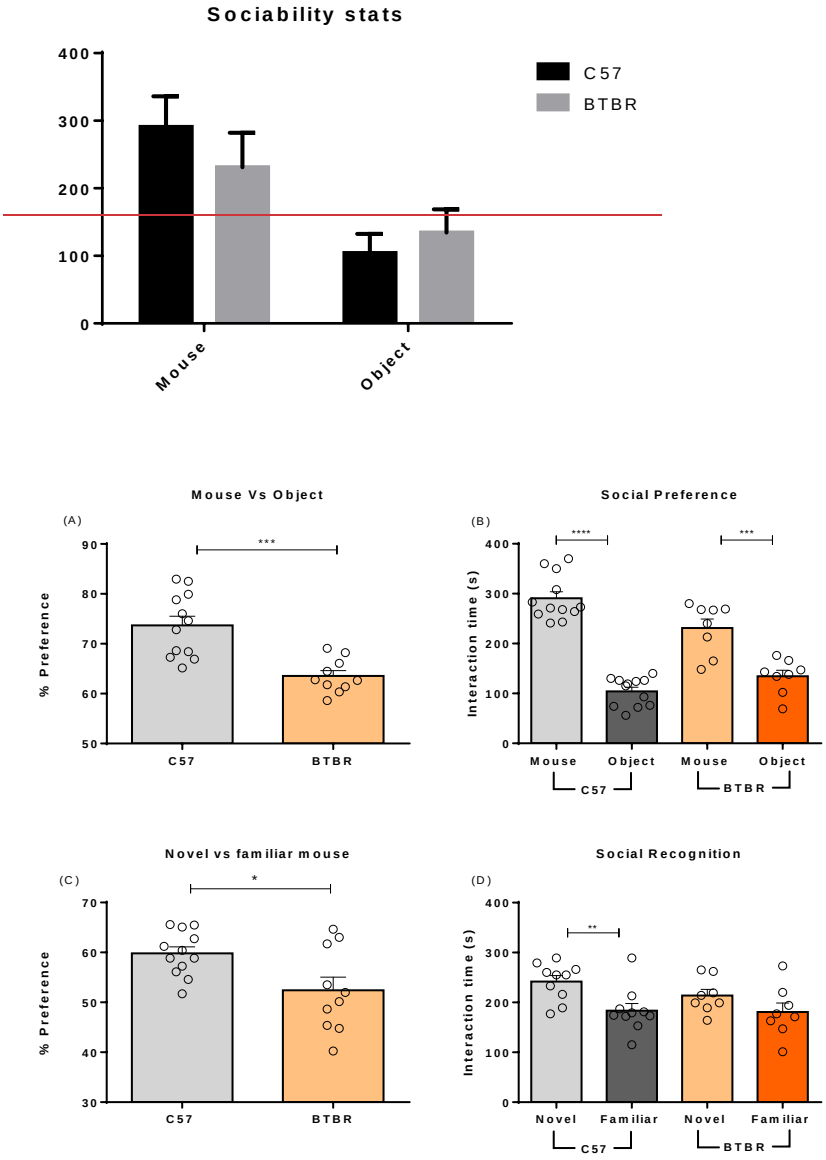

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Fig 4: Aged BTBR mice display impaired social behaviour compared to age-matched C57 mice in the three-chamber test. *(A~~e~~) BTBRmice exhibit a significantly reduced preference for interacting with a novel mouse over an object. Similarly in (B) both groups exhibit a preference for a mouse over an object. (C) BTBR mice have a significantly reduced preference for a novel mouse over a familiar mouse. (D) Only the C57 group display a preference for a novel over a familiar mouse. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test (fig 4A/C) or a two-way ANOVA with Bonferroni post-hoc analysis (fig 4B/D). *p<0.05,**p<0.005, ***p<0.001,****p<0.0001.*

4.4.1.4. Novel Object recognition

When the time spent interacting with either the novel or familiar objects was analysed via a two-way ANOVA~~Aged animals in both strains display a preference for interaction with a novel compared to a familiar object, with both spending more time with the novel object~~ (**F~~f~~ig. 5B)** a significant effect of factor (novel/familiar object) was observed $F_{(1,40)=}43.48$, P<0.0001. No significant effect was observed for either factor strain $F_{(1,40)}=0.9194$, P = 0.3434, or the interaction between strain and (novel/familiar object) $F_{(1,40)}=1.599$, P=0.2134.

Following the application of a post-hoc analysis it was observed that both C57 (P<0.0001) and BTBR (0.0005) mice exhibited a significant preference for the novel over the familiar object. ~~($F_{(22)}=5.282,P<0.0001;F_{(18)}=4.212,P=0.0005$).~~ When these results are expressed in terms of a discrimination index (**F~~f~~ig. 5A)** ($T_{(20)}=1.336,P=0.1964$), no differences are observed between the groups.

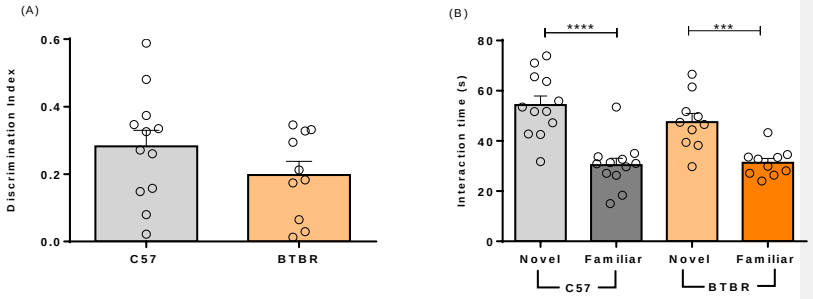

Fig 5: Aged mice of both C57 and BTBR strains display a preference for a novel object in the novel-object recognition test with no difference in discrimination index between the groups. *(A~~a~~) No strain difference was observed in discrimination index in the novel object recognition test. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test (B) Both aged C57 and BTBR mice spend significantly more time interacting with a novel object that a familiar one. Data are expressed as mean ± SEM. Data analysed by means of two-way ANOVA followed by a Bonferroni post-hoc test [C57 n=12, BTBR n=10]~~. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test~~. ***p<0.001, ****p<0.0001.*

4.4.2. Physiological data

Similar to previous reports in much younger mice (Ruskin et al., 2013), aged BTBR mice have a greater body weight compared to C57 mice **(Fig. 6A)** (F(20)=8.716,P<0.001)**.** However**,** cecum weight is greater in the C57 animals **(Fig. 6B)** (F(19)=3.734,P<0.005). Aged BTBR mice also exhibit an increased intestinal transit **(Fig. 6D)** (F(20)=3.346,P<0.005) though it may also be linked to longer colon that was seen in these animals **(Fig. 6C)** (F(20)=2.213,P<0.05)**.**

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Finally, no difference was seen in spleen weight between the two groups **(Fig. 6E)** (F(19)=1.701,P=0.1053).

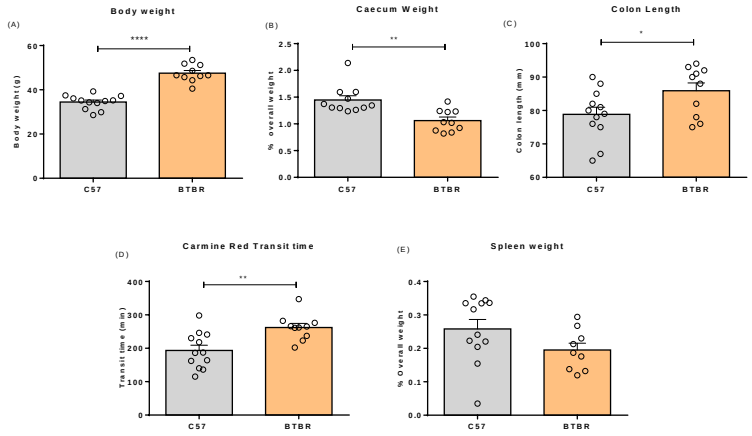

Fig 6: Body tissue weight differences between the two strains *(A) BTBR mice are larger than their age-matched C57 counterparts (B) C57 animals have a greater cecum weight relative to their body weight, however (C-D) both colon length and carmine red transit time were greater in BBTR animals (E) No differences were observed in spleen weight between the groups. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. *p<0.05.*

4.4.3. Flow Cytometry Data

4.4.3.1. Aged BTBR mice display an altered T-cell repertoire

Aged BTBR mice show increases in (CD4+) T helper cells both in MLNs **(Ffig. 7A)** (F(18)=13.69,P<0.0001and the peripheral circulation **(Fig. 7D)** (F(18)=5.908,P<0.0001). In addition, there was a decreased prevalence of (CD8+) cytotoxic T cells in MLNs **(Fig. 7B)** (F(18)=6.045,P<0.0001), but not the circulation **(Fig. 7E)** (F(18)=0.2661,P=0.7932). Overall, this results in an increased CD4/CD8 ratio in both MLNs and blood **(Figs. 7C&F)** (F(18)=23.90,P<0.0001) & (F(18)=6.081,P<0.0001), which is often used as a marker of an

activated adaptive immune system (Amadori et al., 1995).

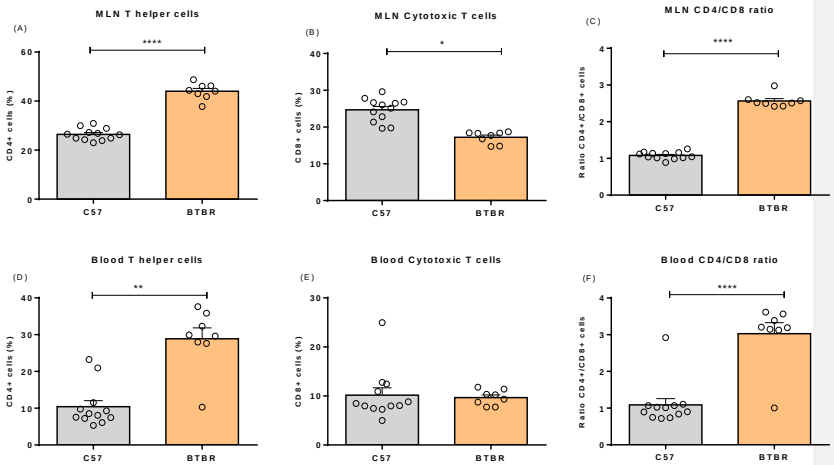

Fig 7 BTBR mice display an altered T-cell repertoire. *(A) BTBR mice have an increase in MLN helper T cells. (B) There is a lower level of cytotoxic T cells in the MLN, however (C) The overall effect is a significantly greater CD4/CD8 ratio in the BTBR animals (D) helper T cells are expressed in greater numbers in the blood in BTBR mice (E) No differences are observed in cytotoxic T cells in the blood (F) The CD4/CD8 ratio is significantly greater in the BTBRstrain. [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. *p<0.05,**p<0,005,****p<0.0001.*

4.4.3.2. Aged BTBR mice express decreased MLN Treg cells.

Aged BTBR mice have decreased levels of MLN Treg cells **(Fig. 8A)** (F(17)=3.120,P<0.005), offering further indication of an inflammatory phenotype. No alterations were seen in circulating Treg cells, however **(Fig. 8B)** (F(16)=1.423,P=0.1738). Interestingly, differences in overall MLN Treg cell levels were explained by decreased levels of peripheral-derived Treg cells (pTreg) **(Fig. 8C)** F(17)=8.527,P<0.0001). This may be linked to the microbiota, as Treg differentiation can be induced by gut microbial metabolites in MLNs, which would be pTregs (Zeng and Chi, 2015). Thymus-derived Treg cells (tTreg) were increased **(Fig. 8D)** (F(17)=3.991,P<0.001).

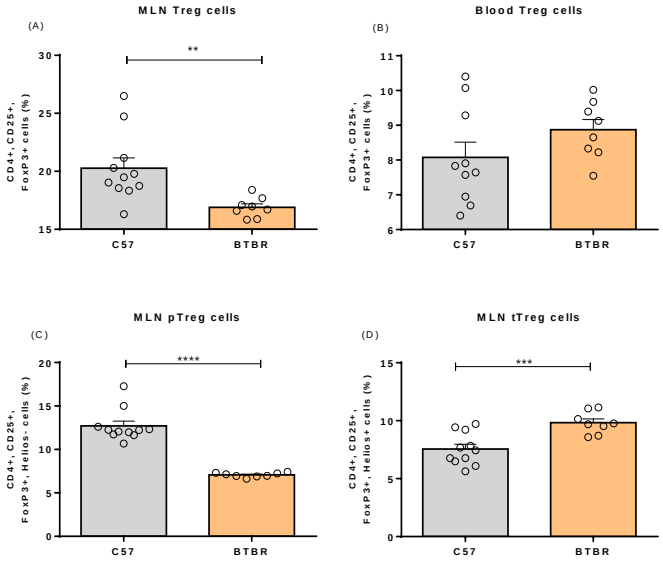

Fig 8. Aged BTBR mice display altered MLN Treg cells. *(A~~e~~) Treg cells are expressed at a significantly greater level in C57 mice in the MLN (B) No differences are observed between the strains in Blood Treg levels (C) MLN tTreg cells are observed at a higher level in BTBR mice with the opposite being true of MLN pTreg cells (D) [C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. **p<0.005, ,***p<0,001,****p<0.0001.*

4.4.3.3 Dendritic cells are decreased in number in BTBR mice

Dendritic cells are well-known inducers of Treg cell differentiation (Zeng and Chi, 2015). In line with the decrease in MLN Treg cell levels, BTBR mice showed decreased levels of MLN dendritic cells (**Fig. 9)** (F(18)=3.012, P<0.005).

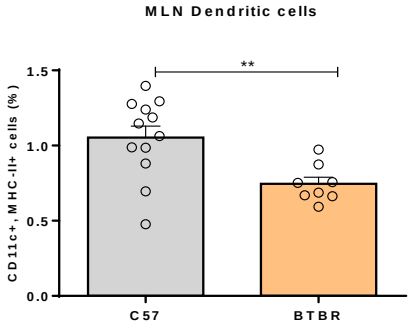

Fig 9: BTBR mice display a decrease in the number of MLN dendritic cels. *[C57 n=12, BTBR n=10]. Data are expressed as mean ± SEM. Data analysed by means of unpaired t-test. **p<0.005*

4.5. Discussion

BTBR mice are a highly utilised, informative and robust mouse model of autism spectrum disorders (Meyza et al., 2013). Previous studies in young animals have shown that they exhibit a number of behavioural abnormalities, compared to control strains. Deficits have been observed for example, in sociability and social withdrawal (Bove et al., 2018), learning and attention (McTighe et al., 2013), stress response (Gould et al., 2014), anxiety and depressive behaviours (Golubeva et al., 2017) as well as repetitive behaviours (McFarlane et al., 2008). Here we characterise these parameters in older animals and show that many of the characteristic behaviours of the BTBR model during early-life and adult are maintained in the ageing animal, however, there are several notable changes in the older animals. We compared behavioural differences between these animals and a C57 aged control, also assessing immune system differences between the two strains.

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The initial tests undertaken in the behavioural battery assessed levels of anxiety-like behaviours in each of the strains. As a model of ASD, BTBR mice generally exhibit a higher level of repetitive and anxiety-like behaviours as has been demonstrated in the marble-burying test (Amodeo et al., 2012; Golubeva et al., 2017), the elevated-plus maze (Golubeva et al., 2017) and grooming behaviour (Golubeva et al., 2017) . We observed that this phenotype was still present in later age in the grooming test where BTBR mice spend significantly more time engaged in grooming behaviour than the C57 controls. As increased engagement in repetitive behaviour is among the most robust behavioural characteristics, this is an important result as it suggests that this core facet of the behavioural phenotype is maintained.

Other tests provided less of a robust anxious phenotype. In the marble-burying test, there was no difference in the number of marbles that were buried between strains. In the elevated plus maze, no differences observed in the number of entries to the open arms, or the amount of time spent in the open arms; BTBR mice spent significantly less time in the closed arms. This test measures the conflict between the motivation of mice to explore a novel area, and their preference for a protected environment (Walf and Frye, 2007). As such,

a reduction in the time spent in the closed arms of the test is regarded as an anxiolytic behaviour, not what would be expected from a mouse model of autism.

An alternative interpretation of the results takes into account the age of the animals. It has been established that aged C57 animals spend significantly less time in the open arms of the EPM (Scott et al., 2017b), thereby increasing the amount of time in the closed arms. These results offer insight into the differential ageing trajectory between the two strains. The anxiety-like behaviour observed in this study is not as robust as what is seen in younger animals, with only the grooming test showing a much increased anxious phenotype. This suggests that there may indeed be a lessening of anxiety-like behaviour in aged BTBR animals .At least in comparison to their C57, counterparts. Assessment against younger BTBR animals in a future study would provide empirical evidence of this.

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Within the forced-swim test, a measure of antidepressant-sensitive behaviours, we observed that BTBR animals exhibit significantly less immobility time than C57 controls. This is consistent with what has been observed in younger animals (Silverman et al., 2010). Furthermore, it is unlikely to be an age-related effect of controls, as aged C57 animals do not display an alteration in the test compared to younger animals (Scott et al., 2017b). While these results are consistent with what has been observed in the past, there is also an inconsistency with what is seen clinically. Both individuals with autism (Hudson et al., 2019), and the elderly (Djernes, 2006) are known to display elevated levels of depression. A further caveat is that the BTBR group exhibited increased locomotor activity in the open-field test, which suggests that the additional levels of activity may be due to a hyperactivity within this group that may mask differences in depressive-like behaviour. Hyperactivity is a factor that is known to be present in animal models of ASD (Fatemi et al., 2012), having also been observed in mice displaying autistic behaviour which lack the synaptic proteins proSAP1 and Shank2 (Schmeisser et al., 2012).

Previous studies have reported social deficits in BTBR mice in young animals (Constantino, 2011). Our data reveals that, while both strains exhibited a preference for a mouse over an object in the test, the C57 animals exhibited a significantly greater preference. This greater preference was also seen in a test for social recognition, where C57 animals exhibited a greater preference for the novel over the familiar mouse, a feature not observed in the BTBR mice who exhibited a similar preference for each. Considering that sociability is regarded as one of the most robust behavioural traits of the BTBR model, and has been demonstrated

on numerous occasions (Golubeva et al., 2017; Pobbe et al., 2010), the maintenance of this phenotype into old age highlights its durability in the model.

Within ASD there exists a wide range of cognitive abilities, ranging from severe disability to high-functioning individuals (Bölte et al., 2009). We undertook the novel object recognition paradigm as our measure of cognitive function. Previous studies have demonstrated a reduced level of performance in BTBR animals in this test compared to other strains (Golubeva et al., 2017; Silverman et al., 2013). Tests of cognition, however are one of the most widely undertaken in studies of ageing and aged animals have been shown on many occasions to preform worse in these tests than younger animals (Stefanko et al., 2009; Wimmer et al., 2012). In our experiment we saw that both strains of mouse exhibited a preference for a novel over a familiar object, and that there was no difference in discrimination index between the groups. It must be noted in this study, however, that there is no young control to which behaviour in the test can be compared. So while BTBR mice perform worse in the test compared to C57 controls in younger animals, it may be that the higher performing C57 groups have a bigger relative decline over their lifespan, explaining the similar performance of both groups in ageing.

Physiological measurements showed a number of differences between the strains of mouse. We observed that C57 mice exhibited a significantly greater cecum weight than their BTBR counterparts as a percentage of overall body weight, and this structure has a high density of bacteria that has been shown to have its own distinct composition (Klein et al., 2016; Newell et al., 2016). Intestinal transit was observed to be delayed in BTBR mice compared to controls, though this also corresponds with an increase in colon length which may be a contributing factor. Previous studies have found corresponding results, however and suggested that the increases may be linked to a reduced intestinal availability of serotonin in these animals and a subsequent alteration in the ability of the neurotransmitter to act on NMDA receptors within the enteric nervous system (Golubeva et al., 2017; Wu, 2017)

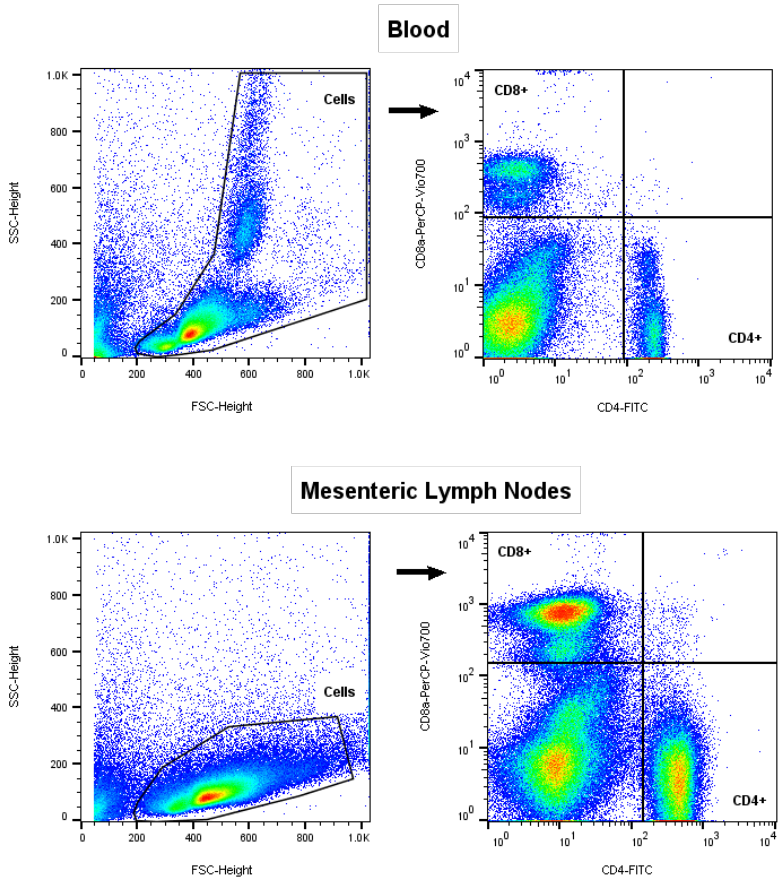
In addition to behavioural and physiological differences between these two strains of animals we also performed flow cytometry analysis in order to determine whether any immune changes were present in these older animals. Here we show that aged BTBR mice display an altered T-cell repertoire to C57 animals. An increase in CD4+ T-helper cells was observed in BTBR mice in MLNs and the peripheral circulation, while CD8+ cytotoxic T cells were decreased in MLNs only. This resulted in an increase in the CD4/CD8 ratio, often associated with an activated immune system (Sainz et al., 2013). Indeed, it has previously

been demonstrated that animal models of ASD (Ashwood, 2013), as well children with the disorder (Ashwood et al., 2011), show higher immune activation. Furthermore, BTBR mice had decreased levels of Treg cells in MLNs, further indicating an inflammatory phenotype (Bettelli et al., 2006). This is in line with previous reports in adolescent BTBR mice (Bakheet et al., 2017). Alterations in Treg cell subtypes in the MLN have been linked to inflammation in the gut (Boschetti et al., 2017), with children with ASD more likely to suffer from inflammatory disorders of the gut than neurotypical controls (Lee et al., 2018b). Our results also reveal that this inflammation may be linked to a deficit in non-thymic Treg production.

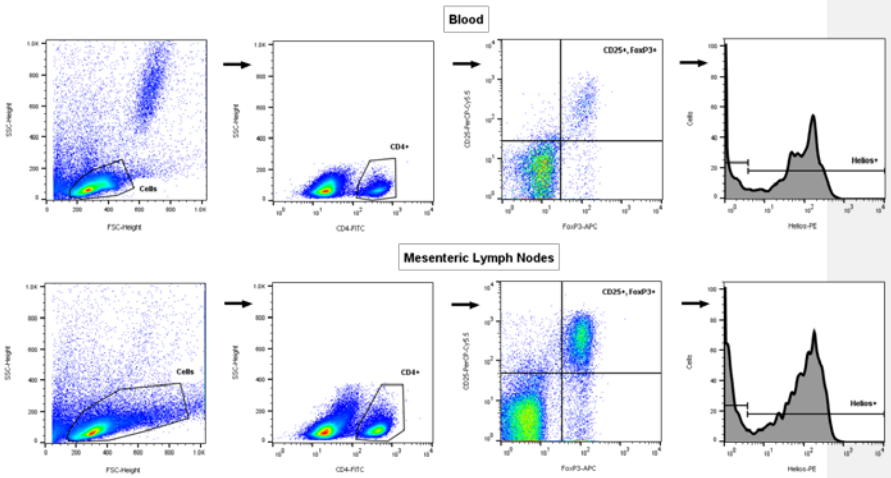
In line with this is a decreased prevalence of dendritic cells in MLNs of BTBR mice, which are known to induce the Treg cell differentiation (Clark et al., 2000). Even though increased levels of dendritic cells were observed in individuals with ASD (Breece et al., 2013). Overall the immune data suggests that aged BTBR mice display a chronically activated T-cell system compared to control C57 animals, suggesting the involvement of auto-immunity in the differences observed between the strains. While there may be some alterations due to the natural disruptions to the immune system in advancing age, the data suggests that the observed increases are in autoimmune activation that has been observed in animal models of ASD (Ashwood, 2013), as well as in humans (Ashwood et al., 2011), and that this phenotype is maintained at a later age.

The results of this study yield information on a novel aspect of the study of ASD in mouse models. The vast majority of preclinical research in the area focuses on young animals where results will be translated clinically to young individuals where much of the focus in the disorder rests. Outside of these studies there are a handful of clinical studies in early middle age (Mukaetova-Ladinska et al., 2012) though this is not the case preclinically. Our study makes an assessment of behaviour during this age in a preclinical cohort. We find that while many of the behavioural characteristics observed in early life are maintained at this later stage others are not, particularly in the case of anxiety-related behaviours. This study provides a platform for an interventional analysis targeting the gut-brain axis. Future studies must focus on interventional studies in this ageing model of altered gut-brain-axis function.

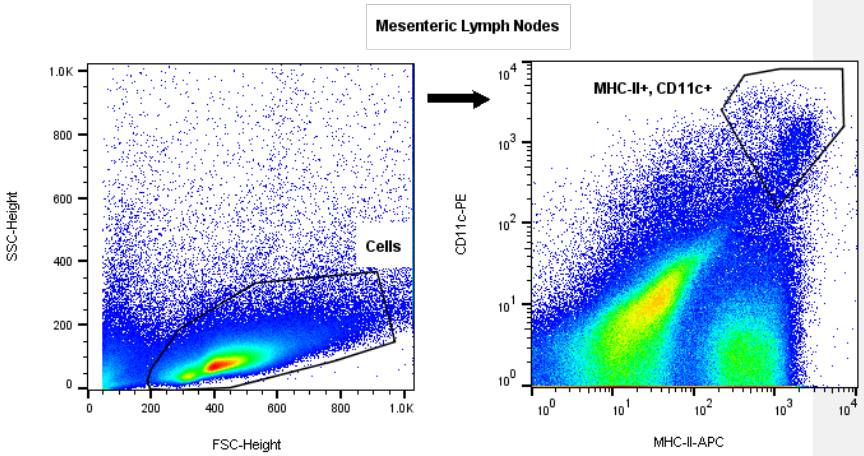
4.6: Supplementary data



sFigure 1. Gating strategy for CD4+ and CD8+ cells. *CD4+ and CD8+ cells were quantified in blood and mesenteric lymph nodes. First, cells were selected based on FSC-Height and SSC-Height, after which cells were selected based on CD4 and CD8 receptor expression.*



sFigure 2. Gating strategy for T regulatory cells (Treg). *Treg cells were quantified in blood and mesenteric lymph nodes. First, cells were selected based on FSC-Height and SSC-Height, after which T helper cells were selected based on CD4 receptor expression. Treg cells were subsequently selected based on CD25 and FoxP3 expression. Finally, Treg cells were investigated for their origin (i.e. thymus or non-thymus), based on helios expression.*



sFigure 3. Gating strategy for dendritic cells. *Dendritic cells were quantified in mesenteric lymph nodes. First, cells were selected based on FSC-Height and SSC-Height, after which dendritic were selected based on CD11c and MHC-II receptor expression.*

Chapter 5

Modulation of hippocampal synaptic signaling through direct application of gut microbial metabolites.

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5.1 Abstract

The gut microbiota is increasingly being recognized as a key component of a signaling network that not only affects behaviour but also has the ability to modulate the development of the central nervous system. Modifications to this system can influence learning and memory and synaptic signaling within the hippocampus. Of the myriad microbial metabolites the short-chain fatty acids (SCFAs) are among those that have demonstrated effects on the central nervous system. As such, we utilised the multielectrode array (MEA) as a tool to investigate whether SCFAs can influence synaptic signaling in hippocampal slices. Results suggest that application of the highest concentration of sodium acetate tested (700μM) leads to deficits in long-term potentiation (LTP) when measured post theta-burst tetanus. The lower concentration of sodium acetate (70μM) and the two concentrations of sodium propionate tested (0.5μM and 5μM) do not lead to any alterations in LTP. None of the compounds tested lead to any alterations in basal synaptic signaling, or paired-pulse ratio. We suggest that the observed changes may be due to alterations in local ion channels in the presence of these gut microbiota metabolites, and that elucidation of this mechanism may form a cornerstone of future work regarding the effects of SCFAs on synaptic signaling.

5.2. Introduction

The link between the intestinal microbiota and behaviour is well established (Codagnone et al., 2019; Cryan and Dinan, 2012b; Fulling et al., 2019; Hechler et al., 2019) with these bacteria having been shown to influence factors from sociability (Sherwin et al., 2019) and mood (Dinan and Cryan, 2013) to cognition (Gareau, 2014), among numerous other aspects of brain and behaviour (Cryan et al., 2019b). The term 'psychobiotics' has been coined to describe the potential for gut microbiota modulating therapies to positively impact these conditions (Dinan et al., 2013b).

More recently, studies have begun to focus more on the mechanisms underpinning these behavioural effects. Such research has shown that the gut microbiota is able to exert an influence over central nervous system factors including blood-brain-barrier permeability (Braniste et al., 2014a; de Cossio et al., 2017), prefrontal cortex myelination (Hoban et al., 2016c; Radulescu et al., 2019), neurogenesis (Mohle et al., 2016; O'Leary et al., 2018), and microglial maturation (Abdel-Haq et al., 2019; Erny et al., 2015b). While each of these factors individually may not be sufficient to directly influence behaviour, that the gut microbiota is exerting an influence over such an array of central nervous system processes suggests that alterations in behaviour are a likely outcome.

Another aspect of development that been shown to be under the influence of the microbiota is synaptic structure (Luczynski et al., 2016). Dendritic morphology in both the amygdala and the hippocampus was shown to be altered in germ-free compared to conventionally colonized mice (Luczynski et al., 2016). The existence of such effects leads us to question whether modulation of the gut microbiota can influence synaptic signaling with possible consequences for behaviour. Early studies showed that probiotic administration could protect against age-related decreases in hippocampal long-term potentiation (LTP) (Distrutti et al., 2014a; Talani et al., 2019), the activity-based increase in synaptic strength that is considered to be the corollary one of the major cellular mechanisms of learning and memory (Bliss and Collingridge, 1993)) as well as reversing deficits in LTP in a mouse model of diabetes (Davari et al., 2013).

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Furthermore, high-fat diet administration (a known modulator of gut microbiota composition (Wan et al., 2019)) can also negatively impact both structural and electrical hippocampal synaptic plasticity (Chunchai et al., 2018a), effects which are reversed following psychobiotic administration. Maternal high-fat diet has been shown to lead to a disrupted microbiota in offspring along with alterations in synaptic plasticity in the ventral tegmental

area (Buffington et al., 2016). Both of these effects were reversed following administration of the probiotic *L. reuteri* (Buffington et al., 2016), again highlighting the close link between gut microbiota composition and synaptic plasticity. We also demonstrate in chapter two of this thesis that maternal penicillin administration in early life leads to a decrease in hippocampal expression of BDNF (O'Connor, IN PRESS), a key regulator of synaptic plasticity (Bekinschtein et al., 2008).

A number of bidirectional routes of communication between gut and brain exist that allow compositional changes in the microbiota to signal to the brain. These allow for such changes to be integrated in the central nervous system, while also meaning that changes in mental state can influence conditions in the gut and thereby impact microbiota composition (Carabotti et al., 2015). Routes of communication include the immune system (Petra et al., 2015) and the HPA axis (Cryan et al., 2019b). Bidirectional communication via the vagus nerve also plays a key role in this communication (Forsythe et al., 2014a). Furthermore vagal nerve stimulation has been shown to potentiate LTP in rats (Zuo et al., 2007a), suggesting that it may play a role in in learning and memory specifically.

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In addition, gut microbiota metabolites allow for communication via the gut brain axis (Stilling et al., 2014). The most widely studied of these are the short-chain fatty acids (SCFAs)(Dalile et al., 2019a), produced through bacterial fermentation of host-indigestible fibres (Macfarlane and Macfarlane, 2003). SCFAs can also influence the brain, with links having been made between SCFA concentration and neurological conditions including Parkinson's (Unger et al., 2016) and Alzheimer's disease (Zhang et al., 2017).

The gut microbial-derived SCFA acetate has been shown to reach the brain and trigger signaling in the peripheral nervous system (Frost et al., 2014a; Nagashima et al., 2010). Effects of butyrate have been demonstrated on LTP induction in hippocampal slices (Levenson et al., 2004a), with both butyrate and trichostatin A, (A histone deacetylase inhibitor), capable of enhancing LTP induction. This has been highlighted in subsequent research examining the effects of HDAC inhibitors on synaptic function in disease models where normal signaling activity is disrupted (Basu et al., 2019).

The aim of this study is to utilize the novel, multielectrode array (MEA) technology to probe the effect of SCFA application to hippocampal slices. We highlight its potential by making simultaneous recordings at two distances from the stimulation site. Following further optimization, it will be possible to use this technology to record from many additional electrodes in other areas of interest, vastly increasing the data generated from a given slice.

We assessed whether different SCFAs can exert differential effects on signaling, as well as determined whether such effects are dose-dependent. We then assessed whether different aspects of signaling, including basal synaptic transmission, input-output, short-term plasticity and long term plasticity are affected following SCFA application. The ubiquity of these compounds means that they should form a cornerstone of future research in studies investigating the microbiota-gut-brain axis be it though modulation of their expression through probiotic or antibiotic administration, direct supplementation with dietary SCFAs or through application of the compounds to *in-vitro* and *ex-vivo* preparations.

5.3. Materials and Methods

5.3.1 Animals

All experiments were conducted in accordance with the European Directive 2010/63/EU and under authorization from the Health Products regulatory authority of Ireland and approved by the animal ethics committee of University College Cork. C57BL/6 mice were sourced from Envigo (UK). Animals were group house and had *ad-libitum* access to food and water as well as cage enrichment. The animal holding room was under a 12-hour light/ dark cycle (lights on at 08:00h) with a temperature of 21°C ± 1°C and a humidity of 55% ± 10%.

5.3.2 Tissue Preparation

Mice were decapitated using a tungsten-carbide surgical scissors (F.S.T 14501-14). The scalp was cut with a straight 11.5cm iris scissors (WPIINC 401758). The skull was bisected using the sagittal suture as a landmark with a heavy blade-dissector scissors and the skull was reflected away. Optic nerves were severed to ensure a smooth removal of brain before extraction with a micro-spatula and a rat-toothed forceps (WPIINC 15918).

Once the brain was removed it was placed on an ice-cold platform dorsal-side up on top of a Whatmann filter paper membrane saturated with ice-cold artificial cerebrospinal fluid (ACSF). The brain was immediately doused with fresh, carbogenated, ice-cold ACSF prior to preparation.

The brain was cut along the midline using a single-edged razor blade (VWR international – 233-0156) to produce two symmetrical hemispheres which were isolated and gently gripped by narrow-pattern forceps (FST-11002-12) and excess cerebellar tissue was slowly separated from the cortex (taking care not to damage the hippocampus when separating the cerebellum). The hippocampus was then isolated by a coronal cut to the rostral third of the

cerebellum to provide a flat surface for the mounting of the tissue to an agar block on the slicing stage using ethyl-2-cyano-acrylate glue.

Taking care to minimise physical impact, each hemisphere was positioned on the vertical face of an agar block with the medial side adhering to the agar, ventral side glued to the stage and dorsal surface facing up towards the blade. Ethyl-2-cyano acrylate glue was then applied to the slicing platform with the agar block being placed on top and bathing in ice-cold cutting solution (see section 2.4 for composition) to avoid the brain being coated in glue upon submersion into the ACSF bath in the vibratome cutting chamber.

The slicing stage was placed in and attached to the vibratome (Lecia VT1200, Lecia Microsystems) with the cortical/ lateral side of the tissue facing the blade. Ensuring that the platform was submerged in the buffer tray, surrounded by carbogenated (95%CO_2, 5% O_2) cutting ACSF at 2°C-4°C. The blade was angled at 18-20° from the horizontal plane with the temperature and carbogen saturation being maintained throughout the cutting process.

The vibratome was set at 1.5mm amplitude and 0.28mm/s speed and slices were made simultaneously from both hemispheres of 300µm thickness. The orientation of the agar block ensured that slices were cut consistently in a dorsal through ventral order. Once the first slice containing hippocampal tissue was identified the pivotal slices used are the 5^{th}/6^{th} subsequent slices – this results in a consistent dorsal-intermediate hippocampal recording location.

Slices were then transferred to ice-cold, carbogenated cutting solution in a petri-dish using a disposable plastic Pasteur pipette. Hippocampal slices were isolated from the cortical tissue at the subiculum using a scalpel (F.S.T 10004-13) and fine forceps (F.S.T 11412-1) to pin the cortical tissue, while the scalpel is used to rotate the cortical tissue in a gentle manner, allowing for separation at the subiculum and isolation of the pure hippocampal slice. This method ensures the very minimal contact with the hippocampus, reducing the potential for damage.

Isolated sections were then transferred to a water bath containing carbogenated cutting ACSF at 32°C for at least one hour to allow slices to recover (Lein et al., 2011).

Recording slices were chosen based on dorsal-intermediate location and slice health which was visually determined based on structural integrity, as well as quantity of residual cortical tissue attached (less cortical tissue being preferred). Dorso-intermediate sections were

consistently chosen as recent literature suggests that there are differences in LTP inducibility along the dorso-ventral axis of the hippocampus (Schreurs et al., 2017).

Slices were then selected and transferred to the MEA and submerged in the housing well of the MEA chip. If positioning alterations were required they were undertaken gently using a fine paint brush taking care not to damage tissue with the paint brush or on the 3D chip surface electrodes. Slice positioning was visualised with an upright light microscope (Olympus IX-70).

Once the slice was in the desired position with a stimulating and recording area located in the stratum radiatum (precise location being determined using a number of factors including: visual location, stimulus response, waveform shape, proximity to internal reference electrode **(fig. 1)**) a glass and aluminium/ nylon mesh anchor was inserted into the ACSF at an angle to the surface in order to cause as little disruption as possible and placed on top on the slice to ensure optimal contact with microelectrodes. It was possible to further refine slice position on the MEA chip after anchor placement. Carbogenated ACSF inflow and outflow were then initiated via peristaltic pump allowing for fresh nutrients to reach the slice. Flow rates were 2.5mL/min in and 3.5mL/min out (Dondzillo et al., 2015). Slices were allowed to recover and acclimate to the new environment for at least 90 minutes prior to any stimulation.

5.3.3 Electrophysiological recording in the MEA

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Recordings were performed on an MEA2100 Minisystem (Multichannel Systems, Germany) using a Microelectrode Array Chip (60MEA200/30iR-Ti) with an internal reference electrode. Spacing between adjacent electrodes was 100µM. Fresh carbogenated ACSF was delivered to the slice via a temperature controlled perfusion canula (PH-01), which warmed the incoming ACSF to 32°c prior to entry into the well. A heating element with an internal reference thermocouple heated the MEA chip to 32° from within the headstage, allowing for a more consistent temperature control.

Data acquisition was governed by MC Rack 3.2.1.0 software. Data was digitised and sampled at 25000Hz and analysed using Clampfit 10 software (Molecular Systems). Recordings were filtered in real-time using a 4th order low pass Bessel filter to preserve wave shape in filtered signals. Biphasic rectangular pulses (200µs in duration – 100µs positive, 100µs negative) were used for stimulation. Amplitude, frequency and duration of stimulation were controlled by MC_Stimulus II software. Rectangular stimulus waveforms were used to increase

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electrode longevity. The descending slope of the field excitatory post synaptic potentials (fEPSPs) were measured and used as the primary response variable throughout all experiments, an 80/20 ratio was used when defining the descending slope of the fEPSP (ie the first 10% and last 10% of the descending slope was discarded in order to determine a more consistent value).

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Biphasic stimulation of Schaffer collateral/ commissural fibres was performed by selecting stimulation points at the medial/ distal part of the stratum radiatum near the CA1/CA3 border. Recording was performed on the adjacent electrode in the anterograde direction. Although we recorded from all electrodes, only the 200 and 400µm from the stimulation site electrodes in the anterograde direction of the performant path in the CA1 were chosen for analysis **(fig. 1)**.

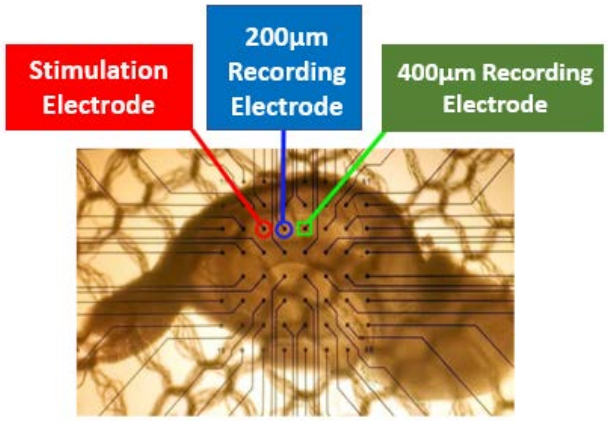

Fig 1: *Recordings at 100µm (blue) and 200µm (green) from the site of stimulation were analysed.*

Stimulation protocols were determined on a slice by slice basis based on the half maximum as measured by input/output (i/o) curves (Shaban et al., 2017b). Paired pulse facilitation (PPF) protocols were run as a measure of short-term potentiation. Post hoc parameters were set in Clampfit to analyse data.

Baseline stimulation consisted of a single pulse stimulus to a single electrode repeated at 30 second intervals. LTP was induced by a Theta Burst Stimulation (TBS) protocol (Abrahamsson et al., 2016) following 20 minutes of baseline stability, either in the presence or absence of short-chain fatty acids.

5.3.4 ACSF preparation and administration of test compounds

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ACSF was prepared daily from 10x stock solutions, which themselves were prepared every three days with the final recording solution containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 KH2PO4, 25 NaHCO3, and 25 glucose, saturated with 95% O2/5% CO2 (pH 7.2–7.3 and 325 mOsm/kg). For the cutting solution additional magnesium was added to the ACSF to bring the final magnesium concentration to 4mM. This was to preserve slice health during the physically stressful cutting process.

Experimental compounds were dissolved in ACSF to the desired concentration and added to circulation by moving the input cannula to a falcon tube containing the compounds once baseline measurements had been recorded. The 'sweep' in recording during this switch was made, was noted, to accurately represent any changes due to addition of the compound.

The following is the list of all compounds used: Sodium Acetate – (W302406 - Sigma-Aldrich); Sodium Butyrate – (W303410 – Sigma Aldrich); Sodium Propionate (P1880 – Sigma Aldrich); Sodium Formate (W71539 – Sigma Aldrich); Magnesium Sulphate – (M7506 Sigma-Aldrich); Sodium Chloride – (S7653 Sigma-Aldrich); Monosodium Phosphate – (S8282 Sigma-Aldrich); Potassium Chloride – (P9333 Sigma-Aldrich); Sodium Bicarbonate – (S5761 Sigma-Aldrich); Calcium Chloride – (C1016 Sigma-Aldrich); D-Glucose – (D9434 Sigma-Aldrich); Ascorbic Acid – (A1300000 Sigma-Aldrich).

5.3.5 Data Analysis

Axon Binary Files (*.abf) were generated from recording electrodes on MC_DataTool (Multichannel Systems, Germany), they were opened by Clampfit where users set post hoc parameters to analyse fEPSPs. The descending slope of the fEPSP was measured for all traces with an 80/20 ratio (ignoring the first and last 10% of the descending slope). Input/Output (IO) curves (see Fig. 2.9) were used to determine half max stimulation intensities. (Stimulation intensities for IO curves were set to 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200µA.

PPF ratios were generated to measure short term plasticity by measuring the ratio of the slope of the secondary pulse to the primary. Inter-pulse intervals were set to 25, 50, 75, 100, 150 and 200ms. Data was exported and analysed in SPSS (IBM, CA) to determine stimulation parameters specific to the test slice(s).

5.3.6 Statistics

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Data are presented as mean +/- standard error of the mean. Data was tested for normal distribution using the, Shapiro-Wilk test & Kolmogorov-Smirnov test. One-way ANOVA

analysis was performed on Paired-pulse ratio data and on the bar-chart summary of LTP data. Two-way ANOVA was undertaken on input-output and LTP data. Bonferroni post hoc analysis was used in both cases to correct for multiple comparisons. P, t and F values are presented in the results section, n values are provided in the figure legends. P<0.05 was considered statistically significant. (*P<0.05, **P<0.001, ***P<0.0001,****P<0.00001). Graphpad prism 6 (LaJolla CA) software was used to perform statistical analyses and for generating graphical representations of data.

5.3.7 Optimizations to the multi-electrode array system

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At the beginning of experimentation the MEA apparatus and had not previously been used in the lab. It was necessary, therefore, to optimize the equipment in order to achieve robust, repeatable, and reliable results. Since electrophysiological experiments in general, and MEA experiments in particular, consist of multiple variables over many stages, numerous optimizations were undertaken. These have been categorized into sub-headings, but consisted of a large number of alterations over several years. During the optimization process, every effort was made to ensure that only one aspect of the process was altered on any given day. This allowed us to determine specific effects of each alteration, removing the ambiguity that would be present when multiple alterations were performed. Only optimisations that improved the quality of recording are listed. Numerous additional modifications were attempted without success.

5.3.7.1 Slicing, ACSF, and recovery optimizations.

- Initially, slicing utilized the protocol by Chong et. al (Chong et al., 2011). Briefly, following removal of the brain, the prefrontal cortex and cerebellum were removed as described earlier. Following numerous iterations, the following method was determined and has numerous benefits relative to initial methods.
    - Increased processing of the brain prior to slicing means a more efficient and reliable isolation of the hippocampus post-slicing.
    - This processing also leads to more reliable and uniform slice retrieved from animal to animal, reducing variability.
    - It is now possible to obtain a greater number of slices from an individual brain, increasing the likelihood of viable slices.
    - Simultaneous slicing of hippocampi from multiple brains is now possible due to the removal of excess cortical tissue – this allows more animals to be assessed on a given day.

- Selection of slices from one animal was initially based on finding a slice that elicited a suitable degree of response. Following refinement, only dorsal-intermediate slices were selected for recording. This allowed for a greater degree of reliability between experiments, as different regions of the hippocampus are known to produce different electrophysiological responses (Nguyen et al., 2015).
- Recovery time following movement of the slice to the MEA chip was increased. While slices were always allowed to recover from room temperature to 37°C for at least 90 minutes prior to recording, initial experiments began recording almost immediately following the transfer of the slice to the chip. By allowing the slice to acclimatize to the conditions present in the chip for a greater amount of time (90-120 mins) before recording, much larger and more stable responses were observed.
- Increasing recovery time and the subsequent increase in response size following stimulation means a significant improvement in signal-to-noise ratio. This allowed the stimulus intensities employed in the initial tests of synaptic response (baseline and input-output) to be greatly reduced. This lead to a significant reduction in stimulation stress placed on the slice, thereby improving longevity of recording.

5.3.7.2. Flow-rate optimizations

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- Consistent replacement of inflow, outflow, and pump tubing every four weeks or equivalent usage, in combination with a more rigorous post-experiment rinsing protocol (involving warm purified & deionised water and 70% ethanol), reduces the probability of unwanted compounds (dissolved salts, test compounds, bacterial & fungal growths) coming into contact with the test slice and potentially altering the observed results.
- Reduction of ACSF flow rates to try and minimize the formation of bubbles on the slice anchor, an issue that occurred particularly following the prolonged recording durations and can result in the anchor being lifted and contact between recording slice and recording electrode being compromised. This issue was also addressed by extending the aluminium inflow cannula using perfluroalkoxy alkane (PFA) tubing (reducing turbulence, and therefore electrical noise) and replacement of the outflow tip with a finer cannula (allowing for better vacuum generation and a more even outflow)
    - Other optimisations to this end were also undertaken:
        - Removal of bubbles with a hypodermic needle
        - Reduction of carbogen saturation

- Modulation of anchor (anchor material, shape, and mesh material altered)
- Pre-heating of ACSF to 32°C (recording temperature) and maintaining this temperature via regular mild warmings throughout the day.

5.3.7.3. System optimizations

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- High levels of noise even from empty chips indicated an issue with an internal functioning of the MEA; as such, the system was sent to the manufacturer for reconditioning. This resolved the issue.
- Regular replacement of chips and ensuring a consistent layout in order to ensure consistency and quality. In this case, chips manufactured by Quane biosciences (in both an 8*8 layout and 5*13 layout) were replaced by 8*8 chips manufactured by multichannel systems (OEM 60MEA200/30iR-T).
- As well as replacement of the chips, spare tungsten-carbide, gold-plated pins were obtained which can be replaced as required should any issues with recording quality be observed.
- Fortnightly cleaning of MEA chips with the detergent 'tergazyme' to ensure removal of tissue detritus from the chip, leading to greater contact between chip and slice improved of signal-to-noise ratio.
- Validation of TC_01 software display temperature using external thermometer

5.3.7.4 Other optimizations

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- Following optimization of the slicing procedure and isolation of individual hippocampi, it became possible to record from two hippocampi on the same chip simultaneously. Through careful placement of each hippocampus, and with each CA1 orientated towards the centre of the MEA chip it became possible to record CA3→CA1 signaling from two slices simultaneously. This allows an increase in the number of data points that can be generated from an individual animal. Combined with the ability to slice multiple brains simultaneously, means that slices from two animals may be recorded under identical conditions, particularly useful for increasing robustness of data following application of compounds to these hippocampal slices. The two-slice method leads to an altered electrical profile on the MEA, however so consistency needs to be maintained within a given study.

5.4. Results

5.4.1: Effects of sodium acetate perfusion on basal synaptic signaling

We initially examined whether sodium acetate has an effect on basal synaptic responses. A standard input-output protocol (increasing stimulus intensities of 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200µA) was run and the application of sodium acetate at either 70µM or 700µM did not yield a statistically significant difference in synaptic response at the electrode 200µm **(Fig. 1A)** ($F_{(24,353)}$=0.05396, P>0.9991) or 400µm **(Fig. 2A)** ($F_{(24,117)}$=0.04071, P>0.9991) from the stimulation site.

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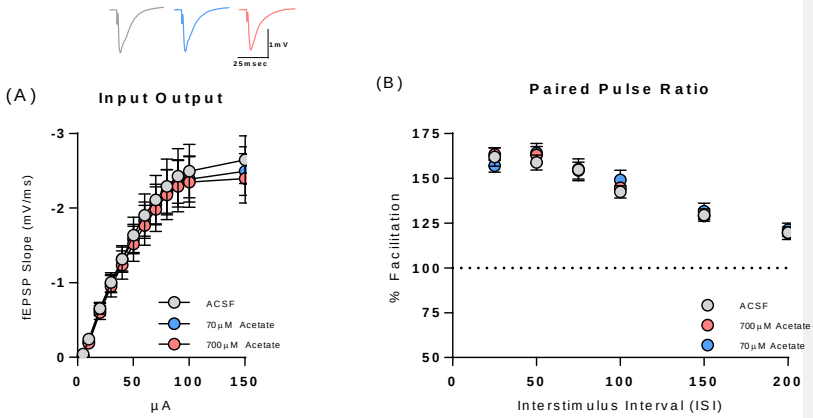

Fig 1: Effects of sodium acetate application on Input-output and Paired pulse ratio 2100µM from the stimulation site. *(A) No alteration was observed in Input-output following application of either concentration of acetate Data expressed as mean ± SEM with two-way ANOVA and Bonferroni post-hoc test for each time point. (B) Paired pulse ratio was also unaffected by the application of sodium acetate. Data expressed as mean ± SEM with one-way ANOVA and Bonferroni post-hoc test for each time point. [ACSF N=9, 70µM N=10, 700µM N=10]. Representative traces are shown above the graphs are from an individual slice included in the data set taken at half the maximum stimulus intensity.*

Similarly, we examined the paired pulse ratio in each of these slices as a measure of short-term plasticity. Again, application of acetate at either concentration (70µM or 700µM) did not alter the paired-pulse ratio at either the 2100µM **(Fig. 1B)** ($F_{(2,15)}$=0.097, P=0.9848) or 4200µM **(Fig. 2B)** ($F_{(2,15)}$=0.1317, P=0.8776) distance from the stimulation site.

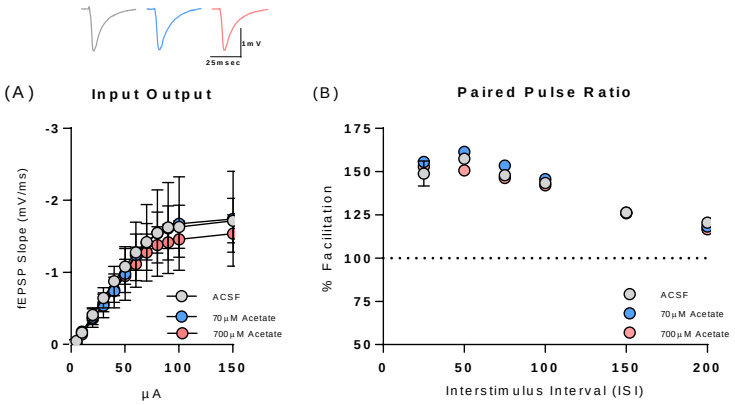

Fig 2: Effects of sodium acetate application on Input-output and Paired pulse ratio 4200µM from the stimulation site. *(A) No alteration was observed in Input-output following application of either concentration of acetate Data expressed as mean ± SEM with two-way ANOVA and Bonferroni post-hoc test for each time point. (B) Paired pulse ratio was also unaffected by the application of sodium acetate. Data expressed as mean ± SEM with one-way ANOVA and Bonferroni post-hoc test for each time point. [ACSF N=7, 70µM N=9, 700µM N=9]. Representative traces are shown above the graphs are from an individual slice included in the data set taken at half the maximum stimulus intensity.*

5.4.2: Effects of sodium propionate on basal synaptic signaling

The same tests were undertaken in the presence of four different concentrations of the SCFA sodium propionate. Again, we observed here that that no differences were present in the basal synaptic response measured using a standard input-output protocol compared to control in the presence of either 0.5µM, 5µM, or 50µM propionate. This was the case both at the recording electrode 2100µM from the stimulation site **(Fig. 3A)** ($F_{(36,104)}$=1.071, P=0.3833) or the electrode 4200µM from the recording site **(Fig. 4A)** ($F_{(36,104)}$=0.1653, P>0.9991).

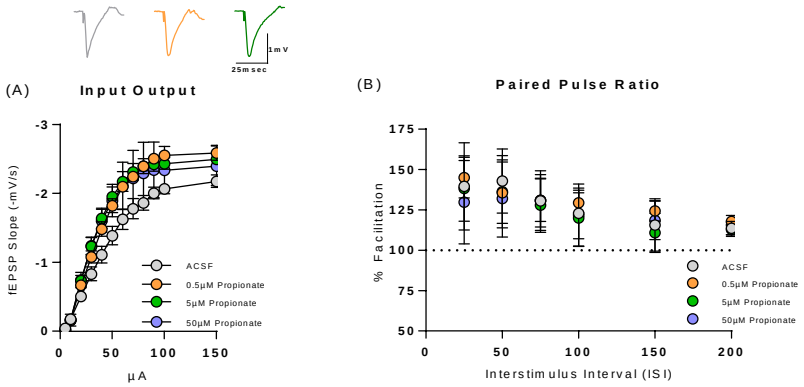

Fig 3: Effects of sodium propionate application on input-output and Paired pulse ratio 2~~1~~00µM from the stimulation site. *(A) No alteration was observed in Input-output following application of any of the propionate concentrations tested. Data expressed as mean ± SEM with two-way ANOVA and Bonferroni post-hoc test for each time point. (B) Paired pulse ratio was also unaffected by the application of sodium propionate. Data expressed as mean ± SEM with one-way ANOVA and Bonferroni post-hoc test for each time point. [ACSF N=9, 0.5µM N=9, 5µM N=9, 50µM N=9]. Representative traces are shown above the graphs are from an individual slice included in the data set taken at half the maximum stimulus intensity.*

Again, the paired pulse ratio was also measured in each of the concentrations used in the experiment. Similar to what was observed following application of acetate, we found that there were no changes in the paired pulse ratio following propionate perfusion at either the 200µm **(Fig. 3B)** ($F_{(3,20)=}$1.184, P=0.7041) or the 400µm **(Fig. 4B)** ($F_{(3,20)=}$0.4022 P=0.9878) recording sites.

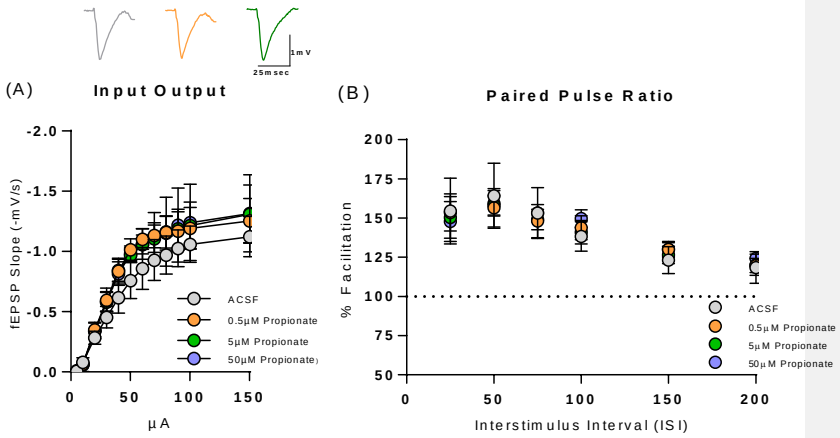

Fig 4: Effects of sodium propionate application on Input-output and Paired pulse ratio 42~~00~~µM from the stimulation site. *(A) No alteration was observed in Input-output following application of any of the propionate concentrations tested. Data expressed as mean ± SEM with two-way ANOVA and Bonferroni post-hoc test for each time point. (B) Paired pulse ratio was also unaffected by the application of sodium propionate. Data expressed as mean ± SEM with one-way ANOVA and Bonferroni post-hoc test for each time point. . [ACSF N=9, 0.5µM N=9, 5µM N=9, 50µM N=9] Representative traces are shown above the graphs are from an individual slice included in the data set at half the maximum stimulus intensity.*

5.4.3: Effects of Short-chain fatty acids on Long-term synaptic signaling.

Prior to the assessment of long-term potentiation, we ran the previous tests (input-output and paired-pulse ratio) again, in order to determine that there were no differences in signaling between the slices before stimulation. This ensures that all slices are performing equivalently and that any changes in potentiation are due to application of the test compounds and not due to differences in slice performance or slice health. No differences were observed between any of the groups (data not shown).

When the effects of LTP were measured in the presence of acetate differences were observed in levels of LTP between treatment groups. This was seen at both electrode distances. At the 200µM electrode differences were observed **(Fig. 5)** ($F_{(5,52)}$=0.2855, P<0.01) with post hoc-analysis revealing that there was a statistically significant increase in signalling in the ACSF group (P<0.01) and the 70µM group (P<0.01) following stimulation, but that no difference was seen in the 700µM group. Similarly, differences were also observed at the 400µM electrode **(Fig. 6)** ($F_{(3,20)}$=0.4022 P=0.9878). Once again, a post-hoc analysis revealing that there was a significant increase signal strength following stimulation in the ACSF group (P<0.001) and the 70µM group only.~~e,~~

~~it was observed that application of sodium acetate at the higher concentration (700µM) resulted in a reduction of LTP compared to control and the lower concentration. This effect was observed whether recordings were made at a distance of 100µM **(Fig. 5)** ($F_{(5,52)}$=0.2855, P<0.01), or 200µM **(Fig. 6)** ($F_{(3,20)}$=0.4022 P=0.9878) from the stimulation site.~~

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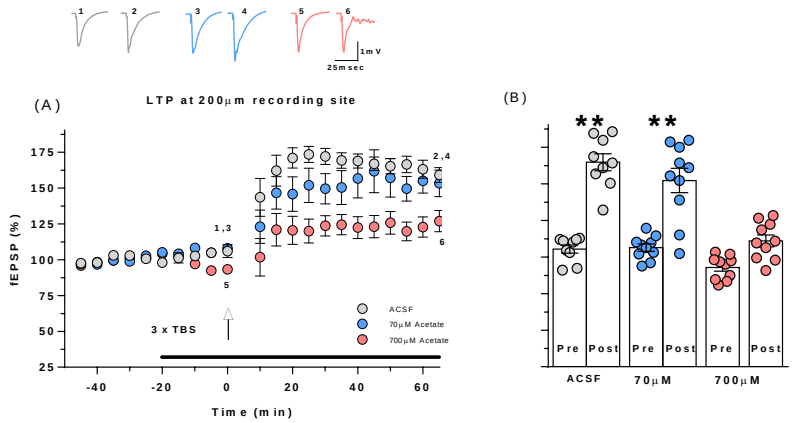

Fig 5: Effects of acetate on Long-term potentiation ~~2~~400µM from the stimulation site. *(A) LTP was induced following TBS in control and 70µM acetate, however, this effect was attenuated in the 700µM group. Data presented as mean ± SEM, analysed using a two-way ANOVA with Bonferroni post-hoc (B) Summary of the data presented in (A) measured in the 10 minutes prior to stimulation (-10 – 0mins) and 10 mins prior to one hour post-stimulation (+55 - +65mins). Data presented as mean ± SEM, analysed using a one-way ANOVA with Bonferroni post-hoc test. **p<0.01 [control N=9, 70µM N=10, 700µM N=10].. Representative traces are presented above the graphs, taken from one slice within each group and measured at a time represented by the corresponding number on the LTP graph.*

While the effects are smaller when measured at the ~~2~~400µM recording site, there remains a reduction in LTP following bath application of 700µM sodium acetate **(fig. 6)**

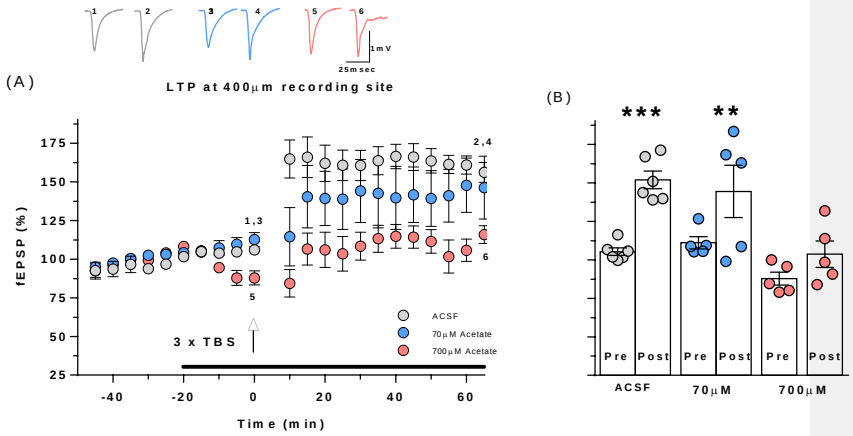

Fig 6: Effects of acetate on Long-term potentiation ~~4~~2~~1~~00µM from the stimulation site. *(A) LTP was induced following TBS in control and 70µM acetate; however, this effect was attenuated in the 700µM group. Data presented as mean ± SEM, analysed using a two-way ANOVA with Bonferroni post-hoc (B) Summary of the data presented in (A) measured in the 10 minutes prior to stimulation (-10 – 0mins) and 10 mins prior to one hour post-stimulation (+55 - +65mins). Data presented as mean ± SEM, analysed using a one-way ANOVA with Bonferroni post-hoc test. **p<0.01. ***p<0.001. [control N=6, 70µM N=5, 700µM N=5]. Representative traces are presented above the graphs, taken from one slice within each group and measured at a time represented by the corresponding number on the LTP graph.*

The effects of sodium propionate on long-term potentiation were also assessed. Once again, baseline measurements were made of the slices used in order to ensure that observed differences were due to the application of the compounds being tested. No differences were observed in input-output or paired-pulse ratios between the groups (data not shown). The effects of two concentrations of propionate on LTP were measured (0.5µM and 5µM); however, no differences were observed following the application of either concentration of the compound.

When response was measured ~~This was evident at both the~~ 2~~1~~00µM ~~recording~~ from the stimulation site **(Fig. 7)** differences were observed after high-frequency ~~stilulation~~stimulation ($F_{(5,22)}$=2.842 P=0.0397). Post-hoc analysis revealed that this was the case in all groups: ACSF (P<0.01), 0.5µM (P<0.05) and 5µM (P<0.01).~~,~~ Similarly, differences were seen at~~and~~ the 4~~2~~00µM recording site **(Fig. 8)** ($F_{(5,28)}$=2.342P=0.0028). Again post-hoc analysis indicating a significant increase in fEPEP size following high-frequency stimulation in all groups: ACSF (P<0.05), 0.5µM (P<0.05) and 5µM (P<0.05).~~)~~.

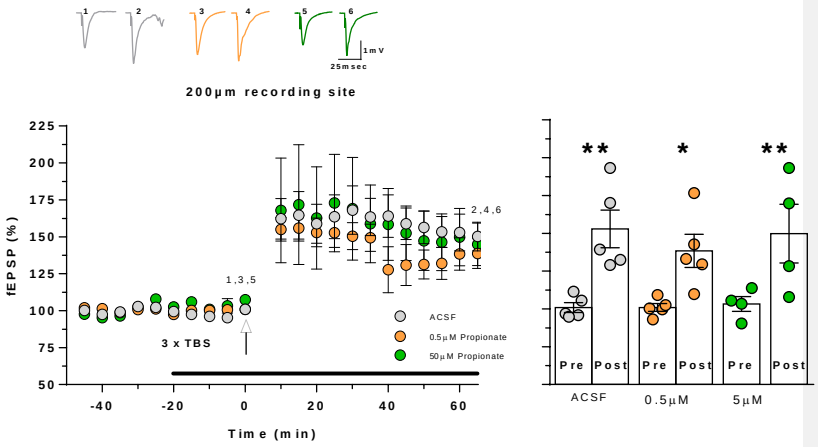

Fig 7: Effects of propionate on Long-term potentiation ~~2~~1~~0~~0µM from the stimulation site. *(A) LTP was induced following TBS in all groups. Data presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni post-hoc test (B) Summary of the data presented in (A) measured in the 10 minutes prior to stimulation (-10 – 0mins) and 10 mins*

*prior to one hour post-stimulation (+55 - +65mins). Data presented as mean ± SEM, analysed using a one-way ANOVA with Bonferroni post-hoc test. *p<0.05, **p<0.01 . [control N=5, 0.5µM N=5, 5µM N=4]. Representative traces are presented above the graphs, taken from one slice within each group and measured at a time represented by the corresponding number on the LTP graph.*

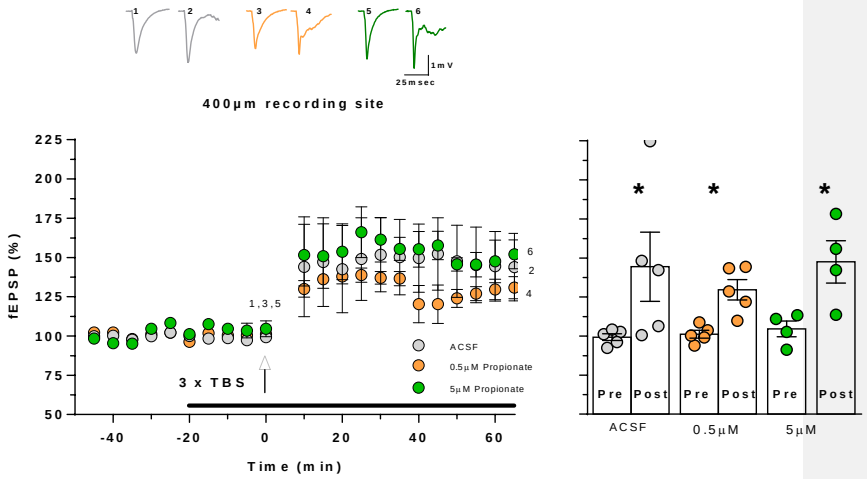

Fig 8: Effects of propionate on Long-term potentiation 400µM from the stimulation site. . *(A) LTP was induced following TBS in all groups. Data presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni post-hoc test (B) Summary of the data presented in (A) measured in the 10 minutes prior to stimulation (-10 – 0mins) and 10 mins prior to one hour post-stimulation (+55 - +65mins). Data presented as mean ± SEM, analysed using a one-way ANOVA with Bonferroni post-hoc test. *p<0.05. [control N=5, 0.5µM N=5, 5µM N=4]. Representative traces are presented above the graphs, taken from one slice within each group and measured at a time represented by the corresponding number on the LTP graph.*

5.5. Discussion

The importance of the microbiota to the learning and memory has been the subject numerous behavioural studies in rodents. Germ-free mice, for example display memory deficits in the novel object recognition test (Gareau et al., 2011; Luk et al., 2018). Disruption of the microbiota following antibiotic administration has also been shown to lead to memory deficits in the same behavioural assessment (Desbonnet et al., 2015a; Guida et al., 2018b; Mohle et al., 2016).

The underlying changes that occur in the brain, and in particular within the hippocampus, have only recently begun to be addressed, with links made between probiotic supplementation and expression of LTP in the hippocampus (Davari et al., 2013; Distrutti et al., 2014a). Here, we aimed to build on these studies through assessing the impact that

exposure to SCFAs, a key metabolite of the gut microbiota, has on synaptic signaling in the hippocampus using a modern electrophysiological testing tool, the MEA. We assessed the effects of exposure to sodium acetate and sodium propionate and looked to tease apart where any potential alterations may be occurring through assessment of different aspects of synaptic plasticity.

Initially, we observed that none of the concentrations of the compounds tested altered basal synaptic signaling when measured through assessment of the input-output response. Similarly, no differences were observed in the paired pulse ratio. This result suggests that any observed alterations in plasticity will be generated postsynaptically, as the PPR elucidates the origin of synaptic plasticity alterations (Shaban et al., 2017b). For example, no change in PPR in combination with an alteration in LTP suggests that postsynaptic mechanisms are at play for observed effects.

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Following assessment of LTP, we observed that levels of LTP similar to control animals were observed following perfusion with the lower concentration (70µM) of acetate. The higher concentration (700 µM) however, inhibited LTP induction. No differences were observed following perfusion with acetate, both of the concentrations tested resulted in the formation of LTP.

The loss of LTP in the high concentration (700µM) acetate group is a noteworthy finding. While we believe this concentration to be super-physiological, it is not thought to be toxic, as reported in a recent study (Yang et al., 2019). Here, the researchers exposed human neural progenitor cells (hNPCs) to increasing concentrations of SCFAs. Initially it was found that physiologically relevant levels (µM) of SCFAs increased the growth rate of hNPCs significantly and induced more cells to undergo mitosis. Furthermore, only very high (mM) concentrations of SCFAs led to increases in toxicity (48mM for acetate & 12.8mM for propionate) (Yang et al., 2019), all of which are significantly higher than any concentrations used in our study. The much lower concentrations of propionate measured in this study are reflected in the lower concentrations used in our assessment. This is due to the fact that concentrations of propionate in the circulation are significantly lower than those of acetate (Yang et al., 2019).

Rather than having an effect on the viability of neurons at this higher concentration, we believe that alterations in LTP may be due to a local ion channel effect. This is due to the fact that the observed effects on potentiation are immediate meaning that the activation of cellular signaling processes or transcription factors which are likely to play a role in late-stage LTP (Bozon et al., 2003; Nguyen et al., 1994) are unlikely to be responsible. They may be an

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important factor in longer-term changes, however. The recording of synaptic response over a more prolonged period is an important addition that could be made to future experiments with the aim of establishing the impact on synaptic signaling later in the LTP process.

The effects on longer-term aspects of synaptic signaling (over a period of 3-5 hours or longer) may, however, play an important role beyond the observed changes in our experiment. Acetate (Soliman and Rosenberger, 2011), propionate, and butyrate (Silva et al., 2018) are known to behave as HDAC inhibitors, and through this activity may have a role to play in long-term memory formation (Abraham et al., 2019). Following the release of neurotransmitters from the presynaptic neuron and activation of postsynaptic NMDA receptors there is an influx of calcium into the postsynaptic neuron and subsequent activation of downstream signaling pathways, which converge on the ERK (extracellular receptor kinase) cascade (Adams and Sweatt, 2002). ERK is central to a number of functions relevant to enhancing memory formation, these include regulation of a number of transcription factors, including CREB and Elk-1 (Davis et al., 2000). It has been shown that within the central nervous system, alteration of chromatin structure enables more robust changes in gene expression via these pathways (Guan et al., 2002). Chromatin structure can be effectively targeted via histones, which when acetylated lead to changes in chromatin structure (Lalonde et al., 2014), hence the potentially significant role of SCFAs in memory. Indeed exposure of hippocampal slices to 300µM butyrate for 40 minutes has been shown to lead to elevated levels of histones (Levenson et al., 2004b). Furthermore, this exposure has also been shown to lead to enhancement of LTP (Levenson et al., 2004b).

Whether the hippocampus is exposed to these levels of SCFA *in vivo* in order to exert direct effects on the brain is currently unknown. It is believed that SCFAs may be able to interact with brain physiology through direct interactions. Acetate is known to be present in cerebrospinal fluid (Nagashima et al., 2010); furthermore, gut-microbial derived acetate has been shown to reach the brain and trigger peripheral nervous system signaling (Perry et al., 2016). Acetate that reaches the hypothalamus can cause appetite suppression through a direct effect on neuroglial cycles (Frost et al., 2014b).

Considering the relatively low levels of propionate and butyrate in circulation, whether these SCFAs can directly impact the brain is still very much uncertain. However, it is important to note that a propionate concentration as low as 1µM can affect the blood-brain barrier, indicating that physiologically relevant levels of SCFAs could potentially impact the brain (Hoyles et al., 2018), albeit via different mechanisms to those explored here. There is

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considerable scope for research into the potential for these compounds to reach the brain in sufficient concentrations to exert a significant impact on synaptic signaling.

Antibiotic administration is another method of microbiota modulation that may be important in determining the role of SCFAs in synaptic signaling. Antibiotic administration has been shown to decreases in levels hippocampal BDNF, a key regulator of hippocampal synaptic plasticity (Leal et al., 2017), while also altering spiking in the CA3 of the hippocampus when electrophysiological measurements were made in the region (Guida et al., 2018b). Similarly, administration of a different antibiotic cocktail was linked to reduced performance in a task of hippocampal-dependent memory while also reducing levels of hippocampal BDNF (Fröhlich et al., 2016). Interestingly, in this study, these changes were linked to a significant reduction in the concentration of the SCFAs acetate, propionate and butyrate in the colon (Fröhlich et al., 2016). Also, supplementation with butyrate has been shown to enable hippocampal-dependent memory in conditions that are normally sub-threshold for memory formation, with these effects being dependent on upregulation of hippocampal BDNF (Intlekofer et al., 2013). While these studies highlight the positive effects of these microbial metabolites and their links to expression of neurotrophic factors, they do not make explicit links to synaptic signaling. But they do suggest that future studies investigating the effect of SCFAs on hippocampal signaling may benefit from the addition of electrophysiological recordings of LTP.

Supplementation of animals with SCFAs as potential treatment is another investigative approach that may be utilised in future MEA studies. Supplementation with a mixture of SCFAs has shown, for example, to improve stress response, anxiety-like behaviour and antidepressant-sensitive behaviours (van de Wouw et al., 2018a). Measurement of SCFA concentrations in different brain tissues as well assessing electrophysiological alterations following this supplementation would be hugely informative to the field.

The results of this research are an attempt to provide a springboard in order to make analysis of electrical signaling a cornerstone of future studies of the microbiota-gut-brain axis. Gradual modification of the setup has led to increased reliability and a significantly improved signal-to-noise ratio. As such the results presented can be regarded as replicable and reliable. We can say with confidence that short-chain fatty acids can influence synaptic signaling within the hippocampus. Furthermore, these studies will provide the basis for assessment of the effects of SCFAs on synaptic signaling in hypothalamus for example, a region that is known to be exposed to circulating propionate (Frost et al., 2014c). In addition to these

studies, the MEA platform is well positioned to add an additional level of screening for future interventional studies (prebiotics, probiotics, FMT, antibiotics, etc.) of the microbiota-gut-brain axis (Shaban et al., 2017b), as well as models of neurological and behavioural alterations (e.g. the BTBR mouse model) attempting to shed light on a potential mechanism involved in behavioural and neurochemical changes that may be observed.

Chapter 6

General discussion

6.1. Summary of findings

In this thesis we have explored the potential for alterations in gut microbiota to affect brain and behaviour throughout the lifespan, with an interest in synaptic plasticity as a mechanism that we believe may become a cornerstone of the field in the future. Mode of delivery, antibiotic administration and psychobiotic treatment were utilised to modulate the gut microbiota. Their effects on the gut-brain axis were measured throughout the lifespan via behavioural, neurochemical, physiological and immunological assays. The data generated in this thesis highlight the importance of early life in shaping the development of the gut-brain axis, and the major implications for mental health through the lifespan.

The widespread use of antibiotics throughout life is a cornerstone of modern medicine, with global rates increasing by 65% between 2000 and 20015 (Klein et al., 2018). With this trend projected to continue there is an increased risk, not only of antibiotic resistance, but also of negative effects following disruption to a healthy microbiota (Desbonnet et al., 2015b). Established links between the gut microbiota, the brain, and behaviour (Cryan and Dinan, 2012a) have led to the widespread use of antibiotics as research tools in preclinical models. This preclinical use is subject to significant variation however, in terms of age of administration, specific antibiotics administered, duration of administration and route of administration (Desbonnet et al., 2015b; Leclercq et al., 2017a; Russell et al., 2012). We compared the effects of maternal administration of a single penicillin antibiotic to a cocktail of antibiotics. Early life was chosen due to its clinical relevance based on guidelines on maternal use during this period (Cunningham GF, 2005) as well as the importance of this time to development of brain and behaviour (Sheridan and McLaughlin, 2014). Furthermore, with the only difference between the groups being the antibiotics administered we were able to compare the effects of different levels of microbiota disruption on behaviour throughout the lifespan.

In early life, administration of the antibiotic cocktail led to significant disruptions in social behaviour in the homing test. In adulthood, both antibiotic interventions led to significant increases in anxiety-like behaviour. The penicillin antibiotic had a greater effect on social behaviour, while there was a more significant impact of the antibiotic cocktail on learning and memory behaviours in adulthood. We also hypothesised that alterations in the expression of synaptic plasticity genes in the hippocampus may be a contributing factor to these behavioural changes, observing that maternal penicillin led to a decrease in BDNF in offspring in adulthood. The results demonstrate the importance of microbiota composition

during early life and the potential knock on effects on behaviour later in life. The differences between the groups also highlights the specificity of effects that are present depending on the administered antibiotics. This is a factor that should be of particular note in the design of future preclinical antibiotic studies.

In order to explore the effects of early-life alterations in microbiota composition following birth via C-section, we utilised a model of murine C-section delivery that has previously been developed in our lab (Morais, 2018). It was observed that birth via C-section led to adverse outcomes in anxiety-like behaviour, cognitive behaviour, sociability, and antidepressant sensitive behaviour. This is very much in line with what has previously been observed (Morais, 2018).

In order to build on this existing data, we further exacerbated the microbiota disruption through maternal administration of a penicillin antibiotic. This led to additional disruption in anxiety-like behaviour, sociability and antidepressant-sensitive behaviours. These effects have previously been observed following antibiotic administration in the perinatal period (Leclercq et al., 2017a), adolescence (Desbonnet et al., 2015b) and adulthood (Bercik et al., 2011a; Guida et al., 2018b). That they can exacerbate the effects of C-section delivery is a striking novel finding with translational relevance given the widespread use of maternal antibiotics following C-section delivery (Chaim and Burstein, 2003).

The potential for psychobiotic administration from weaning was then explored in both of these groups, with some positive effects being observed, particularly in social and antidepressant-sensitive behaviours. Improvements in social behaviour have been observed preclinically following both prebiotic (Burokas et al., 2017) and probiotic (Bharwani et al., 2017) administration, while probiotic administration has shown benefits in antidepressant-sensitive behaviour (Desbonnet et al., 2010b; Dhaliwal et al., 2018b; Liang et al., 2015). Again, the beneficial effects of these treatments following C-section delivery and with additional maternal antibiotic administration makes a striking novel finding with significant translational relevance. When gut microbiota composition was assessed in adulthood no differences were observed between vaginally born and C-section delivered animals in either alpha or beta diversity. Numerous differences were seen between the other groups in each of these measures however, suggesting that the behavioural differences between the groups may be linked to microbiota differences during this critical time for neurodevelopment (Anand and Scalzo, 2000). Assessment of microbiota composition during this key stage will provide important evidence in future studies.

We also sought to explore behavioural and immune differences in aging using a rodent model of autism spectrum disorder. The BTBR mouse model is known to have an altered gut microbiota composition (Golubeva et al., 2017). We compared behaviour in these animals to similarly aged C57BL/6 mice, observing that the majority of behavioural differences between the two groups in young animals are maintained into adulthood (repetitive behaviours, antidepressant-sensitive behaviours, social deficits & cognition). There are more nuanced effects in terms of anxiety in older animals of the BTBR strain compared to C57bl/6 controls. Furthermore, BTBR animals also exhibit an activated T-cell system. As such, these results represent confirmation that ASD-associated behavioural deficits are maintained in ageing, and that that there may be need for differential interventional approaches to counter these impairments, potentially through targeting the immune system.

Finally we utilised a new technology , the multi electrode array, that we hope to integrate further into future studies of the microbiota, to determine the potential effects of common microbiota metabolites on hippocampal synaptic signalling. It was observed that perfusion of the slice with a high concentration (700μM) of acteate led to deficits in the generation of hippocampal LTP, while propionate was not seen to alter LTP expression in any of the doses tested.

Taken together, these data support the microbiota-gut-brain axis as a therapeutic target, throughout the lifespan. Interventions that can disrupt microbiota composition should be administered with caution in early life, and only when the potential benefits outweigh the potential risks. The use of psychobiotics has the potential to restore some of the instigated deficits, however. We also suggest potential links between the gut microbiota, its metabolites, and synaptic plasticity within the hippocampus. This is an avenue that should be explored in greater detail in future studies that attempt to tease apart the mechanisms underpinning the changes seen in the microbiota-gut-brain axis.

6.2: Disruption of the microbiota in early life – lasting effects on the microbiota-gut-brain axis.

The influence of early life microbiota composition over physiology has been known for some time. Clinically, early-life antibiotic use has been linked to the development of asthma (Kozyrskyj et al., 2007; Semic-Jusufagic et al., 2014) and childhood obesity (Cox and Blaser, 2015b; Trasande et al., 2013), with studies in preclinical models showing similar effects (Cox et al., 2014; Russell et al., 2012).

Clinically, the effects of microbiota composition in early life on the function of brain and behaviour have remained somewhat unclear, perhaps due to the multitude of other genetic and environmental influences that make the individual components difficult to tease apart. Preclinically, however, evidence has emerged demonstrating that being born without a microbiota can profoundly influence behaviour and stress response (Sudo et al., 2004b). Furthermore, it was shown that reversal of these effects through microbiota reconstitution could only occur if performed during a critical window (Sudo et al., 2004b). This result highlighted the importance of microbiota composition in early life and provided the springboard for the subsequent explosion of research into the microbiota-gut-brain axis (Cryan et al., 2019a). Here, we attempted to add to this body of research with a particular focus on the role of early-life microbiota composition on the development of the microbiota-gut-brain axis.

6.2.1. Bugs at birth, the brain & behaviour: The effect of C-section delivery through the lifespan

Caesarean section delivery is an essential obstetric procedure, that can be lifesaving for both mother and child. Globally, rates of elective C-section have increased rapidly in recent years (Boerma et al., 2018). These elective procedures bring added risk, a result of complications of surgery, as well as C-section birth being linked to a variety of immune and metabolic disorders in early life including coeliac disease (Decker et al., 2010; Marild et al., 2012), hypertension (Li et al., 2013), type 1 diabetes (Cardwell et al., 2008) and obesity (Li et al., 2013).

Mode of delivery has also been shown to play a key role in early-life microbiota composition. Vaginally delivered babies have a microbiota similar to that of the vaginal microbiota, while C-section babies have microbiota more similar to that of their mother's skin (Dominguez-Bello et al., 2010a). Here we do not observe any differences between groups based on mode of delivery in any of the analyses undertaken (alpha or beta diversity). While this initially appears surprising, it is made less so, when taking into account the fact that measurements are made in adulthood (approx. 12 weeks old). Previously, when murine gut microbiota differences were compared between vaginally and c-section born animals differences were observed up to six weeks of age (Martinez et al., 2017). When separated by sex however, differences were no longer present in males at six weeks of age while still being observed in

females (Martinez et al., 2017). This suggests a possible reason for the lack of divergence in our study. Only male mice were used and microbiota analyses were only made far into adulthood.

When behavioural tests were assessed between the two groups in adulthood birth via C-section was associated with significant increases in anxiety-like behaviour, worsening of learning and memory behaviours, disruptions in social recognition and increases in in antidepressant-sensitive behaviours, suggesting long-term effects of alterations in gut microbiota composition in adulthood.

In early life, alterations in vocalizations following maternal separation have been noted between C-section and vaginally delivered animals (Castillo-Ruiz et al., 2018b). Here we show that these alterations in early-life social development are maintained to adulthood. Furthermore, alterations in anxiety-like behaviour, social recognition, cognition and stress response have been observed in adult rodents following C-section delivery (Morais, 2018).

When effects on cognition, in particular, are assessed there may be additional mechanisms at play, whose links to the microbiota are unclear. Dendritic arborization in hippocampal pyramidal neurons is underdeveloped at birth in CS mice compared to vaginally born (at P0.5, though this effect is no longer present at P1.5) (Chiesa et al., 2018). Additionally, uncoupling protein 2 (UCP2) which regulates neuroprotection and synaptogenesis in the adult brain, is downregulated in the hippocampus of CS born mice compared to naturally born controls, potentially leading to changes in plasticity in later life (Seli and Horvath, 2013; Simon-Areces et al., 2012).

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These data highlight the impact that birth via caesarean section can have on brain and behaviour throughout the lifespan. They also provide the basis for future studies where additional focus should be placed on the sex differences as well as regular microbiota analysis throughout the lifespan to determine how variations are expressed at different timepoints. Investigations may also be made into the mechanisms underlying behavioural changes. Are levels of UCP2 linked to mode of delivery in these animals? How does this relate to behavioural changes? Do alterations in cognition correspond to electrophysiological alterations?

Furthermore, these studies raise interesting translational questions regarding how such potential effects may be avoided. One suggestion is the exposure of c-section born neonates to vaginal microbes via 'seeding' through exposure to vaginal fluids (Dominguez-Bello et al.,

2016) with a partial restoration of microbiota composition having been observed. Some caution must be advised with this technique as it also increases the potential for transfer of pathogens. Larger trials are currently underway that aim to further elucidate the benefits and pitfalls of the technique (Reardon, 2019).

Other methods to reduce any potential adverse effects of C-section delivery include limiting the procedure to cases where it is medically necessary and promoting breast feeding wherever possible due to its positive impact on microbiota composition (Mueller et al., 2015). This is a field of research in its infancy however, and more trials both clinical and preclinical are required to tackle the many questions that remain surrounding the long-term effects of C-section delivery on brain and behaviour.

6.2.2. Early-life antibiotic exposure: A necessary evil?

As mentioned, the presence and composition of the gut microbiota in early life plays a key role in stress response (Sudo et al., 2004b), behaviour (Morais, 2018) and neuronal development (Chiesa et al., 2018). In addition to colonization with commensal bacteria during this critical period, neonates are also at risk of exposure to pathogens. The ability to mount an immune response to these pathogens is impaired during this early stage of life, which can lead to significant mortality, particularly in developing countries (Osrin et al., 2004). In such cases, treatment with antibiotics is a lifesaving measure. Even in situations where the new born is able to mount an immune response, there can be long-term consequences of infection including effects on cognition and microglial response later in life (Bilbo and Schwarz, 2009), and as such prompt treatment with antibiotics may be able to limit such adverse effects.

In addition to direct administration of antibiotics to the neonate, it is often necessary to administer antibiotics to the mother following birth to prevent maternal postpartum infection that may occur as a result of trauma sustained during delivery, post-surgical complications following C-section, or physiological changes that occur during pregnancy (Cunningham GF, 2005). A wide variety of antibiotics are used in such cases (Chaim and Burstein, 2003) and mothers are recommended to continue breastfeeding during their treatment (Amir and Academy of Breastfeeding Medicine Protocol, 2014). The antibiotics used are considered safe for nursing infants with no reported adverse effects on the neonate being observed during early life (2001; Kaiser et al., 2007). Monitoring of adverse events in the offspring following maternal antibiotic administration is generally confined to the duration of treatment and a short period thereafter, neglecting to account for any potential

long-term consequences (although links have been made between maternal antibiotic use in early life and development of asthma later in life (Stokholm et al., 2014)).

Recently, preclinical data has shown that perinatal antibiotic administration can lead to alterations in anxiety and social behaviours in adulthood, along with an increase in aggressive behaviours (Leclercq et al., 2017a). Furthermore, changes in cytokine expression in the frontal cortex and blood brain barrier permeability were affected (Leclercq et al., 2017a) highlighting the ability of antibiotic exposure during this critical window to influence both brain and behaviour.

We aimed to build on existing data to determine whether maternal antibiotic treatment can cause effects on brain and behaviour in offspring. Initially, comparisons were made between administration of a single penicillin antibiotic (penicillin V) and a cocktail of antibiotics to the mother in order to determine whether a more comprehensive cocktail will lead to greater alterations in brain and behaviour.

When behaviour was assessed in early life, the group exposed to the maternal antibiotic cocktail displayed reductions in attachment to maternal bedding. As this test is linked to development of social behaviours (Brennan and Kendrick, 2006), we were interested to know if such behaviours would continue to later life.

In adulthood, both antibiotic groups displayed increased anxiety-like behaviour compared to control animals. Interestingly, the penicillin-treated group exhibited more disrupted behaviour in tests of sociability, while the groups receiving the antibiotic cocktail were more affected in tests of learning and memory. Furthermore, reductions in hippocampal BDNF were see in the penicillin V-treated group that suggest that alterations in hippocampal function may play a role. None of the other plasticity-related genes were affected, however. While studies have observed alterations in hippocampal BDNF following the administration of an antibiotic cocktail (Bercik et al., 2011a; Frohlich et al., 2016), antibiotic treatment failed to affect levels of GRIN2B, however (Frohlich et al., 2016). These results mirror the observations in our study. The variations in makeup of the cocktails as well as duration and age of administration in these studies mean that this is an area that demands more research in order to suggest definitive effects of antibiotic treatment on expression of plasticity-related genes.

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In addition to comparison of the effects of maternal administration of different antibiotics, we also gauged the impact of maternal antibiotic administration following c-section delivery.

This addition adds a high degree of translational relevance to the research as clinical guidelines recommend maternal antibiotic administration as best practice following C-section delivery (2018) in order to prevent maternal infection at the site of incision.

Since gut microbiota composition between vaginally born and C-section delivered mice differ at P9 (Morais, 2018), we theorised that the addition of maternal antibiotics to these animals in the week leading up to this point would lead to further disruption of the microbiota that may exacerbate behavioural alterations.

Anxiety-like behaviour in C-section born animals was amplified following the addition of early-life maternal antibiotics. While disruptions in social recognition were restored to baseline following synbiotic administration (which will be discussed further in the subsequent section), disruptions could not be reversed by synbiotic treatment when animals had received antibiotics, indicating an exacerbation of mode of delivery effects following antibiotic administration. Significant increases in antidepressant-sensitive behaviour and stress response were also observed following the addition of maternal antibiotic treatment to C-section born animals. In addition to these behavioural alterations, significant disruptions in microbiota were observed in C-section born animals who received antibiotics to those who did not. Both the Shannon index of alpha-diversity as well as overall beta diversity differed between the groups.

These results highlight the significant impact that early-life antibiotic administration can have on brain and behaviour, with anxiety-like behaviours and social behaviours both significantly affected in both studies in which penicillin antibiotics were administered. Of particular note is the fact that both of these behaviours were altered in the previous study of perinatal administration of a penicillin antibiotic (Leclercq et al., 2017a). That maternal penicillin administration led to microbiota disruption both in their study (Leclercq et al., 2017a) as well as in our C-section animals suggests that microbiota composition may have a significant role to play.

That the antibiotic cocktail had more robust effects on tests of cognition is of note. In a study that aimed to determine the effects of administration of an antibiotic cocktail on cognition, they observed significant effects of the cocktail on behaviour in the novel object recognition test (Fröhlich et al., 2016) similar to our study. These changes in behaviour were strongly linked to alterations in levels of SCFAs, with acetate, propionate and butyrate all significantly reduced. Levels of these compounds may therefore play a key role in these behaviours and

interestingly, supplementation with the SCFA butyrate has been shown to reverse deficits in the novel object recognition test in a mouse model of autism (Takuma et al., 2014).

Future studies seeking to investigate links between maternal antibiotic administration and the gut-brain axis should pay greater attention to the effects that the specific protocol that is chosen may have on results. In our study we show that even when two different antibiotic cocktails are administered under identical conditions, effects on behaviour later in life are different. Regular monitoring of gut microbiota composition throughout the experiment to see how alterations in bacterial composition or the presence of bacterial metabolites would also aid these studies greatly.

Furthermore, within studies of maternal antibiotic administration more details should be sought with regards to the specific antibiotic exposure of the offspring. It is known that penicillin V enters the breast milk following administration (Leclercq et al., 2017a) and it is therefore able to exert a direct effect on microbiota composition of the offspring. Due to its negligible penetration of the blood-brain barrier (Andrew and Miltefosine, 2010) as well as its rapid renal clearance (Andrew and Miltefosine, 2010) accumulation in the serum is negligible and direct effects on the brain should not occur. The same cannot be said for components of the antibiotic cocktail such as metronidazole, however. This antibiotic can both enter the breast milk (Passmore et al., 1988) as well as penetrate the blood-brain barrier (Jokipii et al., 1977). As such, direct effects on the brain cannot be ruled out as a mechanism of action. In order to combat such issues, it may be worthwhile to screen breast milk to determine the concentrations of antibiotics that offspring are exposed to. Additionally, intraperitoneal injection of the same cocktail can aid determination of whether effects of antibiotics are due to effects on the microbiota or due to direct effects of the antibiotics themselves, this has been undertaken successfully in the past (Tochitani et al., 2016).

There is a fine line to tread therefore, when it comes to antibiotic administration in early life. There are certainly situations when its administration is justified and indeed essential. This must be weighed up, however, with the potential risks. In general the age-old mantra of the lowest effective dose for the shortest effective duration makes absolute sense in this scenario.

6.2.3: Early-life – a window of opportunity for microbiota based interventions.

Based on the evidence previously discussed, there are numerous factors that may lead to a disruption of microbiota composition in early life, not least mode of delivery (Dominguez-

Bello et al., 2010a), antibiotic administration (Koch, 2019), maternal antibiotic administration (Arboleya et al., 2015) and even environmental factors such as exposure to pets (Tun et al., 2017). These influences have also been shown to impact gut-brain axis function (Leclercq et al., 2017a; Morais, 2018). As such, the use of microbiota-based interventions during this critical time of development has become an enormous area of interest.

As is often the case, these novel interventions are attempting to replicate what is seen in nature. Breast milk contains human milk oligosaccharides (HMOs), carbohydrate polymers which serve as natural prebiotic compounds (German et al., 2009). HMOs have particular beneficial effect on specific strains of bacteria, most notably *Biffidobacteria infantis* (German et al., 2009). Interestingly, when this bacteria was administered as a probiotic, beneficial effects were observed in tests of cognition (Ceccarelli et al., 2017) as well as in antidepressant-sensitive behaviours (Desbonnet et al., 2010b) highlighting the importance of encouraging its growth by choosing to breastfeed whenever possible.

When new-born rats were supplemented with the HMO 2"-fucosyllactose until weaning, it was observed that the supplemented groups performed significantly better in behavioural tests of learning and memory, as well as displaying significantly greater levels of LTP when behavioural tests were performed at 1 year of age (rodent middle age) (Oliveros et al., 2016a). This highlights the benefits of targeting the microbiota during early life in order to achieve enduring neurobehavioural effects.

The maternal separation model has been used in rodents as a model of depression and generally involves removing pups from their mothers nest for three hours daily from P2-P14 (Vetulani, 2013). When maternally separated rat pups were administered probiotics during the separation period, they exhibited a reduction in HPA axis activity at P20 (Gareau et al., 2007). Interestingly, if the mothers were administered probiotics during the same period instead of pups there was a similar reduction in HPA response in the offspring (Cowan et al., 2016; Cowan and Richardson, 2019). Furthermore, the beneficial effects of administration of probiotics during this period have even been shown to restore behavioural deficits in the offspring of maternally separated animals (Callaghan et al., 2016)

Here, we aimed to assess the effects of microbiota-based interventions (prebiotics, probiotics and synbiotics) administered to two groups; animals born via C-section and animals born via C-section who had been exposed to maternal antibiotics in early life.

When these interventions were administered to animals born via C-section, a number of effects were observed. Treatment with prebiotics or synbiotics were able to reverse social recognition deficits in these animals. Probiotic treatment did not have any beneficial effect, however. In the forced swim test, synbiotic treatment led to a significant reduction in antidepressant-sensitive behaviours, though the other groups had no effect. It is interesting that the treatments containing the prebiotic component had a greater effect on these animals, perhaps suggesting that the species whose growth was encouraged by the prebiotic element played a more significant role in controlling these behaviours than the probiotic strains administered – more detailed analysis of microbiota composition may be able to shed interesting light on this.

In addition to the effects of these compounds on behaviour, there was a significant increase in alpha diversity following synbiotic treatment in all groups, though not with either of the individual components. Beta diversity differences were observed between the C-section and the prebiotic and between C-section and synbiotic groups, but not between CS and the probiotic group, again, this may be linked to the greater beneficial effects on behaviour that were observed in the prebiotic containing groups.

When synbiotic treatment was administered to C-section born animals exposed to early-life antibiotics and behaviours assessed, the most significant effects were observed in antidepressant-sensitive behaviours and in corticosterone response to stress. It is interesting to note that the synbiotic treatment also exerted a significant effect in the non-antibiotic treated C-section animals, suggesting that the specific synbiotic combination used in the study may have particular potential for the treatment of antidepressant-sensitive behaviours.

Synbiotic administration led to a significant increase in all measures of alpha diversity compared to maternal antibiotic treated C-section animals. No differences were seen in beta diversity however, which suggests that while synbiotic treatment has a beneficial effect on diversity within each group, the same strains may be altered in each of the groups.

The current data demonstrates, therefore, that treatments targeting the microbiota in early life may have positive effects on the gut-brain axis throughout the lifespan. With some of the behaviours assessed not becoming apparent until after this critical window, treatment during this period may be more of a prophylactic nature. Some general advice may be possible, however. Breastfeeding has many advantages due to this early prebiotic exposure, in addition to numerous other beneficial effects on the immune system and long-term health

(Jackson and Nazar, 2006). Further probiotic interventions may also be beneficial in situations where there are additional disruptions to the microbiota such as following antibiotic treatment of either mother or offspring, while targeting either group may lead to positive outcomes.

6.3. The Microbiota-gut-brain axis in ageing – the microbiota as a target for novel synolytic therapies.

The protective effects of targeting the microbiota during the early-life mark it out as an excellent target in order to yield beneficial effects across the lifespan. It may also be desirable however, to target the microbiota at other stages in life to slow, or reverse adverse events throughout the lifespan.

Increased longevity and reduced birth rates in western countries have meant an increase in the global aged population (Lutz et al., 2008). With increasing age comes an increased prevalence of disease, as well as an overall deterioration of homeostatic function (Lopez-Otin et al., 2013). In addition to these changes, the relatively stable microbiota composition that is present in adulthood deteriorates in older individuals (Claesson et al., 2011). This marks out the aged population as an excellent target for future microbiota-based therapies.

Recent studies have demonstrated potential beneficial effects of these therapies in an older population. For example, in the SAMP8 mouse model of premature ageing (Flood and E. Morley, 1997), long-term supplementation with *Lactobacillus paracasei* K71 was associated with increased cognitive performance (Corpuz et al., 2018). They also provided a functional link for these changes showing that supplemented animals had enhanced levels of serotonin, BDNF and CREB in the hippocampus, factors that are the hallmarks of sustained neuronal plasticity (Poo, 2001). Furthermore, microbiota-based therapies have shown beneficial effects in preclinical models of diseases associated with ageing such as Alzheimer's (FMT) (Sun et al., 2019) and Parkinson's disease (SCFA supplementation) (Sampson et al., 2016a). Dietary supplementation with omega-3 fatty acids (which are known to increase SCFA production (Costantini et al., 2017)) has been shown to have beneficial effects in a mouse model of Parkinson's disease.

Indeed, administration of microbiota-targeted diets to prevent the age-associated decline of beneficial *Bifidobacterium* has been found to have positive effects on gut microbiota composition and associated health. A recent study found that a 14-week long dietary intervention with prebiotics increased both *Bifidobacterium* and *Akkermansia* in middle-

aged mice (Boehme et al., 2018; Johnson et al., 2011). *Bifidobacterium* species have been shown to be negatively correlated with pro- and anti-inflammatory cytokine levels in humans indicating that modulation of Bifidobacteria may represent a target for reducing the inflammatory response (Ouwehand et al., 2008).

6.3.1. Inflammaging – the immune system and the gut-brain axis in later life.

Altered gut microbiota compositions in ageing have also been proposed to contribute to "inflammaging" (Thevaranjan et al., 2017), the heightened proinflammatory status and decline in adaptive immunity progressively expressed in older age (Franceschi et al., 2000). Inflammaging contributes to the speed of the aging process and may progress the development of age-related diseases (Xia et al., 2016), from neurological disorders like Alzheimer's disease (Giunta et al., 2007) to metabolic and other physical disorders like heart disease, osteoporosis, and type II diabetes (Boren and Gershwin, 2004; Franceschi et al., 2001; Lencel and Magne, 2011). Both aging and stress weaken the integrity and function of the gastrointestinal barrier (Kelly et al., 2016a; Thevaranjan et al., 2017) and negatively affect blood-brain barrier permeability (Esposito et al., 2002; Montagne et al., 2015) which potentially accelerates inflammaging.

When analysis was undertaken of changes in microbiota composition in elderly individuals with intestinal inflammation, compositional differences were noted (Guigoz et al., 2008). Furthermore, reduced levels of *Akkermansia* following FMT from old mice into young germ-free mice was associated with an inflammaging phenotype in the recipient young mice (Fransen et al., 2017). An important component of the immune system and potential contributor to inflammaging are microglia, the brain's resident immune cells. A key component of the immune response of the central nervous system, these cells also play a role in neuronal wiring and activity, synaptic plasticity, phagocytosis and support the survival of neurons and neuronal progenitors via the secretion of growth factors (London et al., 2013; Tay et al., 2017). However, during aging, microglia develop into a highly reactive and unbalanced state promoting cognitive dysfunction including altered brain plasticity and neurodegeneration (Boehme et al., 2014; Lui et al., 2016; Tay et al., 2017; von Bernhardi et al., 2015). Recent studies have shown that germ-free mice display deficits in microglia maturation and function, while recolonization or administration of key gut microbiota metabolites such as SCFAs restore microglial function (Erny et al., 2015a). Such studies

suggest that targeting microglia could present an interventional approach to ameliorate neurodegenerative disease (Boehme et al., 2018; Matt et al., 2018).

We compared aspects of the immune system in aged mice comparing BTBR and C57BL/6 controls at 21 months of age demonstrating that aged BTBR mice display an altered T-cell repertoire to C57 animals. BTBR mice had an increased CD4/CD8 ratio, often associated with an activated immune system (Sainz et al., 2013). Furthermore, BTBR mice had decreased levels of Treg cells in MLNs, further indicating an inflammatory phenotype (Bettelli et al., 2006).

The immune data in our research suggests that aged BTBR mice display a chronically activated T-cell system compared to control C57 animals, suggesting the involvement of auto-immunity in the differences observed between the strains studied. While there may be some alterations due to the natural disruptions to the immune system in advancing age, the data suggests that the observed increases are in autoimmune activation which has previously been observed in animal models of ASD (Ashwood, 2013), as well as in humans (Ashwood et al., 2011), and that this phenotype is maintained at a later age.

Overall, this data in combination with what is already known regarding inflammaging suggests that ageing represents a particularly promising target for microbiota-based interventions that target the immune system. With immune dysfunction a key factor in the pathophysiology of autism spectrum disorders (Onore et al., 2012) our data suggests that targeting the microbiota in order to combat these effects may be an effective strategy in an older population with ASD.

Furthermore, the strong data linking ageing to behavioural deficits, altered microbiota composition, and an increased inflammatory profile in the gut (Scott et al., 2017b) mean that targeting the microbiota to reduce inflammatory status should be a cornerstone of future therapies. This may be achieved through the administration of prebiotics or probiotic strains that have been shown to positively modulate the immune system (Klaenhammer et al., 2012) or through supplementation with microbiota metabolites that have the potential to reduce inflammation both peripherally (Parada Venegas et al., 2019) and centrally (Erny et al., 2015a). Such questions will be a key focus in future studies of the microbiota-gut-brain axis in ageing.

6.3.2. Neurodevelopmental disorders in later life – an understudied but pivotal area of research for the future?

The prevalence of neurodevelopmental disorders, most particularly autism spectrum disorders (ASD) and the impact they have on the individual and the family has been recognised in recent years (Baxter et al., 2015). While ASD is a disorder that affects individuals throughout the lifespan, the majority of research in the disorder to date has focused on children and young adults (Wallace and Rogers, 2010). There remains a vastly unmet clinical need for additional focus on older individuals. This research is urgently required not only to improve our understanding of the mechanisms underpinning the disorder in these individuals, but also to improve the quality of life for older people living with ASD. Access to interventions and clinical supports for this population are needed however these needs are widely unmet (Mukaetova-Ladinska et al., 2012).

What is recognized, is that there is inadequate support for individuals with ASD in general practice (Krauss et al., 2003) and the fact that older patients with ASD have more problems than adults (Pary, 1993) suggests that even more resources will be required for this population. It is also recognized that older individuals with ASD have a poorer quality of life compared to non-ASD older individuals, including increases in anxiety and depression (Mukaetova-Ladinska and Stuart-Hamilton, 2016).

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In our research we assessed behavioural changes in a mouse model of autism-spectrum disorder (BTBR) in old age compared to a similarly aged control strain (C57BL/6). We observed that while many of the alterations in behaviour that are observed in young animals are maintained (repetitive behaviours, antidepressant-sensitive behaviours, social deficits & cognition) there are reduced effects in terms of anxiety in older animals of the BTBR strain compared to C57bl/6 controls. These results in a preclinical model are informative as they suggest that ASD behaviour persist from youth into old age. They are also consistent with the few behavioural assessments that have been undertaken in clinical groups (Totsika et al., 2010), with the clinical studies suggesting that the effects in ageing may even be more severe than in younger subjects.

With the gut-brain axis being the focus of considerable investigation for its role in the generation of ASD-related behaviours, the prospect of this axis as a therapeutic target has also been raised (Li and Zhou, 2016). Probiotics have been shown to be effective clinically in the treatment of certain childhood disorders associated with GI symptoms and inflammation (Xu et al., 2015). Consequently, a role for probiotics was suggested in the treatment of children with ASD. Preclinical and clinical studies have suggested that probiotics may

improve the gut microbial, gastrointestinal, and atypical behaviour in children with ASD (Slattery et al., 2016).

It has been proposed that an important aspect of the efficacy of probiotic treatment in ASD is through their effect on regulation of the immune system. The complexity of the interactions between gut microbiota, systemic cytokines and CNS inflammation means that there remains a great deal of research to be undertaken in this area (Critchfield et al., 2011). Preclinical data suggests that behavioural, neuropathological and inflammatory disruptions can be restored following treatment with the probiotic *Bacteroides fragilis* with a particular impact on the proinflammatory cytokine IL-6 in the gut (Hsiao et al., 2013a).

Our study found an increase in immune activation in ageing in a mouse model of ASD, along with the mounting evidence for 'inflammaging'. Our findings further support the potential of the microbiota as a target for treatment of neurodevelopmental disorders in older life. More research needs to be undertaken both epidemiologically as well as preclinically and clinically regarding older individuals and ASD. Tied to this there should be a greater focus on how the microbiota may serve as a suitable target for the symptomatic improvement of this condition potentially through targeting this increased immune activation.

6.4: The gut microbiota and synaptic plasticity – a cornerstone of the microbiota-gut brain axis?

With the ability of the microbiota to influence the brain and behaviour now firmly established (Cryan et al., 2019a) the focus of much of the current research in the field is into understanding the mechanisms underpinning the observed effects.

The complexity of the interactions between the gut and the brain make a single mechanism instigating these effects unlikely. More probable is that myriad alterations in physiology are working in tandem to exert these changes. To date among the mechanisms that have been suggested to play a role are: inflammation (Bercik and Collins, 2014), vagal signalling (Forsythe et al., 2014b), alterations in response to stress (Sudo et al., 2004b), the influence of microbiota metabolites (Dalile et al., 2019b), altered myelination (Hoban et al., 2016d) and blood-brain-barrier integrity (Braniste et al., 2014b).

Another mechanism that is believed to play a key role is that of synaptic plasticity. Early studies of the microbiota-gut-brain axis began to suggest such changes when alterations in the levels of key chemical messengers involved in synaptic plasticity processes including

BDNF (Neufeld et al., 2011b; Sudo et al., 2004b), PSD-95 (Diaz Heijtz et al., 2011; Vazquez et al., 2015) and synapsin (Williams et al., 2016) were disrupted in conditions of altered microbiota composition. In addition to this, many studies had noted that expression of NMDA receptor subunits was altered following modulation of the microbiota (Fröhlich et al., 2016; Sudo et al., 2004b; Wang et al., 2015). Given the key role that these receptors and their subunit expression plays in synaptic plasticity, all this evidence pointed towards a key role for the microbiota in the modulation of synaptic plasticity.

More recent research has extended this link between the gut microbiota and synaptic plasticity by demonstrating alterations in dendritic morphology in the hippocampus and amygdala of germ-free mice (Luczynski et al., 2016) and demonstrating links between gut microbiota composition and electrical signalling in the brain (Buffington et al., 2016; Distrutti et al., 2014a; Talani et al., 2019). The strength of this evidence led us to add additional focus on the links between gut microbiota composition and synaptic plasticity in our research.

6.4.1. SCFAs – the likely mechanism behind their effect on synaptic plasticity

As mentioned, SCFAs are a key metabolite of the gut microbiota and have been the target of much research into the microbiota-gut-brain axis. Supplementation with SCFAs has shown to have beneficial effects on stress response (van de Wouw et al., 2018a), learning and cognition (Arnoldussen et al., 2017; Val-Laillet et al., 2018) as well as reward-associated behaviors (Byrne et al., 2016). Once again, however, questions remain as to the mechainisms underpinning these effects and we belive that alterations in synaptic plasticity may be playing a role.

SCFAs have been shown to ameliorate deficits in microglia morphology and immaturity in GF mice (Erny et al., 2015a) while mice deficent in the SCFA receptor FFAR2 have severely malformed microglia even though microglia do not express FFAR2 receptors, indicating an indirect role for SCFAs in microglial maturation (Erny et al., 2015a). With the role of microglia in synaptic plasticity being increasingly recognized (Morris et al., 2013) modulation by SCFAs may play a key role in normally functioning synaptic plasticity.

In addition to effects through activity on microglia, other structural changes may also be under the influence of SCFAs. In pigs supplemented with butyrate, increases were observed in hippocampal granular cell layer volume and hippocampal neurogenesis (Val-Laillet et al., 2018). In diet-induced obese, low-density-lipoprotein receptor transgenic mice which display decreased hippocampal cerebral blood flow and functional connectivity (measured using

fMRI), spatial memory, and increased hippocampal microglia activation, each of these factors could be reversed following supplementation with butyrate (Arnoldussen et al., 2017).

It was the results of such studies that led us to question whether exposure of hippocampal slices to differing concentrations of SCFAs could affect synaptic signalling in the hippocampus. We found that while neither of the SCFAs tested (acetate and propionate) affected basal synaptic signalling or led to an altered paired-pulse ratio, perfusion with the higher concentration of acetate tested (700μM) did inhibit the expression of LTP in the hippocampus.

How SCFAs are exerting this influence over synaptic signalling is a factor that will need to be given greater consideration in future research. Increases in hippocampal LTP have been observed following application of butyrate, as well as the histone deacetylase inhibitor, trichostatin A (Levenson et al., 2004b). This result was observed following in-vitro exposure of hippocampal slices to these compounds. While SCFAs have been shown to enter the brain and exert effects on hunger though activity in the hypothalamus (Frost et al., 2014c) it is not yet known whether they are present in the hippocampus in concentrations sufficient to exert direct effects on synaptic signalling. Such investigations will need to form a key part of research into the effects of SCFAs on synaptic signalling. An additional mechanism that should be given attention is that of vagal nerve signalling. Activation of the SCFA receptor FFAR3 on the vagus nerve innervating the portal vein results in increased neuronal activity in the dorsal vagal complex, parabrachial nuclei and hypothalamus (De Vadder et al., 2014). Oral administration of butyrate to fasting mice results in decreased neuronal activity in the NTS and dorsal vagal complex, as well as decreased activity of orexigenic NPY-positive neurons in the hypothalamus, indicating a dynamic regulation of SCFAs on hypothalamic neuronal circuitry (Li et al., 2018b). It is clear, therefore, that SCFA-induced vagus nerve signaling has a role to play the activation of various neurons in the central nervous system (though further experimentation is required to link these results to what is observed in this thesis). More research needs to be performed to investigate which specific neuronal populations are activated by SCFAs-induced vagus nerve signaling throughout the brain and how this relates to behavior.

6.4.2. What next for electrophysiology in the study of the gut-brain axis?

While the wide range of methods discussed allow for measurement of plasticity-related changes occurring in the brain that can be linked to the microbiota, the gold-standard for

measuring synaptic plasticity changes are electrophysiological recordings (Shaban et al., 2017b). This technique provides real-time, highly-sensitive measurements of communication between neurons in living tissue (Koch, 2006). Measurements can be made from within the neuron of interest, at the surface of the neuron to measure current flow at ion channels of interest, or to record a the summed activity in a relevant brain area (Koch, 2006).

To date, the majority of studies addressing the link between the gut microbiota and synaptic plasticity have addressed extracellular recording of hippocampal LTP. This makes sense as this is the most studied area of the brain for such measures and provides excellent information on potential alterations in learning and memory (Garcia, 2001). Links have been made between probiotic treatment and protection from age-related impairments in LTP (Distrutti et al., 2014a; Talani et al., 2019), diabetes-related impairments in LTP (Davari et al., 2013) and high-fat-diet-induced impairments in LTP (Chunchai et al., 2018b). Recently, extracellular recordings have been made in the VTN that also demonstrate the protective effects of probiotic administration in a rodent model microbiota disruption through maternal high-fat-diet administration.

In our recordings we aimed to utilize a novel electrophysiological tool in to determine the effects of exposure of hippocampal sliced to a microbiota metabolite. Using this technique we were able to expand on the traditional patch-clamp model in that it was possible to make assessments of the effects of SCFAs on synaptic signaling at multiple distances from the site of stimulation, increasing the information that can be generated from an individual hippocampal slice.

Such an addition gives a glimpse at the potential that additional electrophysiological techniques could bring to the field of study of the microbiota-gut-brain axis. These include:

- Electrophysiological recordings at the NTS (Nucleus Tractus Solitarii). As the vagus nerve is a key node in microbiota-gut-brain axis communication (Breit et al., 2018; Fülling et al., 2019) a hugely important question that needs to be asked is what changes in electrical signalling occur at the NTS following modulation of microbiota composition. Previously, in vitro electrophysiological recording in the NTS has allowed the determination of distinct neuronal circuits involved in feeding behaviour influenced by vagal signalling (Roman et al., 2016). In-vivo recordings from the NTS are also possible (D'Agostino et al., 2016). An example of such a study would be perfusion of the distal gut with a SCFA-containing solution and observing the real-time changes that occur in signalling at the NTS.

- Electrophysiological recordings may be of use when determining the direction of signalling from the NTS to the rest of the brain following vagal nerve stimulation. For example, it is known that corticotrophin-releasing factor (CRF) production and secretion might result from a direct stimulatory effect, transmitted from the vagus nerve through the NTS to the paraventricular nucleus of the hypothalamus (O'Keane et al., 2005). Through the placement of electrodes in each of these areas it may be possible to measure associated alterations in electrical signalling
- Previous studies have assessed the ability of bacterial components to alter the survival of cultured neurons (Lukiw, 2016). MEA technology allows for the culturing of hippocampal neurons in a dish containing multiple electrodes (Qi et al., 2019). and this technology could be harnessed in order to observe the effects of these bacterial components on electrical signalling in cultured neurons.
- Multi-electrode array technology may be of use in the determination of long-term effects of gut microbiota modulation. Recent developments in technology (Huijing et al., 2017) have meant that 64 channel recordings can be made in the awake, mobile rodent following chronic implantation in the hippocampus (Huijing et al., 2017). This means that alterations can be made to the microbiota through probiotic or antibiotic administration, for example and long term differences can be measured in signalling between treatment groups and control groups. Such a vast array of data over the lifespan of the animal would go a long way to elucidating the mechanisms linking the gut microbiota to electrical signalling in the brain.
- Similarly, analysis of alterations in synaptic signalling in hippocampal slices of animals which have undergone microbiota modulation (C-section, antibiotics, prebiotics etc.) or which display an altered microbiota composition (BTBR) may be made ex-vivo, utilising the vast data-generating potential of the multi-electrode array.

Such advancements are within the realms of possibility of current technologies and with the appropriate resources could be added to plans for future studies of the microbiota-gut-brain axis. Many questions remained to be answered regarding the link between gut microbiota composition and neuronal signalling but with advancements in technology and increased interest in the gut brain axis the answers to these questions are getting closer.

6.5: Final Conclusions

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In this thesis we sought to assess the impact of microbiota modulation during a critical window in early life, the effects that such modulation can have on brain and behaviour throughout the lifespan, and whether the microbiota presents a target for novel treatments to address these disruptions. We also sought to elucidate the mechanisms underpinning the observed changes, investigating neurochemical, immune and particularly electrophysiological alterations.

Our data show that gut microbiota disruption in early life through C-section delivery or early-life maternal antibiotic administration can have lasting effects on brain and behaviour, some of which can be reversed through psychobiotic administration. We also observed that behavioural alterations in an animal model of ASD persist into old age and that such changes may be linked to immune system alterations, suggesting a potential target for microbiota-based therapies. Finally we observed that microbiota metabolites are able to affect synaptic signalling in the hippocampus, using a novel electrophysiological technique.

Future studies should aim to build on these results through paying particular care to the critical window of development of that is early life. Any alterations made during this period should be carefully noted and its potential as a therapeutic target should be studied in further detail. Furthermore, future studies should continue to address the mechanisms underpinning the microbiota-gut-brain axis in the hope that this greater understanding may pave the way for therapeutic breakthroughs.

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