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*Department of Anatomy & Neuroscience*

***Head of Dept. John F. Cryan***

# Enduring Neurobehavioural Effects Induced by Early-life Microbiota-gut-brain Axis Alterations

***Thesis presented by***
***Livia Hecke Morais, M.Sc.***
**under the supervision of**

**Prof. John F. Cryan**

**Prof. Timothy G. Dinan**

for the degree of Doctor of Philosophy

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Microbiome Ireland
Interfacing Food & Medicine

Department of Anatomy & Neuroscience
University College Cork

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## 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.

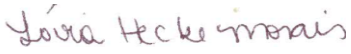
Dr. Paul Kennedy designed and performed the human research. Angela Moya-Pérez performed behavioural experiments with prebiotics and probiotics together. Gerard Moloney and Dr. Anna Golubeva assisted with corticosterone analyses, splenocyte and thymocyte stimulation. Dr. Orla O'Sullivan and Dr. Silvia Arboleya performed mice microbiota samples. Dr. Sahar El Aidy assisted with analyses of mice microbiota samples. Dr. Kieran Rea assisted with data analyses. Dr.Yuliya Borre, Dr. Karen Scott, Dr. Ana Paula Ventura-Silva and Rachel D. Moloney assisted with behavioural experiments. Dr. Elaine Patterson performed SCFA analyses. Dr. Roman Stilling and Dr. Alan E. Hoban assisted with brain transcriptomic data analysis. Ana Marta Sequeira and Sasja Beers helped with qPCR experiments. David Mullins and Paul W. O'Toole performed human microbiota analysis. Ingrid B. Renes, Shugui Wang, Jan Knol provided prebiotics and probiotics. R. Paul Ross, Paul D. Cotter, Catherine Stanton, Timothy G. Dinan & John F. Cryan discussed the data and assisted in writing the manuscript.

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Dr. Anna Golubeva and Gerard Moloney trained the student and assisted with samples collection, Dr. Karen A. Scott and Dr. Ana Paula Ramos Costa helped with behaviour, Sophie Casey assisted with qPCR experiments Timothy G. Dinan & John F. Cryan discussed the data and assisted in writing the manuscript.

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## Publications and presentations

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### Papers under review

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**MORAIS, LH**, FELICE, D., GOLUBEVA, A., MOLONEY, G., DINAN, T., CRYAN, J. Strain Differences in the Susceptibility to Gut-Brain Axis and Neurobehavioural Alterations Induced by Maternal Immune Activation in Mice. **Behavioural Pharmacology**. Apr,29(2 and 3 - Special Issue):181-198, 2018.

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**Oral presentations**

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## Abbreviations

Adrenocorticotropic hormone (ACTH)

Autism spectrum disorders (ASD)

Autonomic nervous system (ANS)

Basolateral amygdala (BLA)

Bimuno-GOS (B-GOS)

Blood brain barrier (BBB)

Brain-derived neurotrophic factor (BDNF)

Caesearean section (C-section)

Central nervous system (CNS)

Cholecystokinin (CCK)

Contactin-associated protein-like 2 (*Cntnap2*)

Corticotropin-releasing factor (CRF)

CRF receptor 1 (CRHR1)

Cross-fostering (CF)

Double-stranded RNA (dsRNA)

Elevated-plus maze (EPM)

Enteric nervous system (ENS)

Enteroendocrine cells (EECs)

Experimental autoimmune encephalomyelitis (EAE)

Faecal microbial transplantation (FMT)

Fluorescein Isothiocyanate–dextran (FITC-dextran)

Fluorescent *in situ* hybridization (FISH)

Forced-swim test (FST)

Fragile X Mental Retardation 1 knockout (Fmr1−/− )

Free fatty acid receptors 2 and 3 (FFAR2 and FFAR3)

Fructooligosaccharides (FOS)

Galactooligosaccharides (GOS)

Gamma-Aminobutyric acid (GABA

Glucagon-like peptide-1 (GLP-1)

General adaptation syndrome (GAS)

Germ-free (GF)

Gastrointestinal (GI)

Glucocorticoid receptors (GRs)

Hypothalamic-pituitary-adrenal (HPA) axis

Hypothamic-pituitary-adrenal (HPA)

Interleukin-10 (IL-10)

Inflammatory bowel syndrome (IBS)

*I*rritable bowel disorder (IBD)

L. rhamnosus GG (LGG)

Lypolysaccharide (LPS)

Marble-burying (MB)

Maternal immune activation (MIA)

Maternal high-fat diet (MHFD)

Melanoma antigen gene expression L2 (Magel2)

Microbe-associated molecular patterns (MAMPs)

Mineralocorticoid receptors (MRs)

Monocyte chemoattractant protein 1 (MCP-1 )

N-methyl-d-aspartate receptor (NMDAR)

Non-obese diabetic (NOD)

Nonobese Diabetes-Resistant (NOR)

Nucleus of the solitary tract (NTS)

Obsessive-compulsive disorder (OCD)

Oxytocin (OXT)

Paraventricular nucleus of the hypothalamus (PVN)

Perceived stress scale (PSS)

Peptide YY (PYY)

Polyinosinic-polycytidylic acid (poly (I:C))

Polysaccharide A (PSA)

Regulatory (reg)

Schizophrenia (SZ)

Segmented filamentous bacteria (SFB)

Serotonin (5-HT)

Social transmission of food preference (STFP)

Short-chain-fatty-acids (SCFA)

Supraoptic nucleus of the hypothalamus (SON)

T helper 1 (TH1) and T helper 2 (TH2)

## Abstract

There is a growing appreciation of the importance of the bidirectional communication between our gut and brain on regulating the function and development of multiple physiological systems, including the central nervous system. Recently, the gut microbiota was demonstrated to interact with the gut-brain axis to regulate behaviour which *has* driven a paradigm shift in our understanding of neuropsychiatric disorders. An individual's microbiota starts to develop mainly upon birth and continues to change throughout life. This initial colonization has a significant impact on development and maturation of the immune system. Conversely, disruptions of early-life microbiota have been implicated to long-lasting effects on stress, social behaviour and anxiety. Understanding the importance of early-life for shaping the gut-brain axis will further contribute to better strategies for disease prevention and treatment.

This thesis investigated the impact of the gut-brain axis disruptions in early-life and the neurobehavioural consequences in two different scenarios: birth by C-section and maternal immune activation with polyinosinic-polycytidylic acid during pregnancy. To this end, here we developed a mouse model of C-section in NIH Swiss mice and demonstrated for the first time that the mode of delivery at birth can alter the stress-response, social behaviour, anxiety-like behaviour and cognition across the lifespan. These neurobehavioural deficits were associated with marked changes in the gut microbiota composition and diversity in early-life and adolescence with special decrease in *Bifidobacterium spp.* Further, we demonstrated that some deficits in social behaviour and cognition induced by the mode of delivery are reversed by targeting the gut microbiota in early-life through prebiotic, probiotic treatment, microbial transfer, and by pharmacological treatment with oxytocin. Complementary to the findings in mice some aspects of stress-related behavioural and physiological changes were also observed in a cohort of young-adults individuals.

To further interrogate the importance of early-life for priming the gut-brain axis function in a different animal model, we investigated whether maternal immune activation with polyinosinic-polycytidylic acid (poly I:C) at gestational day 12.5 could

behavioural, physiological and molecular aspects relevant to neurodevelopmental disorders in offspring of an outbred (NIH Swiss) and an inbred (C57BL6/J) strain. By looking at these two different strains we were able investigate whether gene and environment can interact in the susceptibility to develop gut-brain axis phenotype. We demonstrated that these strains differ in anxiety and depression-like behaviours with the effects being more pronounced in NIH Swiss mice. These strain-specific behavioural effects in the NIH Swiss mice were associated with marked changes in important components of gut-brain axis communication: stress and gut permeability.

Taken together, these data suggest that gut-brain axis alterations in early-life may underpin altered programming of the developing brain and behaviours. Moreover, genetic background is a critical factor in susceptibility to the gut-brain axis alterations in certain conditions. Further efforts into understanding the factors that contribute to the major role for the gut-brain axis on programing brain health in early life may allow the development of new treatment strategies.

# Chapter 1. General Introduction

## 1.1 The Gut Microbiota as a Key Regulator of the Gut-Brain Axis

### 1.1.1 The Gut-Brain Axis

Gut-brain interactions have long been invoked in our language to express emotions, feelings and intuitions in our daily lives (e.g., gut feelings, gut-wrenching, gut instinct, gutted, gutsy, it takes guts, butterflies in one's stomach). Now we are beginning to understand the biological mechanisms underlying the bi-directional communication between gut and brain (Foster et al., 2017). The first comprehensive scientific description of gut-brain cross-talk was in the 1880s, when William James and Carl Lange critically associated emotions such as fear, anger, love to visceral responses. Further, in the 1920s, Walter Cannon added an important contribution to the field by proposing a pivotal role for the brain in regulating gastrointestinal functions. These bidirectional interactions between gut and the brain have been recognised for many years, and are referred to as the gut-brain axis (Cryan and Dinan, 2012; Mayer, 2011).

The emergence of the gut-brain axis is closely associated with the discovery of the third branch of the autonomic nervous system (ANS), the enteric nervous system (ENS). The ENS can act independently from the central nervous system (CNS); however, both systems are inter-connected by a complex network involving afferent and efferent pathways of the parasympathetic and sympathetic nervous systems (Furness et al., 2013). The human ENS contains between 200 and 600 million neurons – as many neurons as in the spinal cord. Most of the cell bodies of these neurons are grouped into small ganglia that are connected within the myenteric plexus and the submucous plexus (Goyal and Hirano, 1996). The interactions between ENS and CNS are important for maintenance of individual homeostasis and were studied by prominent psychologists, psychiatrists and physiologists of the late nineteenth and early twentieth centuries (Mayer, 2011). Over the last few decades, researchers have started to reveal the mechanisms underlying this cross-talk, which likely involves complex interactions between the afferent and efferent pathways of the vagus nerve, neuronal networks of the ANS, the hypothalamic-pituitary-adrenal (HPA) axis, the

immune system and metabolic pathways. Recently, a key role of the gut microbiota in governing this communication has been demonstrated (Cryan and Dinan, 2012; Cryan and O'Mahony, 2011).

The bidirectional nature of this communication allows the brain to control gastrointestinal functions (such as satiety, hunger, motility, epithelial permeability, mucus production/secretion) and modulate mucosal immune function (such as cytokine production and release). At the same time, the presence of specific intestinal contents can influence brain neurochemistry and influence the maturation and activation of CNS resident immune cells (Rea et al., 2016). These complex and multi-system interactions work to maintain homeostasis within the gastrointestinal tract and other physiological systems with consequences on mood, stress perception, behaviour, function and cognition (Foster et al., 2017). Therefore, disruptions in gut-brain axis signaling have the potential to disturb homeostasis, and adversely affect mental health (Cryan and Dinan, 2012; Cryan and O'Mahony, 2011; Foster et al., 2017; Grenham et al., 2011). The clinical relevance of this association is clearly evident, in the frequent comorbidities of gastrointestinal disorders and psychiatric illnesses; for example, irritable bowel disorder (IBD) and inflammatory bowel syndrome (IBS) very often manifest with stress and mood disorders (Farrokhyar et al., 2006; Minderhoud et al., 2004; Simrén et al., 2002). Pathological stress conditions are also known to disrupt gut epithelium barrier and mucosal immune system. In its turn, gut barrier dysfunction can lead to a "leaky-gut," triggering the translocation of luminal contents such as inflammatory cytokines and bacterial products into the peripheral circulation that can further cross the blood-brain barrier (BBB) and reach the brain (Bischoff et al., 2014; O'Mahony et al., 2009).

Finally, perturbations of gut-brain pathways have been implicated in the pathogenesis of a wide range of psychiatric conditions from schizophrenia (SZ), anxiety, mood disorders, autism spectrum disorders (ASDs), Alzheimer's disease to Parkinson's disease (Sherwin et al., 2017). Thus, elucidating the mechanisms underlying these interactions are crucial for gaining new insight into disease etiology and the development of novel and efficacious therapeutic approaches.

## 1.2 The Gut Microbiota

Humans and other mammals harbour a complex gut microbiota, comprised of organisms from all three domains of life (Archaea, Bacteria and Eukarya) (Donaldson et al., 2016). It is estimated that the human microbiota is populated with $3.9 \times 10^{13}$ bacteria, estimated to be almost a ratio of 1:1 in comparison to the number of cells in the entire body ($\approx 0.3 \times 10^{13}$) (Sender et al., 2016) The life-long host-microorganism associations were established over many eras of symbioses and co-evolution, with a strong mutualistic relationship that enhanced the fitness of both the host and microorganisms (Donaldson et al., 2016). Thus, these important interactions between microorganisms and host have been incorporated in the holobiont (host and its symbionts) and hologenome (gene pool of host and its associated symbionts) concept (Kundu et al., 2017).

This life-long microbial-host realtionship is generally initiated at birth, and is further influenced by a series of environmental factors such as diet, the immune system and exposure to chemical agents or environment in general (e.g. stress; nurturing system; infection etc.) along the individual life-span (Cryan and Dinan, 2012; Donaldson et al., 2016). The notion that microorganisms can confer health benefits to the host was recognized long ago (Dubos, 1941). However, in the past decades, the advent of DNA sequencing, and metagenomic, meta-transcriptomic analyses, coupled with the *Human Microbiome Project* has renewed interest in the relationship between the intestinal microbiome and the host (Stilling et al., 2014). Moreover, the use of animal models for manipulation of the gut microbiome has allowed investigation of microbiome-host interactions in health and disease states. Therefore, research employing germ-free (GF), gnobiotics, and mono-associated animal models, and numerous techniques, including antibiotic-induced microbiota depletion, feacal microbiota transplantation, co-housing, cross-fostering (CF), infection studies and the use of prebiotics and probiotics, have played key roles in unravelling such interactions (Cryan and Dinan, 2012; Ericsson and Franklin, 2015; Luczynski et al., 2016a) (**Figure 1.1**).

### 1.2.1 Microbial Distribution across the Gut

The most abundant bacterial taxa present in the human gut belong to the phylum Firmicutes, which includes species such as *Lactobacillus* spp., *Clostridium* spp., *Enterococcus* spp., and Bacteroidetes, which includes *Bacteroides* spp. (Dethlefsen et al., 2007). Bacteria from other phyla also widely common within the gut ecosystem include species from Actinobacteria (Bifidobacteria), Proteobacteria (*Escherichia coli*), Fusobacteria, Verrucomicrobia, and Cyanobacteria (Eckburg et al., 2005; Qin et al., 2010). There are several factors that can influence microbial distribution and diversity within the gut , such as pH, diet and nutrient availability, antimicrobials, the presence or absence of mucus, adherence to the the gut wall, oxygen levels, and the host immune system, in addition to the presence of founder effects of pioneer colonisers (Tropini et al., 2017). For instance, the microbial community present in the small intestine, comprised of the duodenum, jejunum and ileum, is characterised by fast-growing facultative anaerobes bacteria which are adapted to the acidic pH and higher levels oxygen in addition to the presence of bile acids and antimicrobial substances. These bacteria present in the small intestine use simple carbohydrates as an energy source. In mice, members of Proteobacteria (particularly Enterobacteriaceae) and Firmicutes (members of the family Lactobacillaceae, in particular), are dominant in the small intestine (Donaldson et al., 2016; Sharon et al., 2014) (Figure 1.1). The microbial composition of the large intestine, comprised of the caecum and colon, is much more diverse. The large intestine has lower concentrations of antimicrobials, a slower transit time in comparison to the small intestine, and lacks the simple carbon sources that are suitable for the growth of fermentative polysaccharide-degrading anaerobes. In mice, the caecum is enriched in species of the families Ruminococcaceae and Lachnospiraceae, whereas the colon is enriched in members of the families Bacteroidaceae and Prevotellaceae (Donaldson et al., 2016).

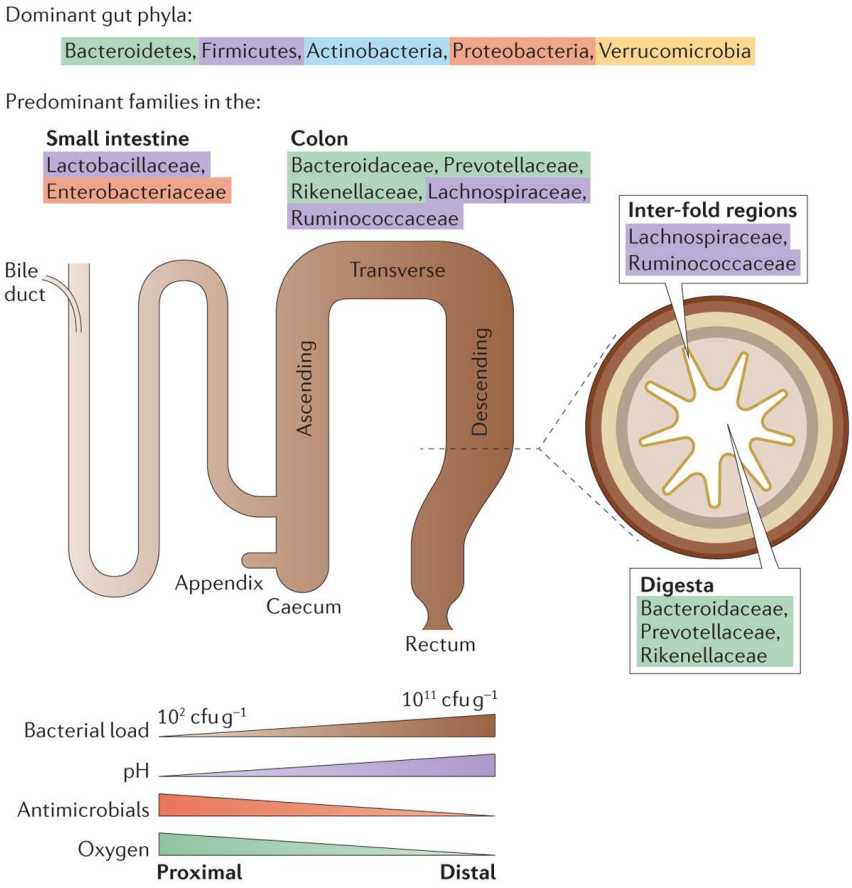



***Figure 1.1 - Microbial distribution within the gut.***

The human gut is mainly populated by bacteria from Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia phyla. Bacterial distribution within the gut reflects physiological differences along its portions. For example, the small intestine is characterised by a high gradient of oxygen, antimicrobial peptides (including bile acids) and lower pH which limits bacterial abundance. Lactobacillaceae and Enterobacteriaceae are the most prevalent in this portion. A lower oxygen gradient, fewer antimicrobial substances, and a higher pH characterises the colon, resulting in a greater bacterial abundance and diversity, including families such as Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae (colours correspond with the relevant phyla).,cfu= colony-forming units. Figure taken from Donaldson et al. (2016).

## 1.2.2 The Establishment of the Microbiome in Early-life

### 1.2.2.1 *Pregnancy & Microbiome*

Maternal microbiota dynamicaly changes over the normal course of pregnancy (Koren et al., 2012). It has been suggested that the biological role for these changes has to due with mother's adaptation to pregnancy and foetal growth and development (Gohir et al., 2015). The microbial diversity normaly increases from early to late pregnancy.

Besides changing in diversity and complexity, maternal microbiome also changes its metabolic profile (Koren et al., 2012). Briefly, in the first trimester of gestation, the gut microbiome of the pregnant mother is similar to that of healthy, non-pregnant women. As the pregnancy develops, increased abundance of Actinobacteria and Proteobacteria and a reduction in alpha diversity is observed (Nuriel-Ohayon et al., 2016). In the third trimester of gestation, levels of *Faecalibacterium*, a butyrate-producing bacterium with anti-inflammatory activities are decreased (Haro et al., 2016). Moreover, By colonizing GF mice with first and third-trimester pregnant mothers, researchers demonstrated that between-subject diversity (beta diversity) is increased in the third trimester associated with metabolic changes including weight gain, insulin insensitivity, and higher pro-inflammatory profile (Koren et al., 2012). Some factors are known to adversely affect the pregnant microbiome with implications to the neonatal health include: preterm birth, cardiometabolic complications of pregnancy such as preeclampsia and gestational hypertension, and gestational weight gain (Dunlop et al., 2015).

#### 1.2.2.2 *Placenta*

More recently, the use of whole metagenomic shotgun sequencing has demonstrated that the placenta has a very distinct microbiome signature, composed of bacteria from the phyla Firmicutes, Tenericutes, Proteobacteria, Bacteroidetes, and Fusobacteria, which most closely resembles the human oral microbiome (Aagaard et al., 2014). Similarities between the neonatal microbial intestinal community and that of the amniotic fluid and placenta were also found in other studies (Collado et al., 2016; Perez-Muñoz et al., 2017). In a longitudinal study, in which the microbiota composition of different bodily regions of newborns from birth to 6 weeks of life was assessed, the microbial content of the first meconium was demonstrated to most similarly reflect the *in utero* environment (Chu et al., 2017). However, given that the overall biomass of microbiota in the placenta is very low (Aagaard et al., 2014) and the methodologies applied in many of these studies do not differentiate between living, dead bacteria (Kliman, 2014), further research is needed to provide a more conclusive consensus on the role microbial infant-mother transmission via placenta (Perez-Muñoz et al., 2017).

### 1.2.2.3 *Early-life*

The most consistent finding in the field is that robust changes of the microbiome occur in the periods during and after birth (Kundu et al., 2017). Upon birth, the newborn is exposed to the maternal vaginal, skin and fecal microbiota, which initiates microbial colonisation in the newborn (Laforest-Lapointe and Arrieta, 2017). The first bacterial communities to grow are facultative anaerobic species (e.g. *Escherichia coli*, *Staphylococcus*, and *Streptococcus)* which will colonise the infant gut, producing an anaerobic environment in the first few days of life. The presence of anaerobic conditions enables other anaerobes, including *Bacteroides* and *Bifidobacterium* spp. to seed in the sequence (Pantoja-Feliciano et al., 2013). The initial microbiota seeding is crucially determined by the mode of birth (Biasucci et al., 2010; Borre et al., 2014; Hill et al., 2017; Moya-Pérez et al., 2017; Mueller et al., 2017). Thus, disrupting the mother-infant bacterial transmission by Caesarean-section (C-section) leads to a different pattern of microbial colonisation within the neonatal gut, resulting in decreased Bifidobacteria and *Bacteroides* genera (Dominguez-Bello et al., 2010, 2016; Hill et al., 2017; Mueller et al., 2017; Pantoja-Feliciano et al., 2013) (The consequences of C-section birth mode to the early microbial seeding will be extensively discussed in the section 1.2.2.4).

The microbial colonisation after birth is strongly influenced by mode of feeding (Walker and Iyengar, 2015). Breastfeeding contributes to the maternal-infant transmission of a unique microbiota rich in Streptococci and Staphylococci (Hunt et al., 2011). Not only does breastfeeding confer protection from harmful bacterial species, via the the transmission of secreted maternal antibodies, breast milk is also a source of oligosaccharides, which may promote the growth of specific, beneficial microbial communities, e.g. *Bifidobacterium* spp. and *Bacteroides* spp,, which confer protection to the general health of the newborn (Castanys-Muñoz et al., 2016). The presence of these specific microbes protect against pathogenic organisms, by acting at the mucosal barrier function and promoting immunological and inflammatory responses (Akbari et al., 2015; Messaoudi et al., 2011). The use of formula-fed nutrition systems also greatly affect infant gut microbiota composition (Bakker-Zierikzee et al., 2005; Hill et al., 2017; Walker and Iyengar, 2015). Exclusive and partial formula-feeding is demonstrated to shift microbial diversity to a

proinflammatory microbiota profile, impair intestinal barrier and decrease the concentration in beneficial short-chain-fatty-acids (SCFA) in neonates (Mueller et al., 2015). These effects were reversed by the use of prebiotic and probiotic supplementation which may represent an important strategy for positively shaping the infant microbiome (Mueller et al., 2015).The gut microbiome continues to develop after weaning and, subsequently, introduction of solid food plays a key role in the maturation of the infant microbiome to a more adult-like profile (Bäckhed et al., 2015). The cessation of breastfeeding results in a rise of butyrate producers such as members of Bacteroides spp. and Clostridium spp.. The microbiome will reach complete functionality at approximately one and a half years of age (Putignani et al., 2014) and complete stability by the age of three years old (Borre et al., 2014).

Some prenatal and postnatal factors that can shape the infant gut microbiota include the delivery birth by C-section (Dominguez-Bello et al., 2010, 2016; Hill et al., 2017), not being breastfed (Azad et al., 2016; Bergström et al., 2014; Hill et al., 2017; Pannaraj et al., 2017), early-life stress (Golubeva et al., 2015; Jašarević et al., 2015a); environment (Marquez et al., 2010), maternal diet (Buffington et al., 2016), gestational age (Hill et al., 2017), host genetic (Rodríguez et al., 2015), maternal medication (de Theije et al., 2014); exposure to infections (both maternal and infant) (Hsiao et al., 2013; Kim et al., 2017), and antibiotic usage (Penders et al., 2013) and hospitalization (Penders et al., 2013) (**Figure 1.2**).

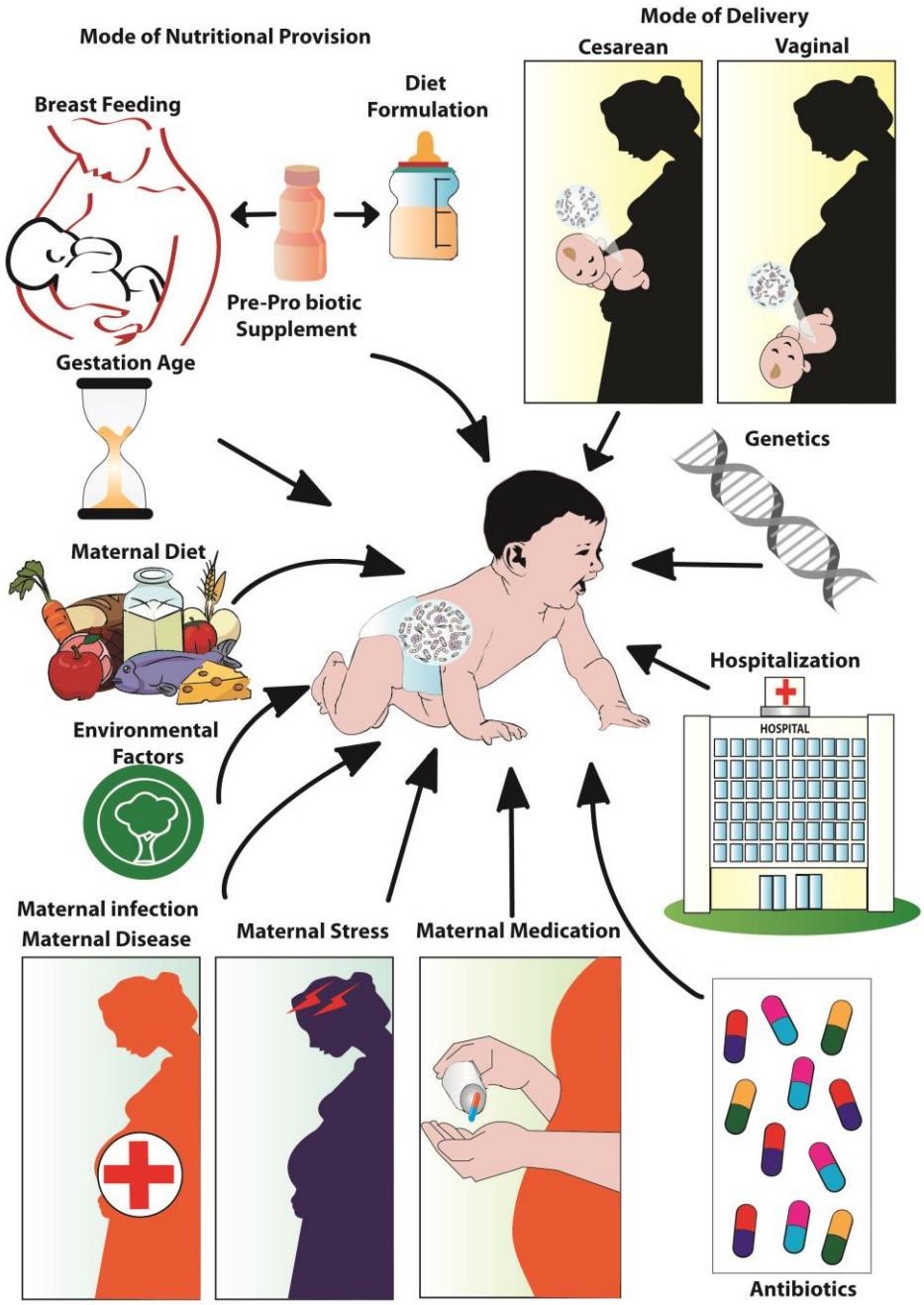



***Figure 1.2- Prenatal and Postnatal factors influencing the gut microbiota development in early-life.*** The individuals microbiome starts to develop mainly after birth reaching complete funcitonality at one and half years of age and complete stability by the age of three years old. The sequence of microbial community stablishment in the infant gut is influence by both prenatal and postnatal factors, including: maternal gestational age, maternal diet, maternal infection and stress, maternal medication, host genetics, hospitalization, birth mode, brestfeeding system and solid food introduction, exposure to antibiotics and additional environmental factors.

#### 1.2.2.4 *The Mode of Delivery at Birth Shapes the Intestinal Microbiota*

Medically-indicated C-section is a life-saving procedure (Deshpande and Oxford, 2012). However, the number of infants delivered by C-section has rapidly increased worldwide over recent years with many territories far exceeding the World Health Organization guidelines of between 10-15% (Betran et al., 2016). For example, Latin America and the Caribbean region shows the highest rates of 40.5%, followed by North America at 32.3%, Oceania with 31.1%, Europe with 25%, Asia with 19.2% and Africa with 7.3% (Betran et al., 2016). In the United States approximately 1 in 3 babies was delivered via C-section in 2013. In Brazil and in China, C-section occurs in 30-85% of births due to several cultural, aesthetic and economical reasons. Conversely, in some areas with severe poverty, there is limited access to medically-necessary C-section (especially in sub-Saharan Africa) (Moya-Pérez et al., 2017).

As mentioned in previous sections, one of the major influences on the composition of the gut microbiota during infancy is the mode of delivery at birth. While vaginally born (VB) infants are colonised by faecal and vaginal bacteria from the mother, infants born by C-section are colonised by hospital (or other) microbial species derived from the external environment (Dominguez-Bello et al., 2010). Thus, infants born by C-section have different gut microbial composition than VB, during the first years of life (Dominguez-Bello et al., 2010; Hill et al., 2017; Korpela et al., 2018; Martinez et al., 2017; Moya-Pérez et al., 2017). Babies born via C-section have less Bifidobacteria and Bacteroides (Biasucci et al., 2010; Penders et al., 2013) and more *Clostridium difficile* in their intestinal microbial community (Penders et al., 2013). A seminal study by Dominguez-Bello and colleagues (2010) characterised the bacterial communities from mothers' skin, vaginal and oral mucosa, and those of their newborns' skin, oral and nasopharyngeal mucosa, and meconium. They reported that distribution of the neonatal microbiota is akin to the delivery mode; *Lactobacillus*, *Prevotella*, *Atopobium*, or *Sneathia* spp. were abundant in samples from VB newborns, whereas C-section babies were mainly colonised by typical skin taxa, including *Staphylococcus* spp. (Dominguez-Bello et al., 2010). Furthermore, a series of follow-up studies consistently demonstrated that C-section born infants are

colonised with fewer *Bacteroides* and *Bifidobacterium (Bäckhed et al., 2015; Makino et al., 2013). Longitudinal* studies suggest that these initial differences remain for the first months of life to years (Bäckhed et al., 2015; Moya-Pérez et al., 2017). However, a recent study by Hill and colleagues (2017) showed that C-section-mediated gut microbiota diversity is restored to that of VB infants by 6 months of age (Hill et al., 2017).

The perinatal period is a crucial developmental window for the establishment of lifelong host health (Borre et al., 2014). Moreover, early-life microbiota perturbations, interfering with Bifidobacterium and Bacteroides levels, have been reported to precede the development of certain disorders including allergies and obesity (Negele et al., 2004; Watanabe et al., 2003). Consistently, C-section delivery mode has been associated to a greater risk of developing immune-related conditions such as asthma and allergies (Kero et al., 2002; Mårild et al., 2012), and metabolic disorders including obesity and type-I diabetes (Blustein et al., 2013; Huh et al., 2012; Hyde and Modi, 2012) and hypertension in young adults (Horta et al., 2013). Although a number of factors can be attributed these findings, one perspective is that the disturbances of early postnatal gut microbiota may prime aspects of physiology which persist across the lifespan (Cho and Norman, 2013). Interestingly, recent studies using animal models have revealed an association between C-section delivery mode on the shaping of the gut microbiota and effects on host health. For example, mice born by C-section exhibit a distinct bacterial profile at weaning, although this does not persist into adulthood mice (Hansen et al., 2014). Moreover, in non-obese diabetic (NOD) mice, which are used to model type 1 diabetes, alterations in the early-life microbiota have long-term systemic effects on priming of the immune system. NOD mice born by C-section exhibit lower levels of $Foxp3^+$ regulatory (reg) T cells, tolerogenic $CD103^+$ dendritic cells, and decreased *Interleukin-10 (IL-10)* gene expression in mesenteric lymph nodes and spleen (Hansen et al., 2014). More recently, C-section was demonstrated to also alter gut microbiota diversity and increase body weight in wild-type mice, with the greatest impact on females (Martinez et al., 2017).

In parallel to the C-section studies discussed above, recent epidemiological findings suggest that C-section delivery is related to a modest increase in rates of some neuropsychiatric disorders such as bipolar disorder, ASDs, and attention deficit

hyperactivity disorder (Curran et al., 2015a, 2016a, 2016b). More thorough interrogation of epidemiological data revealed that associations between C-section and ASDs and psychosis disappear after familial confounds are taken into account; however, an increased in obsessive-compulsive disorder in C-section individuals was observed following adjustment for these confounds (Curran et al., 2016c).

Considering the health implications of early-life microbiota acquisition (including mental health) (O' Mahony et al., 2015), and the increasing numbers of C-sections, the investigation of microbial-based strategies to overcome possible adverse effects of the mode of birth could be of great value (Moya-Pérez et al., 2017). Recently, the inoculation of a neonate with maternal vaginal microbiota, termed vaginal seeding, was proposed as one possible therapeutical strategy (Dominguez-Bello et al., 2016). In this study, C-section-delivered infants were swabbed with maternal vaginal fluid over their entire body immediately following birth, resulting in microbiota compositions similar to VB infants after 30 days (Dominguez-Bello et al., 2016). A major concern about this practice leading accidental infection and sepsis has been highlighted (Cunnington et al., 2016), and additional, more extensive studies employing longitudinal approaches are needed to demonstrate the efficacy and safety of this procedure (Moya-Pérez et al., 2017).

### 1.2.3 Targeting the Microbiota with Pre and Probiotics in Early-Life

The influence of diet on shaping the microbiota composition is well-established (Oriach et al., 2016). In this way, the modulation of the gut microbiota through specific dietary components is a growing area of investigation specially to overcome microbiota-associated deficits in early-life (Moya-Pérez et al., 2017)

#### 1.2.3.1 *Prebiotics*

Prebiotics were initially characterised as "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria already resident in the colon" (Gibson and Roberfroid, 1995). More recently this definition has been expanded upon to describe "a substrate that is selectively utilized by host microorganisms conferring a health

benefit" (Gibson et al., 2017). Prebiotics can act as energectic substrate for probiotic bacteria such as *Bifidobacterium* and *Lactobacillus.* Fermentation of prebiotics by these bacteria results in production of SCFAs, and lactic and acetic acids, which can have profound effects on host metabolism (Moya-Pérez et al., 2017) and also prevent infection by pathogenic bacteria (Slavin, 2013). Moreover, prebiotics are found naturally in various foods such as vegetables, wheat, and soybeans and are typically composed of oligosaccharides or more complex saccharides (Oriach et al., 2016). Prebiotics can be used in conjunction with probiotics (synbiotic) which has been shown to be more effective on modifying the gut microbiota than when used alone. So far, the most commonly studied prebiotic compounds are inulin, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) (Pandey et al., 2015).

Several studies have demonstrated that GOS and FOS have indirect beneficial effects on the host immune-system (Arslanoglu et al., 2008), metabolism (Knol et al., 2005) and gastrointestinal function (Niittynen et al., 2007). The use of GOS/FOS mixtures in infant formula, not only increase Bifidobacteria levels, but also have been reported to enhance the metabolic activity of the entire infant microbiome (Knol et al., 2005). Indeed, GOS/FOS-induced changes in SCFA, lactate and pH were akin to what is observed in breastfed infants, rather than those fed the control formula, lacking the GOS/FOS mixture (Knol et al., 2005). Moreover, dietary supplementation with GOS and polydextrose during the first 2 months of life was demonstrated to reduce crying and fussiness in preterm infants (Pärtty et al., 2013). Moreover, research using aminal models suggests that consumption of FOS during gestation has anti-inflammatory effects on the offspring, resulting in fewer skin lesions (Fujiwara et al., 2010). Another study in mice suggests that adding another prebiotic (GOS, 9:1 ratio) to the maternal diet during gestation and lactation and exposing offspring to the same prebiotic post-weaning, increases offspring muscle mass (Desbuards et al., 2012). However, there is limited data available on the effects of prebiotic supplementation in infants with disrupted microbiota seeding in early-life.

Only recently, has investigation of the effects of prebiotics on behaviour and CNS function begun (Burnet, 2012).One of the first studies to report central effects of prebiotics involved administration of Bimuno-GOS (B-GOS) and FOS formulation to

rats. This treatment resulted in an increase in hippocampal brain-derived neurotrophic factor (BDNF) and *N*-methyl-D-aspartate receptor (NMDAR) subunits. Both receptors play a critical role in maintaining synaptic plasticity and optimal memory function (Savignac et al., 2013). Administration of the GOS/FOS mixture to adult mice was shown to attenuate chronic stress-induced elevations in corticosterone, proinflammatory cytokines, and depression- and anxiety-like behaviours, in addition to normalising the effects of stress on gut microbiota (Burokas et al., 2017). Moreover, anxiety-like behaviours, impairments in hippocampal-dependent learning and their associated changes in hippocampal genes related to stress circuitry, anxiety and learning, were ameliorated in maternally-separated rats maintained on a diet containing polydextrose/GOS following weaning (McVey Neufeld et al., 2017).

#### 1.2.3.2 *Probiotics*

A probiotic can be defined as "a live organism that, when ingested in adequate amounts, exerts a health benefit." The first scientific evidence of a probiotic effect was described by Metchnikoff in 1908, based on his findings on the beneficial effects of fermented milk products on longevity (Mackowiak, 2013). Although the definition of probiotics is widely accepted, it is important to note that live and non-viable microorganisms can exhert similar effects that probiotics itself (Dinan et al., 2013). The most widely used probiotics are *Lactobacillus* spp. and *Bifidobacterium* spp. Probiotics can induce beneficial effects when they remain viable in the gastrointestinal tract at a concentration of at least $10^6$-$10^7$ CFU/g (Vinderola et al., 2011).

That probiotics can produce neurochemical substances, has been known for nearly a century (Lyte, 2011).However, a new concept recently introduced, focuses on the potential for probiotics to act as psychotropic agents, psychobiotics (Dinan et al., 2013; Sarkar et al., 2016). Probiotics have been proposed as a potential way to augment the conventional therapies for depression (Logan and Katzman, 2005). Psychobiotics were originally defined "a live organism that, when ingested in adequate amounts, produces a health benefit in patients suffering from psychiatric illness" (Dinan et al., 2013). However, this has been expanded to also include prebiotics and other targeted interventions of the microbiome that supports brain health (Sarkar et al., 2016).The ability of probiotics to produce benefits to patients suffering from psychiatric illness

may be, in part, explained by their ability to produce and release neurotransmitters. For example, strains from *Lactobacillus* spp. and *Bifidobacterium* spp. are known to produce *gamma*-Aminobutyric acid (GABA), serotonin (5-HT), acetylcholine, and cannabinoids, which are essential neurotransmitters for CNS homeostasis and behavioural function (Dinan et al., 2013). The mechanisms by which probiotics can produce beneficial effects are being actively investigated by both clinical and preclinical studies.

Since manipulation of the microbiota at an early developmental stage can have a long-lasting impact, intervention strategies including probiotic treatment in early-life have been investigated (Moya-Pérez et al., 2017). For example, maternal supplementation with commensal bacteria *L. rhamnosus* GG (LGG) was demonstrated to increase bifidobacterial diversity in infants (Gueimonde et al., 2006). In another study, the the maternal consumption of *LGG* during gestation increased this taxa in newborn infant gut for at least 6 months and, in certain cases, these effects were observed for as long as 24 months (Schultz et al., 2004). However, it is worth noting that in a different study, administration of *LGG* promoted *Bifidobacterium spp.* without increasing microbial diversity of early infant gut (Ismail et al., 2012). Moreover, treatment with *Bifidobacterium breve* to preterm infants significantly decreased aspirated air volume from the stomach and improved weight gain (Kitajima et al., 1997). A series of clinical studies have also demonstrated a positive effect of probiotic treatment, by reducing the incidence of necrotising enterocolitis (Patel and Underwood, 2018). Together, these studies support the use of probiotics for ameliorating the adverse effects of gut microbiota disruptions in early life. Furthermore, the gut microbiota has been suggested as a potential target for the treatment of some CNS disorders as outlined above including ASDs, stress-related disorders, IBS and IBS (O' Mahony et al., 2015).

### 1.2.4 Tools for Investigating the Impact of the Gut Microbiota on Gut-Brain Function

Some of the strategies commonly used for investigating the impact of the gut microbiota on gut-brain function are depicted in the **Figure 1.3** and described in the sub-sections bellow.

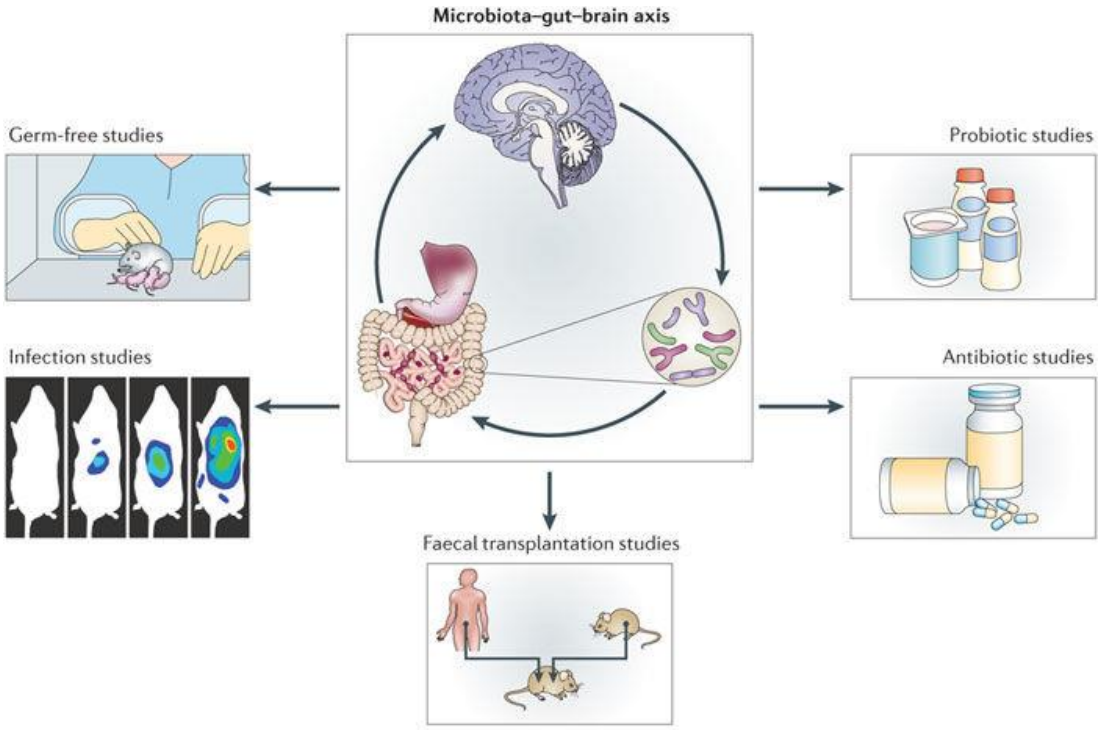



***Figure 1.3- Tools for investigating the microbiota-gut brain axis.***

The use of germ-free animals provide evidences on the functional role of the gut microbiota in comparison to conventional raised animals as wells as allows for the investigation on the effects of by specific bacteria (for example, a probiotic) on the microbiota–gut–brain axis. The administration of antibiotics can deplete the gut microbiota in a temporally controlled and more realistic manner and also consisits in important tool for investigation on the role of microbiota to host behaviour. Moreover, both germ-free animals and antibiotic-treated aniamls can be re-colonized with human microbiota, by feacal transplantation. Figure taken from (Cryan and Dinan, 2012).

### 1.2.4.1 *Germ-free Mice*

The first successful generation of germ-free (GF) mice was in 1946 by James Reyniers (Reyniers et al., 1946), providing evidence that life is possible without microorganisms. Since then, the use of gnobiotic animals in research has increasingly grown (Luczynski et al., 2016a). GF mouse colonies are generated by C-section surgery where offspring have no contact with maternal microorganisms from the skin or vaginal microbiota. Newly born animals are then raised in sterile conditions inside aseptic isolators and fed with sterile milk. After weaning, GF mice continue to be provided with sterile water, food and bedding. Another way to generate GF rodents is through embryo transfer at the two-cell stage to a pseudo-pregnant mother.

Following generations are more easily generated; GF are interbred and mothers are allowed to give birth by natural delivery without exposing the offspring to microbes (Ericsson and Franklin, 2015; Kirk, 2012; Luczynski et al., 2016a; Reyniers et al., 1946). The lack of microbiota in GF mice has been associated with several alterations in physiology, metabolism, immune system and brain function. Some of the important feautures of the GF phenotype are specified in the **Figure 1.4**.

Mice raised in a controlled, aseptic environment, without any exposure to microbes, that are then colonised by identified microbiota are named gnotobiotics (Luczynski et al., 2016a; Turnbaugh et al., 2009). The term gnotobiotic is derived from the Greek *gnotos*, meaning "known," and *bios*, meaning "life" (Ericsson and Franklin, 2015). Historically, Louis Pasteur proposed experiments using gnotobiotic animals and hypothesised that the presence of microorganisms was necessary for life (Gordon and Pesti, 1971). Therefore, gnotobiotic animals were created to study the importance of different members of the microbiota on host health and disease in a controlled situation (Ericsson and Franklin, 2015).

The colonisation of GF mice with specific microorganisms has provided exciting insights into microbial-host interaction (Luczynski et al., 2016a). For example, GF mice can be colonised by one specific strain of bacteria, generating mono-associated mice, or by two different microorganisms, to obtain di-associated mice. Microbial reconstitution of GF gut microbiota with "cocktails" or consortia of bacteria, virus and Fungi, other mice' gut microbiota, human gut microbiota and from other animal species are also important tools for studying the role of the microbiome (Kirk, 2012).

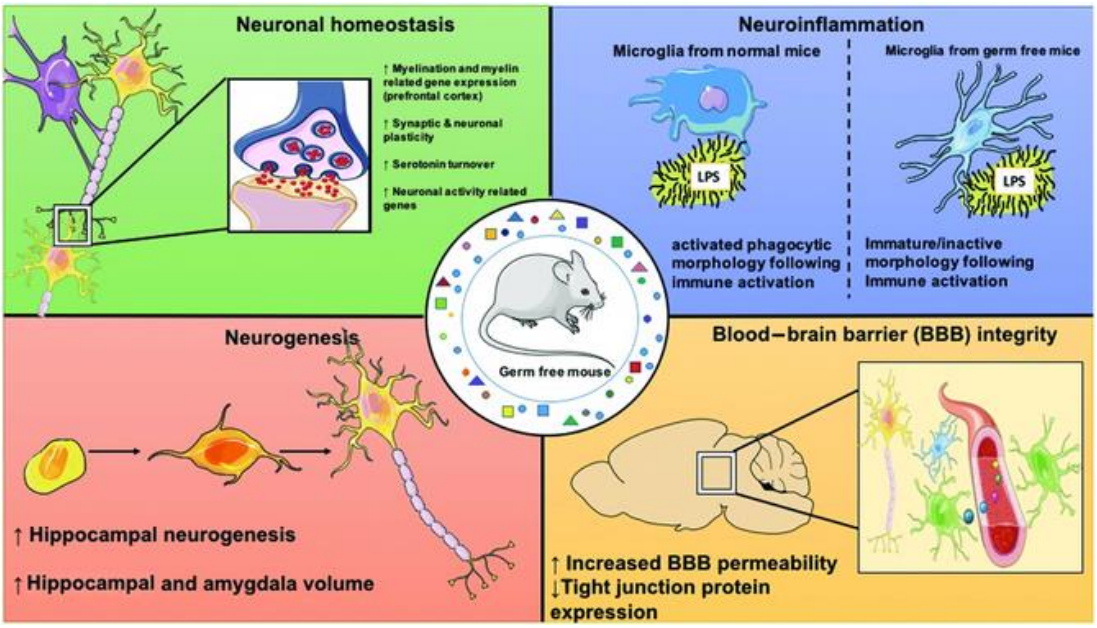



***Figure 1.4- Central nervous system alterations in germ-free mice.***

Mice raised and kept in germ-free conditions present with many alterations in the CNS including: changes in neuronal homeostasis such as altered myelination, synapsis dysfunction, neuronal plasticity and brain neurochemistry; differences in microglia morphology and activation in response to an inflammatory stimuli; increased hippocampus neurogenesis and amygdala volume and blood brain barrier (BBB) dysfunction.

### 1.2.4.2 *Microbial Depletion by Antibiotics*

Germ-free mice have exhibit profound phenotypic differences from "normal" mice due to the developmental effects related to the absence of commensal microorganisms. Therefore, in order to rule out these confounding effects upon developmental trajectories, alternative microbiota depletion strategies may be applied, such as the use of antibiotics in adulthood (Ericsson and Franklin, 2015; Hoban et al., 2016a). The use of antibiotics allows for microbiota depletion to be applied during any desired period of time during an animal's lifespan. Importantly, manipulation of the gut microbiota by antibiotic administration is shown to induce changes in behaviour and brain neurochemistry (Hoban et al., 2016a). However, despite its advantages, some antibiotics can cross the blood brain barrier (BBB) and act directly in the CNS, possibly confounding interpretation of results.

Administration of antibiotics during early-life and adulthood can have long-lasting impact on gut microbiota. For instance, the administration of cocktails of broad-spectrum antibiotics alters microbial diversity, reducing *Firmicutes* and *Bacteroidetes* phyla within the gut for as long as three months (Manichanh et al., 2010). Early-life treatment with vancomycin selectively induces visceral hypersensitivity in rats in adulthood (O'Mahony et al., 2014). Moreover, the administration of antibiotics in mice during adolescence shifts gut microbiota composition, decreases spleen weight, reduces anxiety-like behaviour, induces cognitive deficits, and changes brain gene expression and tryptophan metabolism (Desbonnet et al., 2015). Recently, maternal exposure to antibiotics has been demonstrated to have transgenerational impacts, inducing colitis in IL10$^{-/-}$offspring (Schulfer et al., 2018).

#### 1.2.4.3 *Faecal Microbial Transplantation*

Animals (GF or antibiotic-treated, in particular) can have their microbiota (re)colonised with a desired microbial population through faecal microbial transplantation (FMT) (Manichanh et al., 2010). FMT is also employed in a clinical setting, often referred to as faecal bacteriotherapy; faecal bacteriotherapy has been used to treat *Clostridium difficile* infection for over 50 years. Recently, FMT has garnered attention for its potential to treat numerous clinical conditions including, but not limited to, metabolic, gastrointestinal and psychiatric disorders (Borody and Khoruts, 2011; Shanahan, 2009; Turnbaugh et al., 2009). Experimentally, FMT is an important tool for understanding the complex microbial populations and providing causal relationships between phenotype and microbiota. For example, the link between faecal microbiota and depression has been recently established by transplantation of faecal microbiota from clinically depressed patients to antibiotic-depleted rats. Rats that received faecal microbiota from depressive patients exhibited anhedonia and anxiety-like behaviour, whereas FMT from control subjects to rats had no effect (Kelly et al., 2016). In another example of FMT application in experimental science, microbiota transplantation from obese mice to GF mice resulted in weight gain (Turnbaugh et al., 2006).

#### 1.2.4.4 *Co-housing as a Method of Microbiota Transfer*

Cohabitation has been demonstrated to have a great impact on the human microbiome (Moore and Stanley, 2016). A human study analysing 60 different families concluded that the microbiota composition of members from the same household is more similar than that of individuals from different households (Song et al., 2013). Notably, this occurs between different host species; cohabitation effects on microbiota exchange also extend to dogs and their owners (Song et al., 2013).

In preclinical research using rodent models, microbiota transfer is facilitated by their natural, coprophagic behaviour. Co-housing decreases inter-individual variability and results in a clustering effect. The effect of this co-housing effect on normalisation of the gut microbiota is robust, and therefore, a widely accepted method, eliminating the need for the investigator to perform the faecal transplantation. One of the advantages of this method is that allows for continual seeding rather than a single administration as occurs in most of faecal transplantation protocols (Moore and Stanley, 2016). Interestingly, co-housing is also efficient for transferring the phenotypes associated with certain microbiota profiles. For example, co-housing of obese with lean mice prevented obese mice from gaining weight and returned their metabolic profile to one similar to the lean mice (Ridaura et al., 2013). These effects were associated with the transfer of specific microbes from the lean to obese mice (Ridaura et al., 2013). Recently, co-housing was also shown to ameliorate social and microbial deficits in an animal model of ASD, which was induced by maternal high-fat diet (MHFD) during pregnancy (Buffington et al., 2016).

#### 1.2.4.5 *Cross-Fostering as Method for Transferring Microbes in Early-life*

Cross-fostering (CF) is a widely used laboratory practice used for many different research purposes such as for dissecting genetic and environment effects (Benus & Rondigs 1996) and it consist in an additional method used for manipulation of the gut microbiome. CF consists of the removal of offspring from the birth dam, and transfer to foster dams harbouring the desired microbiota. Different CF protocols has been

applied i.e all-litter fostered, only one pup fostered, etc. This method is particularly relevant for early-life manipulation of the gut microbiome (Moore and Stanley, 2016). Cross-fostering experiments are employed to study the role of maternal transmission of gut microbiota. For example, *TRUC* (T-bet-/- RAG2-/- ulcerative colitis) mice co-housed with the wild-type mice transmitted the phenotype to wild-type control mice (Garrett et al., 2010). In another study, NOD mice fostered by Nonobese Diabetes-Resistant (NOR) mice prevented NOD mice from developing Type-1 diabetes (Daft et al., 2015).

CF methods are also a good tool to access mother-to-offspring transmission of placental and milk compounds (Howdeshell et al., 1999). Recently it has gain a lot of popularity as some knockout strains of mice exhibit low maternal care, CF with other strain that shows high maternal care is an alternative to overcome such bias and to allow their appropriate growing process. CF contribution to behaviour has been studied in different protocols. Of interest, CF mice pups at birth was shown affect male and female adult behavior in a different manner. While female mice exhibit normal behavior and HPA-axis response was unaffected in both sex, CF males showed decreased anxiety. These effects appears to be independent of changes in maternal - offspring interaction (Bartolomucci et al., 2004). The neurobiological effects CF has been also associated to the duration of the separation events (Luchetti et al., 2015).

## 1.3 The Microbiota-gut Brain Axis

### 1.3.1.1 *The Emergence of the Microbiota-gut Brain Axis*

Over a century ago, the British surgeon Sir Arbuthnot Lane and the Nobel Prize-winning microbiologist Ilya (Elie) Metchnikoff introduced the idea that microbes could have an impact on mental health disorders. The studies of Metchnikoff demonstrated that lactic acid bacteria could slow down processes associated with ageing and senescence (Mackowiak, 2013). Additionally, Hubert J. Norman, working at the Camberwell House asylum, and George Porter Phillips Bethlem Royal Hospital, both in London ,suggested that lactic acid bacteria play a key role in psychological aspects of depression (Sherwin et al., 2017). Other contemporary scientists have contributed to the field by further describing associations between commensal

microorganisms and mental health. (See Cryan et al. (2015) for a more complete historical review). Evidence that the microbiome had the potential to regulate brain function resulted in the formation of a new interdisciplinary field of research in the 21st century. Over past two decades, a quickly growing body of research supports the idea that our gut microbiota can profoundly influence the host's CNS function and behaviour. This relationship occurs in a bi and/or multi-directional way with both top-down and bottom-up pathways. Therefore, the term gut-brain axis had been revised to accommodate the key role of the microbiota, and the term "microbiota-gut-brain axis" was introduced (Cryan and Dinan, 2012; Mayer, 2011; Rhee et al., 2009). This new and promising field of research is at its very early stages, and the mechanisms underlying the communication between the gut microbiota and brain are under ivnestigation. A number of mechanisms have been implicated, including afferent sensory neurons of the vagus nerve, neuro-immune and neuroendocrine pathways, microbial-derived metabolites such as SCFA and microbial-derived neurotransmitters (Kennedy et al., 2017) (**Figure 1.5**).

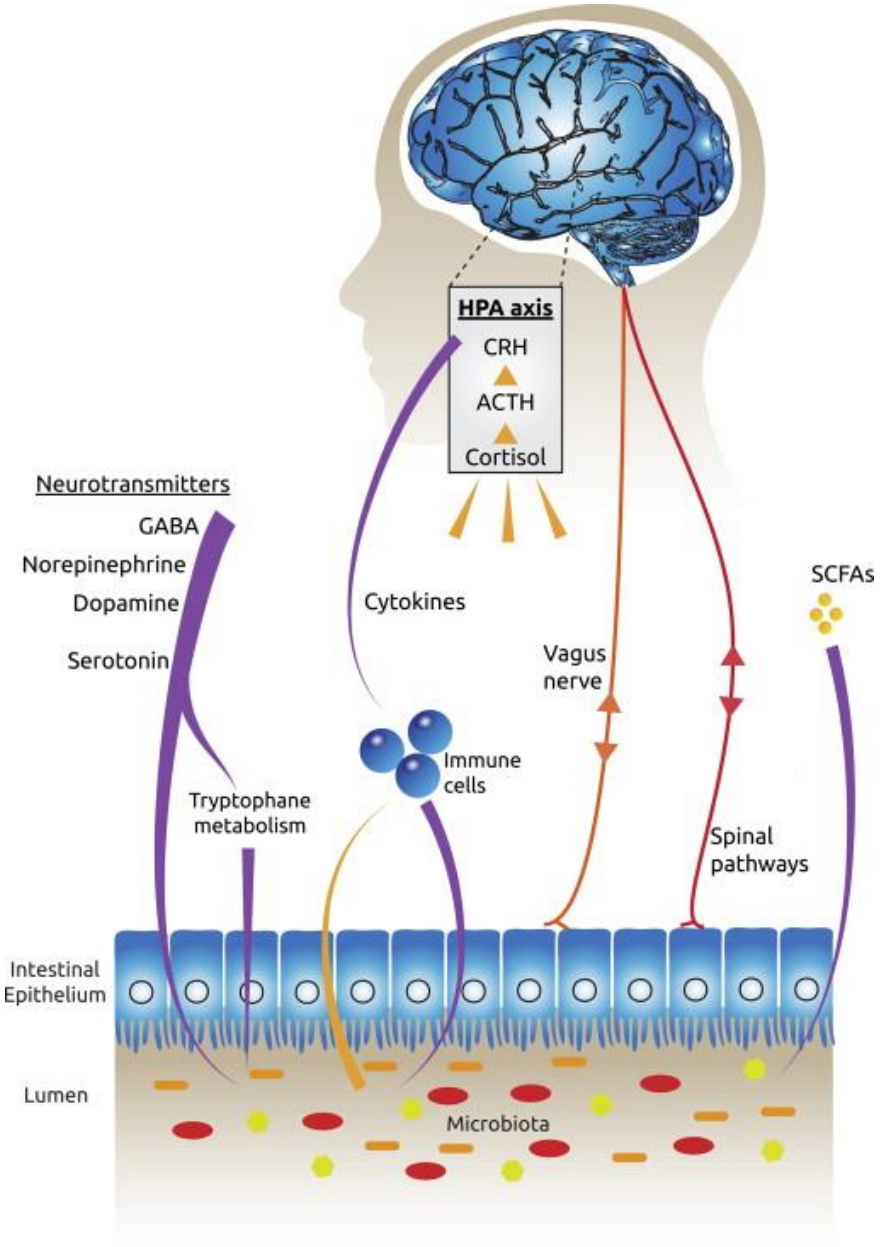



***Figure 1.5- The Microbiota gut-brain axis.***

The biderectional communicaiton between the gut microbiota and the brain is mediated by several direct and indirectpathways. The routes of communication include the ENS and ANS (vagus nerve), the neuroendocrine system and HPA axis, immune system (inflammatory mediators), and various spinal afferents and neurochemical interactions. The gut microbiota can produce neuroactive compounds as neurotransmitters (i.g. gamma-Aminobutyric acid (GABA), noradrenaline, dopamine, serotonin) and microbial metabolites such as short-chain-fatty acids (SCFA) that can either go through portal circulation or act directly on locally in the ENS and vagus nerve (i.g. serotonin (5-HT) produced by enterochromafin cells and bacterial produced SCFA). Figure taken from (Dinan et al., 2015)**.**

### 1.3.2 Microbiota-gut Brain Axis Routes of Communication

#### 1.3.2.1 *The Vagus Nerve Route*

Evidence obtained from preclinical studies has implicated the vagus nerve as the main route of communication between the abdominal cavity to the nucleus of the solitary tract (NTS) and other brain regions. The information from many peripheral organs, including stomach and intestines, is transmitted to the brain via sensory fibres (Forsythe et al., 2014). Earlier evidence pointing to a role for the vagus nerve in mediating behavioural responses comes from studies on animals which demonstrated sickness and depression-like behaviour in association to a pro-inflammatory response followed by administration of the endotoxin LPS (Konsman et al., 2000; Luheshi et al., 2000). However, such effects were abolished after vagotomy (Konsman et al., 2000; Luheshi et al., 2000). The vagus nerve was further demonstrated to sense inflammatory stimulus and sends afferent signals to the brain. Since the gut microbiome modulates many immunoregulatory pathways, it is not surprising that commensal bacteria use this path to act through the CNS and manipulate behaviour (Forsythe et al., 2014).

There is now substantial evidence of vagally-mediated communication between the microbiota and brain, obtained from animal studies. For instance, Bercik et al. (2011) demonstrated that the administration of the probiotic *Bifidobacterium longum* NCC3001 ameliorated anxiety-like behaviour and associated changes in BDNF expression in an animal model of colitis. These beneficial effects of *B. longum* disappeared following vagotomy. A similar effect was observed in studies with *Lactobacillus* spp.; the majority of *Lactobacillus rhamnosus* (JB-1) effects on emotional behaviour and associated-changes in GABA receptor expression are not sustained in vagotomised mice (Bravo et al., 2011). Furthermore, Perez-Burgos et al. (2013) demonstrated an ex-vivo experiment that the administration of *L. rhamnosus* (JB-1) immediately increased firing in vagal afferent neurons. On the other hand, *Lactobacillus reuteri* was shown to improve wound healing in mice, and that this effect was mediated by the vagus nerve (Poutahidis et al., 2013).

#### 1.3.2.2 *The Neuroendocrine and Metabolic Pathways of the Microbiota-Gut-Brain Axis*

##### 1.3.2.2.1. Microbial-derived Neurotransmitters

Among the different cell types that form the gut epithelium, there are epithelial absorptive cells, Goblet cells, Paneth cells and enteroendocrine cells (EECs). The EECs are distributed across the entire gastrointestinal tract and produce several hormones that are important for regulating multiple physiological functions (Cani and Delzenne, 2009; Gunawardene et al., 2011). Among other secretory products, different EECs types produce gut peptides, hormones and neurotransmitters such as 5-HT, somatostatin, glucagon-like peptide-1 (GLP-1), peptide YY (PYY), glicentin, ghrelin, oxyntomodulin, and orexin. It is now becoming widely appreciated that the gut microbiota can influence the activity of EECs and the release of many neuroendocrine substances (Lach et al., 2018). Moreover, the fact that several of these substances have also receptors within, or signal to the brain suggests an important role for EEC-microbiota cross-talk in brain-gut axis communication (Moran, 2006; Moran et al., 2008). Moreover, dysfunction of EEC cells is associated with many gastrointestinal and psychiatric comorbidities including IBS and obesity (Cani and Knauf, 2016).

Bidirectional neuroendocrine-intestinal signalling to the brain is supported by findings of numerous studies. For example, serotonergic EECs can act as chemosensors within the gut, controlling 5-HT release and the expression of $5\text{-HT}_3$ receptors in the primary afferent nerve fibers which can transduce the signal to the nervous system (Bellono et al., 2017). Furthermore, brain regions that control appetite can also be directly activated by gut hormones. Indeed, ghrelin, predominantly derived from the stomach, is transported across the BBB where it can then interact with mesolimbic brain areas involved in food-reward and appetite control (Banks et al., 2002; Torres-Fuentes et al., 2017). Conversely, the gut also produces anorexigenic hormones such as cholecystokinin (CCK) and peptide YY (PYY), which can induce satiety (da Silva et al., 2012). These microbial-derived molecules can modulate appetite and influence host energy balance through several possible mechanisms that include the activation of pattern recognition receptors in the intestinal epithelium, ENS and vagus nerve signaling and epigenetic mechanisms (van de Wouw et al., 2017).

In addition to neuropeptides, other important neuroactive products are released by gut bacteria, including GABA, which is produced by several *Lactobacillus* and *Bifidobacterium* strains (Barrett et al., 2012). Gut bacteria can also produce precursors of benzodiazepine receptor ligands in a rat model of encephalopathy, increasing GABAergic transmission in the brain (Yurdaydin et al., 1995). The production of the neurotransmitter histamine is also influenced by the gut microbiota (Hegstrand and Hine, 1986), and *L. reuteri* modulates histamine production in the gut (Thomas et al., 2012). Indeed, mice lacking gut bacteria have lower hypothalamic histamine concentrations (Hegstrand and Hine, 1986). Pioneering studies from the 1990's demonstrated that some pathogenic bacteria have the ability to respond to exogenous catecholamines *in vitro* and grow (Lyte and Ernst, 1992). More recently, dopamine and norepinephrine have been detected in the gut lumen of SPF mice, which are absent in GF mice, suggesting that gut bacteria are likely responsible for their production of (Asano et al., 2012).

#### 1.3.2.2.2. Short-chain Fatty Acids

The most intensively studied mechanism by which the microorganisms can influence host health is through the production of short chain fatty acids SCFA. SCFAs, which includebutyrate, propionate and acetate are bacterial metabolites derived from the enzymatic processing of nutrients and prebiotics (non-digestible fibres) (Sharon et al., 2014). They are monocarboxylic acids with a chain length of up to six carbon atoms, and comprise an essential energy source to the host. SCFAs mediate signaling pathways by a number of ways including altering histone acetylation, and by acting as endogenous ligands for free fatty acid receptors 2 and 3 (FFAR2 and FFAR3), which are G-protein-coupled receptors (Morrison and Preston, 2016). SCFAs have a modulatory effect on immunity, physiology and metabolism. They confer protection against infection and inflammation, and participate in the recruitment and maturation of various subsets of immune cells (Smith et al., 2013), including microglia, the resident brain immune cell. (Erny et al., 2015). Several studies provide evidence that SCFAs mediate gut-brain communication (Stilling et al., 2016).Propionate and butyrate have been observed to activate gluconeogenesis through a gut-brain signaling mechanism (De Vadder et al., 2014). These SCFAs can modulate brain functioning, in particular regulation of appetite through release of specific gut neuropeptides such

as PYY and GLP-1 that regulate energy homeostasis and have become a target of many anti-obesity therapies (Byrne et al., 2015).

#### 1.3.2.3 *The Immune System and the Microbiota-Gut-Brain Axis*

##### 1.3.2.3.1. The Gut Microbiota Shapes Immune System Development

It is now well established that the gut microbiota plays a significant role in the development of the immune system. In association with this, the early-life succession of mucosal colonisers occurs in parallel with the development and maturation of the mucosal immune system (El Aidy et al., 2014). The development of the immune system begins *in utero*, and extends into the postnatal period. Recently, it has been reported that the maternal microbiota modulates the growth of the innate immune cells of mouse offspring, preparing the offspring for host-microbial mutualism after birth (Gomez de Agüero et al., 2016).

Early-life is a critical period of host immune system maturation in response to microbiota colonisation (El Aidy et al., 2014; Foster et al., 2017; Rea et al., 2016). Studies utilising GF mice have revealed that the gut microbiota is essential for gut associated lymphoid tissue development and for the aggregated lymphoid follicles forming Peyer's patches (Thaiss et al., 2016). GF mice have lower number cells in the lamina propria of the intestinal epithelium, which is rich in immune cells, and reduced numbers of T-cells (CD4+ and CD8+) (El Aidy et al., 2012; Wei et al., 2010). A proper balance of effector CD4+ T cells, T helper 1 (TH1) and T helper 2 (TH2), each carrying out distinct and opposing activities, are required for the development of a healthy immune system. Notably, colonisation of the GI tract with early-life bacterial colonisers, such as *Bacteroides fragilis* and *Bifidobacterium* spp., can critically influence the maintenance of TH1/TH2 balance, and affect the production of immunomodulatory molecules. Dysregulation of this pathway is associated with allergies, autoimmune and inflammatory disorders (Mazmanian et al., 2005; Round and Mazmanian, 2009). More recently, altered levels of *B. fragilis* were associated with neurodevelopmental disorders (Hsiao et al., 2013). Several recent studies point to a key role of breastfeeding in the development of a healthy immune system, acting through the microbiome, either by directly providing the infant with milk-derived

microbes or through breast milk oligosaccharides, which can be utilised by *Bifidobacterium spp.* as a substrate for growth (Bäckhed et al., 2015; Walker and Iyengar, 2015).

By the age of three years old, a stable microbiome is established (Borre et al., 2014); however, the microbiota continues to interact with the immune system producing and releasing lymphocytes and cytokines. During a healthy state, the gut microbiota can maintain a low-level of activation of the innate immune system that starts in the intestinal mucosa surface and expands to the rest of the body (El Aidy et al., 2015). In the past half-century, as marked decreases in infectious diseases in developed countries were achieved, concomitant increases in allergies and autoimmune diseases were observed (Okada et al., 2010)This paradoxical effect is referred to as "the old friends hypothesis," which posits that proper colonisation with healthy microbiota during early-life may prime the immune system for health, whereas disrupted bacterial colonisation may increase vulnerability to develop immune-mediated diseases (Rook, 2012; Rook et al., 2014).

#### 1.3.2.3.2. Immune Signalling Across the Microbiota-Gut-Brain Axis

Given the well-established immunomodulatory properties of the gut microbiota, it is not surprising that cells of the immune system can act as an intermediary between the gut microbiota and the brain. Immune-signaling mediators, such as cytokines and chemokines, can affect the brain through the vagus nerve, or directly, via circumventricular organs (regions characterised by a permeable BBB). Microbe-associated molecular patterns (MAMPs), which include bacterial peptidoglycan and LPS, components of gram-negative cell walls, are candidates mediating this communication. The host immune system recognises bacterial LPS through Toll-like receptors (TLRs; i.e. TLR-4) which are expressed on monocytes, macrophages and microglia. For instance, LPS can reach the brain via blood circulation and act on central TLRs, initiating a cascade of neuroinflammatory processes (El Aidy et al., 2015). In conditions such as IBS, depression and ASD, increased intestinal permeability leads to translocation of bacteria and/or pro-inflammatory cytokines into the systemic circulation which have the potential to affect the brain (Kelly et al., 2015).

The peripheral immune system can also mediate this communication with the brain. As previously described, intestinal bacteria play a key role in the development of the mucosal and systemic immune system. Notably, peripheral inflammation is present in many CNS disorders, including, but not limited to anxiety and depression, ASDs, and Alzheimer's and Parkinson's diseases). However, nature of these interactions are complex, and poorly understood (Rea et al., 2016). Interestingly, a recent study demonstrated that microbiota colonisation with segmented filamentous bacteria (SFB), epithelial-associated bacteria that promote the development of TH17 cells, is sufficient to promote experimental autoimmune encephalomyelitis (EAE) in comparison to GF mice (Lee et al., 2017). The presence of the commensal bacteria *B. fragilis* can rescue the EAE phenotype (Ochoa-Repáraz et al., 2011). *B. fragilis* is known activates Treg cells in mouse colon via capsular expression of polysaccharide A (PSA), (Round and Mazmanian, 2009) and can rescue autistic phenotype in MIA mice offspring although through a PSA independent-manner (Hsiao et al., 2013).

Until recently, the central nervous system was believed to be isolated from the peripheral immune system, due to the lack of a lymphatic drainage system. However, functional lymphatic vessels lining the dural sinuses were recently discovered, and these are capable of transporting both fluid and immune cells from the cerebrospinal fluid (Louveau et al., 2015). Moreover, the migration of immune cells and mediators is still regulated by the highly selective, semipermeable BBB, which separates the circulating blood from the brain and extracellular fluid of the CNS. In addition to other cell types, the BBB is composed of astrocytes and endothelial cells (Rea et al., 2016). Not only are astrocytes important for regulation of BBB integrity, they are also the most abundant glial cells in the brain. Interestingly, they are known to participate in the gut microbiota-brain communication. For instance, gut microbes can modulate astrocyte activity, as metabolites produced by microbes can interact with type I aryl hydrocarbon receptors present on astrocytes. Consistent with this, depletion of the gut microbiota by antibiotics decreases levels of the aryl hydrocarbon receptor signalling, aggravating symptoms associated with EAE (Rothhammer et al., 2016).

Although the interaction between the gut microbiota and innate immune cells is well-established, the role of the brain's resident innate immune cells has only emerged in

the recent years, shifting the direction of research in the field (Cryan and Dinan, 2015; Foster et al., 2017; Rea et al., 2016). Microglia are the primary resident immune cells of the CNS and are responsible for initiating the cascade of neuronal responses to potential threats (including those arising from peripheral inflammation and stress). Following insult, microglia cells switch from the resting state to an activated state, resulting in morphological changes (Cryan and Dinan, 2015). Recently, a proof of concept study by Erny et al. (2015) demonstrated that a normal microbiota is necessary for healthy microglia development and maturation, activation and morphology, and this effect is mediated by bacterial-derived SCFAs. Indeed, compared to conventionally colonised controls, GF mice have increased numbers of immature microglia in several brain regions (Erny et al., 2015). Interestingly, a further study showed that microglia respond differently to the permanent lack of microbiome in males and females: in males microglia is most affected in embryonic life, whereas in females the most critical period is adulthood (Thion et al., 2018). Recently, a causal association between microbiota, SCFA, and microglia was demonstrated in the development of behavioural and neuropathological changes in α-synuclein overexpressing mice, used to model Parkinson's disease (Sampson et al., 2016). Whether microbial-microglia interactions contribute to symptoms of other neuropathological conditions that involve neuroinflammation and comorbid gastrointestinal symptoms, such as ASDs; Alzheimer's disease, and depression, is an important question to be addressed (Fung et al., 2017; Rea et al., 2016).

### 1.3.3 The Intestinal Barrier

The mucosal surface of the gastrointestinal tract is a primary site of contact between immunogenic particles, environmental toxins, microorganism-derived antigens and the immune system, and is essential for maintaining health. The intestinal mucosa is a complex barrier consisting of different layers of intestinal flora, mucus, columnar epithelia, extracellular matrix and the lamina propria. The lamina propria, the inner layer of the gastrointestinal tract, is where blood and lymph vessels, resident immune cells (i.e. lymphocytes, macrophages, eosinophils, dendritic cells and mast cells) and nerve terminals can be found. It is now well-established that these components can interact using neurotransmitters, neuropeptides, cytokines and chemokines, and neurohormones as mediators (Turner, 2009). With these characteristics, the central

role of the intestinal barrier is to regulate the absorption of nutrients, electrolytes and the secretion of antimicrobial compounds from the lumen into the circulation and to prevent the entry of harmful substances and microorganisms (Fukui, 2016).

One of the first reactions to a noxious stimulus involves the release of mucus containing secretory immunoglobulin A, defensins and other mediators that help the host fight against bacterial invasion (He et al., 2007). The commensal gut microbiota is shown to interact with these processes continuously, and changes in its composition may have implications for the maintenance of these critical barrier functions (Kelly et al., 2015).

The epithelial cells of the mucosal barrier are impermeable to most hydrophilic solutes in the absence of specific transporters. When intact, the epithelial barrier is sealed by the apical junctional complex, which is composed of adherens junctions and tight junctions. Adherens junctions are required for assembly of the tight junctions, which seal the paracellular space. Tight junctions consist of transmembrane proteins such as claudin, occludin, tight-junction protein-1 (TJP-1) and tricellulin that connect with the plasma membrane (Kelly et al., 2015). Transport across tight junctions can occur by at least two routes: (1) The leak pathway, which consists of paracellular transport of large solutes, including limited flux of proteins and bacterial LPS. Cytokines may increase the flux across the leak pathway and (2) Passage through small pores that are thought to be defined by tight junction-associated claudin proteins. These pores have a radius that excludes molecules larger than 4Å (Turner, 2009). Different aspects of intestinal epithelial barrier function have been investigated by utlilising a variety of techniques, by determining markers of physical barrier loss and methods to assess functional barrier loss in both clinical and preclinical research (See Kelly et al. (2015) for more deteailed information on available methodology).

In humans, intestinal barrier development occurs at the end of the first trimester of gestation, with the epithelial cells being formed by week eight and tight junctions by week ten. The development of intestinal barrier function continues postnatally, and is influenced by diet and feeding regimen (Cummins and Thompson, 2002). Interestingly, this process occurs in parallel with maturation of the immune system and the gut microbiota.

The role of gut microbiota in disruption of the intestinal barrier in response to stress and neuropsychiatric conditions, such as IBD, anxiety, ASD and depression is of great interest and an important focus of ongoing research. The interaction of stress, gut microbiota and intestinal barrier function has been demonstrated in numerous animal models. For example, early-life maternal separation in rats, frequently used to model early life stress, disrupts the offspring's microbiota and promotes colonic hypersensitivity, anxiety and a depression-like phenotype (Cryan and O'Mahony, 2011; O'Mahony et al., 2009). Furthermore, exposure to brief maternal separation in a limiting nesting stress protocol disrupts epithelial integrity in the large intestine and increased basal corticosterone on offspring (Moussaoui et al., 2016). Alterations in gut paracellular permeability were also demonstrated in rats submitted to a partial restraint stress, an acute stressor (Ait-Belgnaoui et al., 2005). Interestingly, restoring the gut microbiota with probiotic interventions has a beneficial effect on stress-induced changes in intestinal permeability. In one study, treatment with *Lactobacillus farciminis* prevented the leaky gut and attenuated the hypothamic-pituitary-adrenal (HPA) axis response, and decreased endotoxemia and neuroinflammation in rats exposed to partial restraint stress (Ait-Belgnaoui et al., 2012). Moreover, the same research group demonstrated beneficial effects of another probiotic formulation, a combination of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 (Probio'Stick®), in attenuating HPA axis and ANS responses and restoring gut permeability and tight junction protein levels of rats exposed to chronic stress (Ait-Belgnaoui et al., 2014). Importantly, Corticotropin-releasing factor (CRF) and its receptors act as important mediators of stress-induced gut permeability, but the precise signalling mechanisms remain poorly understood (Overman et al., 2012).

Gut barrier dysfunction is also observed in individuals with ASD and in animal models of the condition. LPS is increased in the serum of the autistic individuals compared with healthy individuals demonstrating intestinal permeability impairments (Emanuele et al., 2010). Another study by Fiorentino et.al , 2016 reported gut barrier impairments in ASD individuals, as demonstrated by decreases in tight-junction proteins (Fiorentino et al., 2016). Increased intestinal permeability in ASD children has also been reported as assessed by the lactulose: mannitol test (de Magistris et al., 2010). Interestingly, preclinical models of ASD have also revealed GI dysfunction. Alterations in gastrointestinal permeability, gut microbiota and social behaviour have

been demonstrated in both the maternal immune activation (MIA) and BTBR $T^{+}Itpr3^{tf}$/J mouse models of ASD (Golubeva et al., 2017; Hsiao, 2014).

Finally, alterations in the CNS, gut permeability and the gut-brain axis are observed in a wide range of conditions including neuropsychiatric disorders (as described above), diabetes and the metabolic syndrome, liver encephalopathy. Although the ultimate pathophysiological mechanism behind these alterations remains to be established, the gut microbiota may play an important role in their development.

## 1.4 Microbiota-gut Brain Axis and Stress

### 1.4.1 Stress

Stress has become engrained in our vocabulary and daily life, used to refer to feelings of anxiety and frustration, that affect our ability to cope. However, this use of the term stress is somewhat ambiguous. The stress response is important and necessary process, that enables one to react and cope with threats. However, chronic overactivation of the stress response can result in damage and pathological conditions (McEwen, 2006). Interest in an organism's ability to adapt to challenges was a major focus of interest in the early 20$^{th}$ century. Walter Cannon was the first to introduce and popularise the ideas of homeostasis (building upon Claude Bernard's theory of the "*milieu interieur*"), the "fight-or-fight" response, and the sympathoadrenal system (Cannon, 1929). Hans Seyle was the first to propose stress as a sickness condition (Selye, 1936). By using animal models, he demonstrated his theory of general adaptation syndrome (GAS) which is characterised by enlargement of adrenal glands, lymph node and thymic atrophy, and the development of gastric erosions/ulcers. Seyle also recognised the major role of glucocorticoids in GAS. He performed extensive structure-activity studies in the 1930s–1940s, resulting in the first rational classification of steroid hormones, e.g. corticoids, steroids/androgens, and folliculoids/estrogens. Finally, Seyle also recognised that individuals can perceive and react to stress in different ways. Sterling and Eyer coined the term allostasis, which means "achieving stability through change," referring to the active process of the body to maintain homeostasis in response to daily events. Considering the importance of acknowledging the protective, as well as the potentially damaging effects of stress, McEwen et al.

introduced the term “allostatic load,” the failure to terminate the stress response when it is no longer needed, and “allostatic overload” to denote the more extreme condition, associated with pathological states (McEwen, 2006).

The brain perceives stressful stimuli and recognises environmental threats, stores memories and adjusts the physiological as well as behavioural responses to the stressors. However, a state of allostasis involves the orchestration of many biological systems, including, but not limited to, the HPA axis, autonomic nervous system and their interaction with the metabolic and immune systems (McEwen et al., 2015) and the intestinal microbiota (Cryan and Dinan, 2012; Foster et al., 2017).

### 1.4.2 HPA Axis, Stress and Stress-related Disorders

The HPA axis coordinates emotional, immunological and autonomic inputs regulating allostasis, transducing these into behavioural, neuronal and endocrine responses. The perception of stress activates hypophysiotropic neurons of the paraventricular nucleus of the hypothalamus (PVN), which secrete releasing hormones such as CRF and vasopressin (AVP) into the brain or the portal circulation. CRF acts mainly through its receptor CRF receptor 1 (CRFR1) which is widely expressed in the anterior pituitary, but also in corticolimbic structures, including the prefrontal cortex, hippocampus, PVN and the basolateral amygdala (BLA). The release of CRH by the PVN triggers the secretion of adrenocorticotropic hormone (ACTH) into the circulation by the anterior pituitary. Circulating ACTH initiates the synthesis and release of glucocorticoid hormones (corticosterone in rats and cortisol in humans) from the adrenal cortex (Ulrich-Lai and Herman, 2009) **(Figure 1.6**).

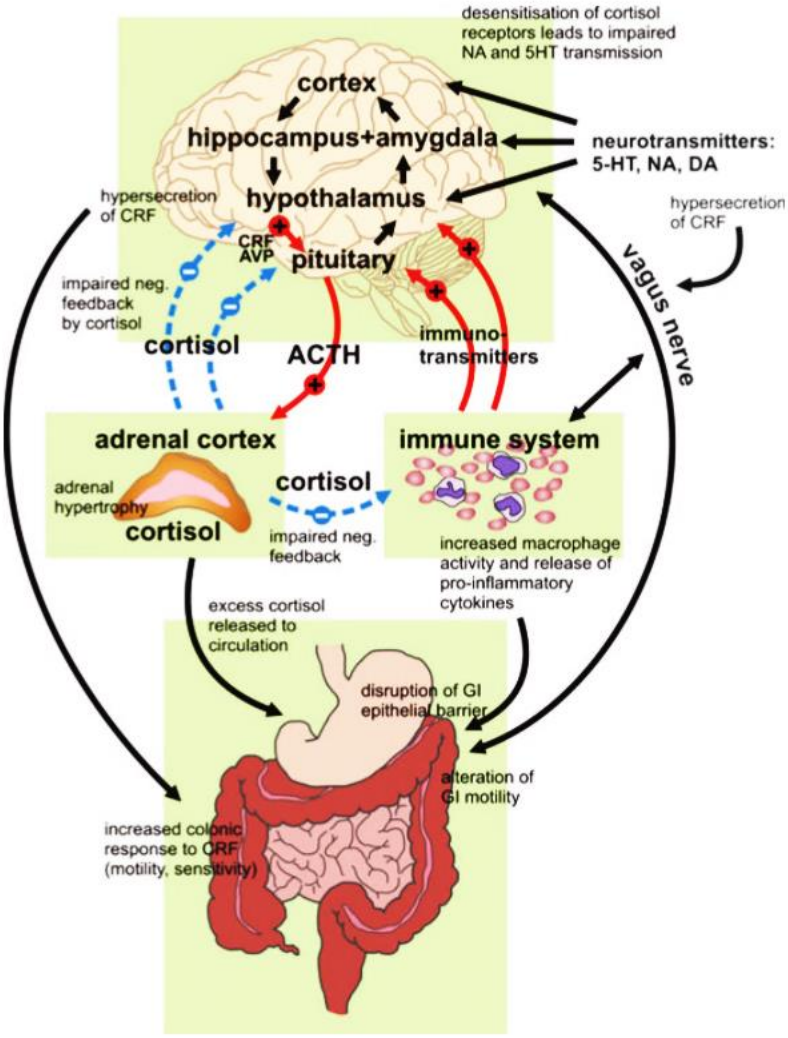



***Figure 1.6- Hypothalamic-pituitary-adrenal axis activation in response to stress and gut-brain axis activity.***

Exposure to a real or perceived stressor stimuli activates parvocellular neurons in the paraventricular nucleus of the hypothalamus that secrete corticotropin-releasing factore (CRF) and vasopressin (AVP) into the brain or the portal circulation. CRH can bind through its receptor CRF receptor 1 (CRHR1) which is widely expressed in the anterior pituitary, but also in the hippocampus, PVN and amygdala. CRH and AVP act synergistically at the pituitary to stimulate release of adrenocorticotropic hormone (ACTH), that stimulates the adrenals to produce and release corticosteroids such as cortisol (humans) and corticosterone (rodents). Dessensitisation of corticosteroid receptors leads to impaired neurotransmitter alterations (e.g. 5-HT, NA, DA) in corticolimbic structures. Activation of this pathways affect immune and gastrointestinal system. Figure taken from (Dinan and Cryan, 2013).

Glucocorticoids can bind to high-affinity mineralocorticoid receptors (MRs) and lower affinity glucocorticoid receptors (GRs). GRs are ubiquitously expressed throughout the brain and periphery, and are richly represented in the limbic system and at all levels

of the HPA axis. MR expression, however, is mainly confined to the limbic regions, with highest expression levels found in the hippocampus. The effects of glucocorticoids within the brain depends on the MR: GR balance (Joëls and de Kloet, 2017). The most well-described mechanism of action of glucocorticoid receptors is through the induction or repression of gene transcription, which occurs within the nucleus. Importantly, glucocorticoids also modulate HPA axis activity via negative feedback; glucocorticoids on receptors within the HPA axis to inhibit further release of ACTH and CRH release within minutes. This rapid feedback control does not depend on gene transcription, and is believed to be mediated through non-genomic actions of glucocorticoid receptors located within the membrane (Herman et al., 2012). Indeed, glucocorticoids were shown to act through G-protein-coupled membrane receptors, rapidly modifying neuronal function and behaviour (Orchinik et al., 1991). However, the underlying mechanisms of GR fast feedback are yet to be fully understood Additional evidence suggests that some limbic regions, such as the hippocampus and prefrontal cortex are also involved in dampening of the HPA axis response, whereas others, such as the amygdala enhance HPA axis activity (Phillips and LeDoux, 1992).

Proper basal and stress-induced function of the HPA axis is of critical importance to an organism's health, and alterations in its circuitry and connectivity underlie emotional expressions of fear and aggression, cognitive performance, motivation, reward and aversion (Joëls and de Kloet, 2017). Indeed, dysfunctional HPA axis function has been associated with a higher risk of two of the most common mental disorders: depression and anxiety, which are often considered stress-related disorders (Lopez-Duran et al., 2009). For instance, patients with major depressive disorder (MDD) are often reported to have elevated basal plasma cortisol and ACTH, whereas successful antidepressant treatment is associated with the resolution of impaired HPA axis negative feedback through normalisation of glucocorticoids receptor in the brain (Pariante, 2004). Moreover, a dysfunctional HPA axis is demonstrated to play a role in the onset of anxiety (Faravelli et al., 2012). A modest, but significantly elevated 1 h cortisol awakening response has been observed in anxious patients, especially in individuals with comorbid depression (Vreeburg et al., 2010). Stress and stress-related syndromes are complex and heterogeneous. Therefore, both diagnosis and treatment depend on taking individual behavioural, physiological or neurochemical

changes, rather than the entire phenotype into consideration. The study of these features in both humans and translational animal models can provide a more clear understanding of stress neuropathology, potentially enabling more effective therapies (Cryan and Holmes, 2005).

#### 1.4.2.1 *Laboratory-based Human Psychological Stress Procedures*

As described in the previous section, psychological stress may change the internal homeostatic state of an individual. Mason (1968) described three main psychological determinants that induce stress responses in humans: a novel situation and/or unpredictability, lack of control, and threat to the ego (Mason, 1968). Although these determinants were further debated by Seyle, studies confirmed that these psychological factors are very specific to stress situations and are quantifiable. Stress can be measured in humans through psychological questionnaires (i.e. perceived stress scale (PSS)); physiological measures (i.e. cortisol can be measured in blood or saliva); autonomic measures (blood pressure, vagal tone, salivary alpha-amylase) (Cozma et al., 2017). Relevant psychological stress measurements and test procedures used in this thesis are discussed in the next two sections.

##### 1.4.2.1.1. Perceived Stress Scale

The PSS is the most widely used psychological instrument for measuring individual perception of stress. It has been described by Cohen et al. as “a measure of the degree to which situations in one’s life are appraised as stressful” (Cohen et al., 1983). The PSS consists of a questionnaire that evaluates the degree to which an individual has perceived life as unpredictable, uncontrollable and overwhelming, and how often they have felt so during the previous month. The PSS also assesses the degree to which external demands seem to exceed the individual's perceived ability to cope (Nielsen et al., 2016).

Higher levels of psychological stress as measured by the PSS correlate with elevated physiological markers including elevated cortisol, biological ageing, and suppressed immune function, resulting in greater susceptibility to infectious disease and slower wound healing (Ebrecht et al., 2004). The questionnaire generic in nature and can be

applied to any sub-population. More recently, research from our laboratory has utilised the PSS and other tests to investigate psychological aspects of the microbiota-gut-brain axis (Allen et al., 2016, 2017a; Kennedy et al., 2017).

#### 1.4.2.1.2. The Trier Social Stress Test

The trier social stress test (TSST) is an acute stress protocol established at the University of Trier and first outlined in the early nineties (Kirschbaum et al., 1993). It has been extensively used in research that aims to investigate the neuroendocrine or psychological effects of stress, as well as in research on how specific factors (such as existing psychiatric disorders or gender differences) can contribute the stress response (Kirschbaum et al., 1995). The TSST combines many salient psychological stressors: public speaking, mental arithmetic, anticipation, social evaluation. Interestingly, studies employing the TSST demonstrate consistent and robust effects on HPA axis responses, with measures of cortisol and ACTH being the most frequently studied (Allen et al., 2017a). Furthermore, the TSST was also demonstrated to induce alterations in peripheral inflammatory markers such as cytokines, in addition to changes in lymphocytes, monocytes, neutrophils, basophils and T cells, T-helper cells and natural killer cells. The TSST protocol can be modified to include repeated measurements (e.g pre and post-stress and pre and post-treatment). In this way, the same participants are exposed to the TSST on more than one occasion (Allen et al., 2017b).

This methodological procedure has been applied in recent studies characterise the stress responses of IBS patients (Suárez-Hitz et al., 2012; Sugaya et al., 2012). Recently, a more comprehensive study used the TSST to investigate the neuroendocrine, immunological and self-reported stress and mood in female patients with IBS. The result of this study indicated a prolonged HPA axis response to acute psychosocial stress, suggesting impaired negative feedback, in addition to an increase in self-reported GI symptomatology indicating a possible use for the TSST on research investigating novel therapeutic approaches in IBS (Kennedy et al., 2014).

#### 1.4.2.2 *Modeling and Testing Stress-related Disorders in Laboratory Animals*

Charles Darwin emphasised that "the difference between the mind of the lowest man and that of the highest animal is immense" in *The Descent of Man* (1871). Nevertheless, he also observed that the expression of emotion in humans and other mammals is phylogenetically preserved: "... the young and the old of widely different races, both with man and animals, express the same state of mind by the same movements..." (Darwin, 1871). These assumptions have brought evolutionary theory close to the basis of the behavioural neuroscience (Campos et al., 2013; Cryan and Holmes, 2005).

Given that understanding emotional disorders in the clinical context has practical and ethical limitations, animal models and tests that recapitulate some aspects of the human emotional condition by correlating the physiological and behavioural changes associated with specific emotional states (face validity), the aetiology of diseases (construct validity), and responses to pharmacological treatments (predictive validity) (Cryan and Sweeney, 2011; Nestler and Hyman, 2010; Slattery and Cryan, 2017; Willner, 1984). However, in light of the differences in cognitive function between humans and animals, some aspects of the human symptomatology associated with stress are more complex and cannot be entirely modelled in animals (i.e. the feelings of worthlessness, suicidal ideation and low self-esteem are impossible to model in rodents) (Cryan and Holmes, 2005).

The majority of the stress tests evaluate behavioural and phsyiological responses induced by exposing the experimental animals to a new environment, which evokes both fear and curiosity, known as an approach-avoidance conflict. Others expose the animals to an inescapable situation leading to physical and psychological stress. Various stress stimuli can be applied in studies involving experimental models of stress-related disorders. The animal's response to stress depends on the state and conditions as well the nature of the stressor itself (e.g. duration, severity and predictability) (Campos et al., 2013).

Animal models of stress-related disorders and stress tests have different concepts: an animal model involves both a manipulation and a readout, whereas the test only focus on the measurement readout (Slattery and Cryan, 2017). Some of the most common behavioural tests used to assess stress responsivitiy include the forced-swim test (FST); tail suspension test, elevated-plus maze (EPM); sucrose preference test and marble-burying (MB). Animal models of stress and stress tests have been extensively reviewed elsewhere (Cryan and Sweeney, 2011; Nestler and Hyman, 2010; Slattery and Cryan, 2017). The following sections will focus on the behavioural tests that employed in this thesis (**Figure 1.7**).

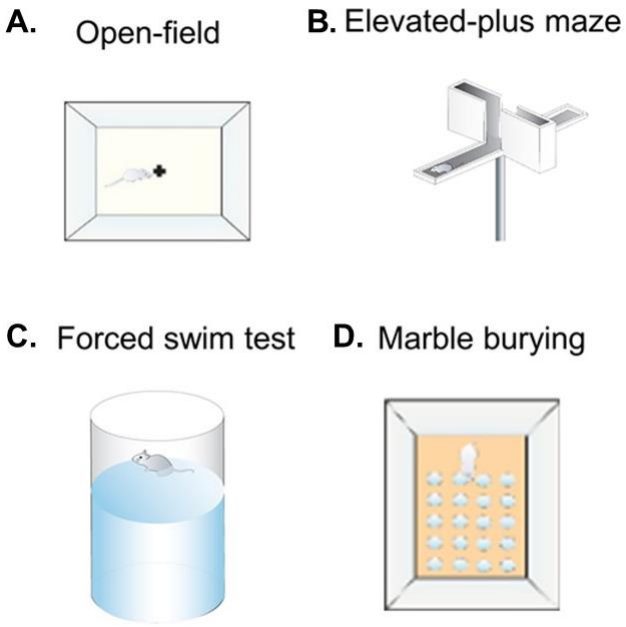



***Figure 1.7- Stress-related behavioural paradigms applied in this thesis.***
**a)** Open-field. **b)** Elevated-plus maze. **c)** Forced swim test. **d)** Marble burying

### 1.4.2.2.1. Forced-swim Test

Immobilization models produce an inescapable physical and psychological stress with a low rate of adaptation. One of the most widely used immobilisation models is the model of stress coping and helplessness, the FST, or despair test, developed by Porsolt and colleagues (Porsolt et al., 1977). This test is based on the observation that rodents placed in an enclosed (inescapable) cylinder filled with tepid water will initially engage in vigorous escape-orientated movements, subsequently followed by

increasing bouts of immobility. This animal model is one of the most frequently used behavioural paradigms and was initially developed for the screening of 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). Other immobilisation tests include the tail-suspension test, foot shock stress and restraint stress. Moreover, after immobilisation stress, animals exhibit higher levels of anxiety in the elevated plus-maze (EPM) and other anxiety-related tests (Campos et al., 2013).

#### 1.4.2.2.2. Elevated Plus-maze

The EPM is possibly the most frequently used test of anxiety-like behaviour, initially developed by Handley and Mithani (Handley and Mithani, 1984) and further validated by (File et al., 1990). 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. In the centre of the plus maze is a centre square, which separates the arms. This test is based on rodents' natural avoidance of open spaces and the conflict between this aversion and motivation to explore a novel environment.

The number of entries into, and duration of time spent in the open versus closed arms is used as a measure of anxiety-like traits. Additional ethological measures such as rearing, stretch- attend postures, and head dipping, behaviours associated with risk assessment, can also be quantified . Some studies also suggest that the number of entrances into the enclosed arm can be used as a measure of locomotor activity (Albrechet-Souza and Brandão, 2010).

The EPM permits rapid screening of anxiety-modulating agents with some distinct advantages over other more complex paradigms that require food restriction, water deprivation or shock administration. However, elevated-plus maze outcomes are sensitive to variations and influenced by a series of factors including age, sex, strain, illumination, handling, housing conditions and time of testing (Campos et al., 2013).

#### 1.4.2.2.3. Open-field

The open field test was originally designed to evaluate locomotor activity, and consists of placing an animal in an unknown environment with surrounding walls. However, it has been further adapted to include measures of anxiety and stress reactivity to a novel environment (Ennaceur and Chazot, 2016). Animals that exhibit higher trait anxiety tend to stay on the periphery of the test arena and avoid the central zone, known as thigmotaxis. They often defecate and urinate more during the test session. To generate a more aversive environment, a bright light can be applied. Bright light exacerbates fear in the novel environment as rodents feel more vulnerable to predators in brightly lit, open environments (Godsil et al., 2005).

#### 1.4.2.2.4. Marble-burying Test

Rodents often engage in a large number of natural behaviours such as repetitive behaviour including grooming and digging, however under stress situations these can be exhacerbated (Deacon, 2006). Repetitive behaviours are characteristic of some human psychiatric condition like obsessive-compulsive disorder (OCD) and ASD. The marble-burying test is a robust and sensitive test for detection of repetitive and anxiety-like behaviours (Angoa-Pérez et al., 2013). Rodents trend manifest neophobia, avoid new objects by burying them. This behaviour is responsive to pharmacological treatments, and is attenuated by low doses of benzodiazepines drugs and by 5-HT reuptake inhibitors (SSRIs), which are often prescribed for anxiety, depression or OCD (Borsini et al., 2002). However, whether marble burying test is an anxiety is not without controversy (Albelda and Joel, 2012; Deacon, 2006); in one of the first publications on the marble burying test, the authors specifically state that “marble burying may be a correlational model for detection of anxiolytics rather than an isomorphic model of anxiety” (Njung’e and Handley, 1991).

### 1.4.2.3 *Early-life Programming of HPA Axis Function and Stress Response*

Although there is great variation among species in the development of the HPA axis, in most mammals, development begins prenatally and continues postnatally. *In utero* development of the HPA axis and the corresponding increase in circulating foetal corticosterone/cortisol, and increased maternal production of prostaglandin, contributes to initiation of the birth process (Challis et al., 2001; Howland et al., 2017). In humans, basal cortisol levels decrease gradually throughout the first year of life and early childhood. From mid-childhood and adolescence, the cortisol circadian rhythm is established. HPA axis development is subject to programming by early life events (van Bodegom et al., 2017) and perturbations during critical periods of HPA axis development can have lasting effects on behavioural and emotional regulatory systems. This can, in turn, influence susceptibility to stress-related disorders which may extend to future generations (Bale, 2015; Golubeva et al., 2015; Jašarević et al., 2015a).

An emerging perspective is that the gut microbiome influences the development and regulation of the HPA axis, participating in the early-life programming of the neuroendocrine stress response system (Foster et al., 2017). In 1974, Tannock and Savage reported alterations in gut microbiota composition, and *Lactobacillus* spp., in particular, of mice exposed to a cage lacking bedding, food and water (Tannock and Savage, 1974). The relationship between stress and microbiota has since been investigated using gnotobiotic mice and various models of early-life stress (Sudo et al., 2004). The first strong evidence that exposure to stressors can impact the gut microbiota came from studies demonstrating significantly reduced *Lactobacillus spp.* populations in the gut microbiota of rhesus monkeys (*Macaca mulatta*) experiencing maternal separation early in life. Interestingly, these alterations in the gut microbiota profile correlated with stress-related behaviours (Bailey, 1999). An important study by Sudo and colleagues (2004) demonstrated that GF mice have an exaggerated stress response to restraint. The authors demonstrated that postnatal microbiota colonisation with *Bifidobacterium infantis*, which is a representative inhabitant of the neonate gut, but not *Bacteroides vulgatus*, blunted this HPA axis hyperactivity. Our group has also

observed hyperactivity of the HPA axis in GF mice exposed to a mild stressor, and this effect is reversed by post-weaning colonisation of the GI tract (Clarke et al., 2013). These and other studies using GF mice highlight the importance of commensal bacteria for proper HPA axis development. Moreover, they also demonstrate that the reversal of HPA axis hyperactivity in GF mice can only occur when colonisation is performed early in life, suggesting that there is a critical period when development is particularly vulnerable to microbiota-based manipulations.

The importance of early-life microbial colonisation in programming of the HPA axis have also been demonstrated in the maternal separation model of early life stress. Maternal separation results in increased corticosterone plasma levels, increased systemic immune response to LPS stimulation, alters visceral pain sensitivity and gastrointestinal motility, and these changes occur in concert with changes in the faecal microbiota (O'Mahony et al., 2009). Importantly, treatment with *Lactobacillus rhamnosus* and *Lactobacillus helveticus* is able to reverse HPA axis hyperactivity associated with maternal separation (Cowan et al., 2016). Additional studies have supported the associations between gut microbiota and the HPA axis by demonstrating that stress exposure early in life (during prenatal and/or postnatal period) leads to long-term effects on adult microbiota composition and priming of the HPA axis response (Bailey et al., 2011; Golubeva et al., 2015; Jašarević et al., 2015a; De Palma et al., 2015).

The logical question that arose from the finding that GF mice have abnormal microbiota development and HPA axis hypersensitivity, was whether the microbiota could also influence stress-related behaviours, such as anxiety and depression. Accordingly, Neufeld and colleagues (Neufeld et al., 2011) demonstrated a reduced baseline anxiety in female GF mice (Neufeld et al., 2011). Furthermore, independent studies reproduced this phenotype in both male and female GF mice (Clarke et al., 2013; Diaz Heijtz et al., 2011). In line with this findings, microbial restoration was able to restore a "normal" anxiety phenotype in GF mice (Clarke et al., 2013). However, conflicting results for anxiety-like behaviour have also been reported (Bercik et al., 2011a). Although the finding that GF exhibit exaggerated HPA axis responses while engaging in fewer anxiety-like behaviours seems contradictory, it is evident that normal gut microbiota is required for proper functioning of the HPA axis

and typical behavioural phenotype (Luczynski et al., 2016a). Moreover, Bercik and colleagues (2011) utilised GF mice to determine whether transfer of the gut microbiota from one strain to another could also alter behavioural phenotype. To this end, they colonised GF male NIH Swiss mice (less anxious mouse strain) with microbiota from SPF Balb/C mice (more anxious mouse strain), and an increased anxiety-like behaviour was observed (Bercik et al., 2011b).

The influence of gut microbiota on anxiety- and depressive-like behavioural phenotypes have been further demonstrated. For example, treatment with *L. rhamnosus* decreases anxiety-like and depressive-like behaviours (Bravo et al., 2011). Recently, a study from our laboratory has further documented the association between depression, anxiety and alterations in the gut microbiota by demonstrating that FMT from depressed patients to microbiota-depleted rats increased depression and anxiety-like behaviours when compared to rats that received microbiota from control donors (Kelly et al., 2016).

#### 1.4.2.4 *Gut Microbiota Coordinates Neuroendocrine-immune Interactions*

As described in the previous section, exposure to physical and psychological stress results in activation of the HPA axis and the neuroendocrine system. Stress is also associated with gastrointestinal disorders that involve activation of both immune cells and the CNS, such as IBS and IBD (Kraneveld et al., 2008). The neuroimmune system may mediate the interaction between gut microbiota and neuroendocrine responses (Rea et al., 2016). In fact, neuroimmune interactions have long been described and can occur by afferent and efferent pathways. Both innate and adaptive activation of the immune system can result in the release of inflammatory mediators, such as cytokines and chemokines, which can in turn modulate CNS activity (Kraneveld et al., 2008).

Additional inflammatory signals may also activate the local immune system including microglia, which are a source of inflammatory cytokines and chemokines, including monocyte chemoattractant protein 1 (MCP-1 ) (Rook et al., 2011). In turn, MCP-1 can recruit monocytes into the brain under the stimulus of LPS or TNFα (Deshmane et al., 2009). Interestingly, glucocorticoid receptors are highly expressed on microglia

throughout the brain (Bellavance and Rivest, 2014), supporting the hypothesised role of microglia in facilitating HPA axis and neuroimmune communication. Microglia activation and the release of inflammatory mediators has been implicated in behavioural changes observed in stress-related disorders (Blandino et al., 2013). Moreover, neuroendocrine hormones are demonstrated to modulate lymphocyte-derived neuropeptides and neurotransmitters production which will, in turn, modulate sensory perception (Kraneveld et al., 2008). Given that the gut microbiota is involved in the activation of the HPA axis by stress and inflammation, the gut microbiota may play an integral role in governing the neuro-immune-endocrine network (Error! Reference source not found.).

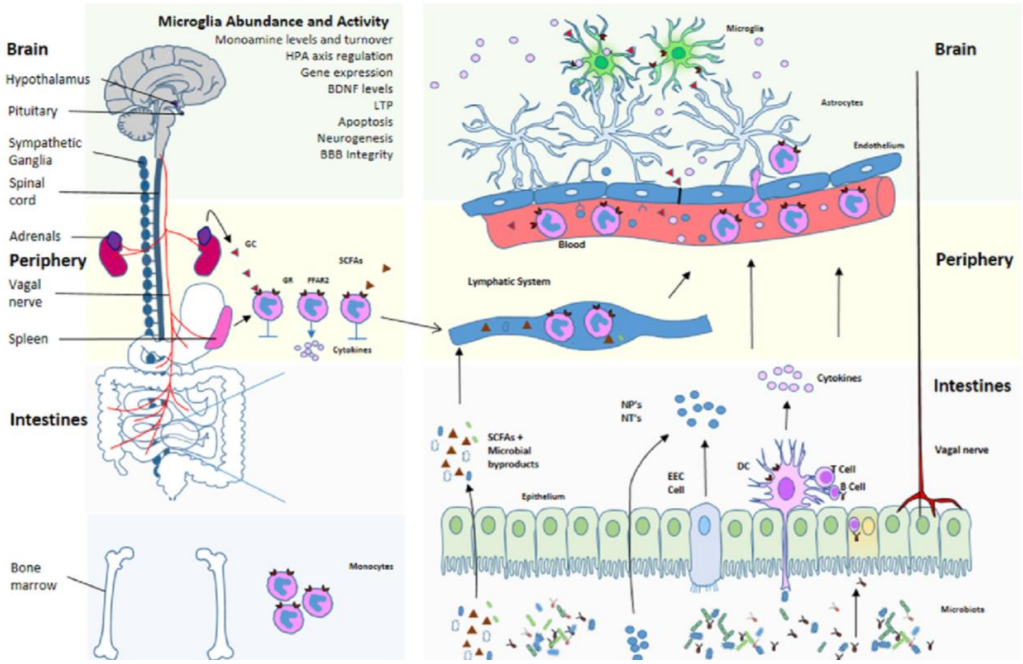



***Figure 1.8- Schematic representation of immune system, Hypothamic-pituitary-adrenal (HPA) axis and microbiota interplay.***

The possible mechanisms of interaction between CNS and the gut include activation of the vagus nerve, release of inflammatory mediators, neuropeptides and neurotransmitters, microbial-derived metabolites such as short-chain-fatty acids (SCFAs), and through lymphatic and systemic circulation. These signals can cross the blood brain barrier and influence the maturation and activation of microglia. Once activated, microglia cells exert its function on protection, synaptic pruning and clearance. The release of glucocorticoids, through activation of the HPA axis, can influence microglia and influence cytokine release and trafficking of monocytes from the periphery to the brain. BDNF Brain derived neurotrophic factor; LTP Long term potentiation; BBB Bloodbrain barrier; GC Glucocorticoids; GR Glucocorticoid receptor; FFAR Free fatty acid receptor; SCFA Short chain fatty acid; NP Neuropeptide; NT Neurotransmitter; DC Dendritic cell; EEC Enteroendocrine cell. Figure taken from Rea et al., 2016.

## 1.5 Microbiota-gut Brain Axis and Neurodevelopment

### 1.5.1 Neurodevelopment

Comparative developmental studies show that brain maturation occurs differently across altricial (early) and precocial (late) birth timing (rodent versus humans) (Workman et al., 2013). However, predictive models demonstrate that the sequence of major neuroembryological events (neurogenesis, axon extension, synaptogenesis, and axonal sorting, brain growth, and early behavioural development) follows the same order across many of the mammalian species (including rodents and humans) (Workman et al., 2013). Therefore, although there are important differences in the development of the neuroanatomical structures in rodents and humans, analogous structures can be identified and the ontogeny of specific behaviours is useful for understanding the process of brain maturation in this both species (Cryan and Holmes, 2005).

The first major neurodevelopmental event occurs during embryogenesis, specifically during neurulation, which refers to the formation of the neural tube at 3-4 weeks of gestation in humans. The cortex also starts to develop *in utero* and continues through approximately 2.5 years of life (Borre et al., 2014). Moreover, the peak of hippocampus neurogenesis is at 8–9 weeks of gestation, but the process continues postnatally. However, primate hippocampal neurogenesis was recently demonstrated to decrease rapidly following the first year of life, and is considered to be extremely rare in adult humans (Sorrells et al., 2018). In humans, the formation of functional synapses starts in the late gestational period and continues to develop into early infancy (Meredith et al., 2012). Synapse maturation continues into adolescence, stabilising in adulthood (Borre et al., 2014). Although the neurobiological mechanisms underlying development of brain function and behaviour remains to be fully understood, it is clear is that childhood and adolescence are critical periods, which are particularly vulnerable to insult, due to the dynamic changes in neuronal organisation (O' Mahony et al., 2015). Thus, perturbations of this process by perinatal factors, which may include as alterations in microbiota, can have drastic programming effects, contributing to the etiology of neurodevelopmental disorders (Kelly et al., 2017).

The use of animal models of neurodevelopmental disorders and/or early-life perturbations are of great value for understanding the mechanisms involved in long-term programming of brain development and function (Bale et al., 2010). The abnormal behavioural phenotypes observed in neurodevelopmental disorders are most likely due to "miswiring" during critical neurodevelopmental windows. Underlying mechanisms may include dysregulation of gene expression (i.e. epigenetic pathways), altered neurocircuitry, changes in the overall input and output of structures, changes in neurotrophic factors (Meredith et al., 2012). Therefore it is imperative to identify the common factors that may influence disease predisposition.

### 1.5.2 Interactions During Critical Developmental Windows

Interestingly, the rapid changes in organisation of the microbiome during the first years of life occurs in parallel with maturation of the neuroimmune and neuroendocrine systems, and development of corticolimbic structures, all of which play critical roles in stress sensitivity and vulnerability. Perturbations of any of these components may induce permanent changes that have lasting effects on stress susceptibility and mental health later in life. In fact, the period from early postnatal period through adolescence is critical for the development of the microbiota-gut-brain axis (**Figure 1.9)**. Although the temporal overlap between organisation of the gut microbiota and neurodevelopment is very clear, the nature of their relationship and possible interaction remains to be elucidated. Addressing the potential mechanisms by which the developing gut microbiota and nervous system interact opens new avenues for the creation of novel microbiota-modulating interventions in early life for the treatments of neurodevelopmental and stress-related disorders.

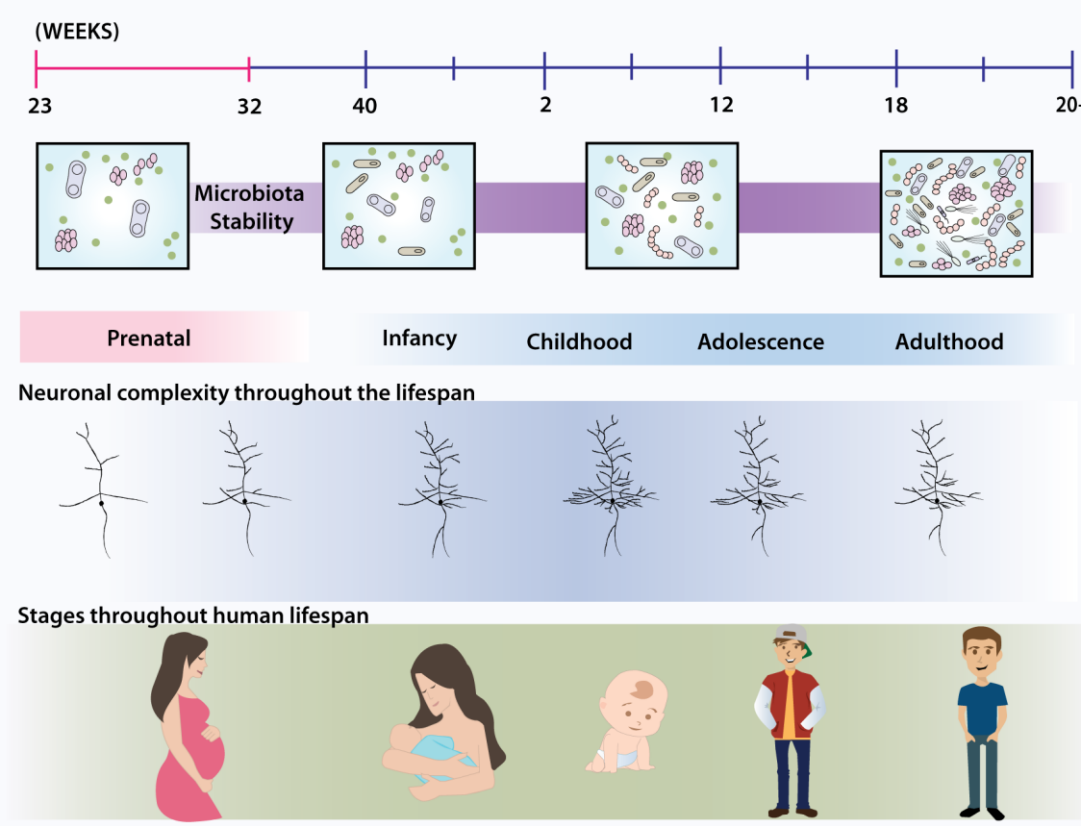



***Figure 1.9- Developmental windows for the gut microbiota and central nervous system***

Temporal profile of microbiome complexity and diversity throghout the lifespan occuring in parallalel to synaptic plasticity and neuronal growth. Early-life and adolescence are critical periods with dynamic period for both processes and windows of oportunity for microbiota-gut-brain manipulation.

### 1.5.3 The Gut Microbiota is Crucial for Development of Social Behaviour

One of the most consistent and surprising findings in the field of microbiota-gut-brain research is the that microbial communities can influence social organization and behaviour (Archie and Tung, 2015). The fundamental hypothesis for this association is based on research suggesting that the established symbiosis between host and microorganisms affects social recognition, partner selection and formation of social bonds and structure (Archie and Theis, 2011). Conversely, the microbial community can be strongly influenced by social relationships (Shawkey et al., 2007). Moreover, social interactions confer advantages to the host, by increasing species fitness, while at the same time increasing contact with symbiotic bacteria. The microbes acquired by

these interactions can be transmitted transgeneratioally, influencing evolutionary costs and species fitness (Stilling et al., 2014).

The influence of social relationships on the gut microbiome are illustrated by the transmission of microbes by co-housing/co-residency as described in section 1.2.4.4. Moreover, there is additional, robust evidence of this effect from research involving numerous species, including invertebrates, rodents and non-human primates (Archie and Tung, 2015). For instance, in newly emerged adult bumblebees, microbial transfer with nestmates' faeces is essential for seeding of the gut microbiome. This confers protection against parasite infection and mediates queen fertility (Koch and Schmid-Hempel, 2011). Moreover, changes within the social hierarchy of baboons results in alterations to both taxonomic structure of the gut microbiome and the structure of genes encoded by the microbiome (Tung et al., 2015). Negative social experiences can also affect composition of the gut microbiome following chronic unpredictable social stress in rodents, resulting in a reduced relative abundance of *Bifidobacteriaceae* and *Coriobacteriaceae* and concomitant increases in *Propionibacteriaceae* (Burokas et al., 2017).

The influence of gut microbiota on social behaviour is clearly evident in GF mice, although the effects are not always universal. In one study of GF mice, which lack gut microbiota, sociability and preference for social novelty was reduced, akin to deficits in social cognition that are present in ASD (Desbonnet et al., 2014). These behavioural abnormalities were then reversed during adolescence by colonisation of the gut with microbiota from SPF control mice (Desbonnet et al., 2014). However, using a similar approach, another group reported that the absence of gut microbiota mice resulted in an increase in social investigation when compared to SPF mice (Arentsen et al., 2015). Decreased social investigation was also observed in GF rats, reinforcing this connection (Crumeyrolle-Arias et al., 2014). Recently, a causal role for the microbiota on regulating social behaviour was demonstrated in a MHFD model of ASD where social deficits were reversed either by microbial reconstitution by co-housing or by administration of *L. reuteri* (Buffington et al., 2016).

### 1.5.4 Autism Spectrum Disorders

ASDs are a group of neurodevelopmental disorders that manifest in early childhood (Lenroot and Yeung 2013). ASD impacts one in every 68 children and its estimated prevalence is higher amongst boys (23.6 per 1,000) than girls (5.3 per 1,000) at 8 years of age (Christensen, Baio et al. 2016). Symptoms are often heterogeneous, changing over time, but are currently characterised by three fundamental domains: social communication, social interaction and repetitive behavioural patterns (Lenroot and Yeung 2013).

In addition to the core symptoms, ASDs often manifest with several psychiatric comorbidities (Plummer et al., 2016). It is estimated that between 37 and 78% of children with ASD also meet criteria for Attention Deficit and Hyperactivity Disorder (Leitner 2014), up to 30% are diagnosed with mood disorders and 56% are experience anxiety disorders (Brookman-Frazee, Stadnick et al. 2017). Other symptoms that often present in conjunction with ASDs include cognitive impairments, seizures, sleep disorders, and gastrointestinal dysfunction (Nadeau, Sulkowski et al. 2011, Clarke, Lupton et al. 2016, Nithianantharajah, Balasuriya et al. 2017).

Although research on the neuropathology of ASDs highlight the heterogeneous nature of the spectrum, some common neuroanatomical features are defined (Abrahams and Geschwind 2010). Some of the most consistent neuroanatomical changes across ASD studies are the presence of aberrant neuronal migration patterns and alterations in cortical mini-column architecture, within the frontal and temporal lobes (Opris and Casanova 2014, Reiner, Karzbrun et al. 2016). These abnormalities may affect the integration of neuronal processing across cortical layers (Ecker, Ronan et al. 2013, Opris and Casanova 2014). Other common neuropathological features are accelerated postnatal growth in specific regions of the brain including frontal and temporal cortex, cerebellum, and amygdala (Carper and Courchesne 2005). Alterations in the amygdala have long been reported in ASD, with reductions in volume and the number of neurons within the lateral nucleus among them (Baron-Cohen, Ring et al. 2000). Additional neuroanatomical changes have been reported in other brain areas, including the Purkinje cells of the cerebellum and lower grey matter volume in the hypothalamus. Importantly, the hypothalamus synthesises the hormones oxytocin (OXT) and

vasopressin (AVP), which have been also associated with alterations in behaviour and neuroendocrine function observed in ASD (Hernandez et al., 2017).

Although the etiopathology of ASD is complex and has not been fully elucidated, several studies support the hypothesis that ASD is a multifactorial hereditary disorder which includes genetic factors, environmental factors, and gene × environment interactions (Tordjman et al., 2014). More recently, a new hypothesis has emerged that implicates the gut microbiota, inflammation, infection, auto-immune disorders, environmental insults and genetic predisposition as triggers for neurodevelopmental disorders (Hsiao, 2014).

#### 1.5.4.1 *Genetic factors*

Several lines of evidence support genetics as a major factor contributing to the development of ASDs (Abrahams and Geschwind 2008). Three main methodologies have been applied to assess this relationship: (1) twin studies, comparing monozygotic twins and dizogotic twins; (2) family studies that evaluate the rate of autism in first degree relatives in comparison with rates in the general population; and (3) research that focuses on rare genetic syndromes with comorbid ASD (Geschwind 2011). Meta-analyses from twin-studies have identified a concordance rate of 98% among monozygotic twins, and 53% among dizygotic twins (Tick et al., 2016). Family studies have also revealed that first-degree relatives of ASD children exhibit a higher prevalence of behavioural or cognitive autistic-like features, in comparison with the general population (Losh et al., 2009). Some individuals carrying rare genetic conditions also manifest autistic-like features. For example, dozens of genetic syndromes including Smith-Lemli-Opitz and Joubert Syndromes, Tuberous Sclerosis, and Fragile X Syndrome lead to autism, although many with less than 50% penetrance. However, these conditions are considered rare cases, and account for less than 1% of ASD cases (Geschwind 2011).

Investigation into the genetic basis of ASDs has been enhanced by the development of cytogenetic studies, microarray analyses of single-nucleotide polymorphisms (SNPs), genome-wide association studies (GWAS), and the assessment of *a priori*-selected candidate genes known to affect brain development or implicated in

pathogenesis (e.g. *NLGN4, NRXN1, SHANK3, MET*) (Eagleson et al., 2011; De Rubeis et al., 2014). A large whole-exome sequencing (WES) study, analysing 16 sample sets comprising 15,480 DNA samples, has identified that gene variants associated with ASD risk disrupt three critical developmental pathways: chromatin remodeling, transcription and splicing, and synaptic function (De Rubeis et al., 2014). Collectively, individual genes account for 10 to 15% of cases, and variants of these and other loci are reported in 2% of children affected by ASD (Abrahams and Geschwind 2010). Due to the extensive variability in the spectrum, ASD is finally gaining recognition as a polygenic disorder with different genes implicated with each subtype of autism (Tordjman et al., 2014).

#### 1.5.4.2 *Environmental factors*

Given that genetics can only explain 10-15% of ASD cases, and that a high number of genetic disorders with epigenetic etiologies present with ASD, it has been proposed that environmental factors are required to trigger the onset of disease (Sealey et al., 2016; Tordjman et al., 2014). One strong possibility is that the origins of ASD are based on events that occur during the critical prenatal and/or early postnatal periods when the brain is most vulnerable to environmental insults (Dietert, 2011). Several environmental factors have been associated with a higher risk of neurodevelopmental disorders, including: maternal infections during pregnancy (Patterson 2002, Meyer 2014, Estes and McAllister 2016), advanced parental age (Durkin, Maenner et al. 2008, Sandin, Schendel et al. 2016), complications of pregnancy and birth such as gestational diabetes (Xiang et al., 2015), the use of antibiotics during pregnancy (Kraneveld et al., 2016) and low birth weight (Schendel and Bhasin 2008). In addition to these, excessive hygiene practices have been linked to higher rates of ASDs (Becker, 2007). Birth by caesearean section (C-section) has also been associated with an increase in ASD rates; however, this was later determined to be due to familial confounds (Curran et al., 2015a, 2015b, 2016c). Exposure to prenatal and perinatal stress (Kinney, Munir et al. 2008), maternal exposure to valproate (de Theije et al., 2014) and to certain dietary constituents (Grabrucker 2012) are also linked to ASD. Although very speculative, a growing body of literature links ASDs to gastrointestinal dysfunction, and alterations in gut microbiota composition and diversity. These

findings support the importance of the gut microbiota in early-life neurodevelopment (Hsiao et al.2013; Kranaveld et al., 2016).

#### 1.5.4.2.1. Gastrointestinal Dysfunction and Microbiota Alterations in Autism

ASD patients commonly report gastrointestinal symptoms (including diarrhoea, constipation, abdominal pain, alterations in barrier function, and/or bloating), which often correlate with the severity of the core sympthoms (Chaidez et al., 2014). For example, the presence of gastrointestinal symptoms is associated with more severe communication deficits (Gorrindo et al., 2012). These GI symptoms in ASD are reported to significantly affect quality of life (Buie et al., 2010). The prevalence of gastrointestinal symptoms in ASD varies from 9-90% (Hsiao, 2014). Although associated gastrointestinal symptoms are well-described, research on microbiota alterations are limited, and data from meta-analyses studies are inconclusive. Despite this, studies employing techniques such as bacterial culture of stool samples, qPCR, 16S rRNA gene sequencing and fluorescent in situ hybridization (FISH), consistently demonstrate increased *Clostridium spp.* in individuals with ASD. Other shifts in microbial composition include elevated Desulfovibrio and altered Bacteriodetes/Firmicutes ratio (Kraneveld et al., 2016).

Most recently, GI dysfunction and microbiota alterations have been demonstrated to have considerable impact on ASD pathogenesis. For instance, mice exposed to valproate *in utero* exhibit alterations in the phyla Bacteroidetes and Firmicutes and the order of Desulfovibrionales, similar to what is observed in the human condition (de Theije et al., 2014). Interestingly, these changes were accompanied by increased levels of the bacterial metabolite butyrate, ileal inflammation, decreased levels of intestinal 5-HT and deficits in social behaviours (de Theije et al., 2014). Altered composition of the gut microbiota and several of its bacterial metabolites have also been observed in the polyinosinic-polycytidylic acid (poly (I:C)) rodent model of ASDs and are associated with gut barrier dysfunction and impairments in social behaviour (Hsiao et al., 2013). Interestingly, the injection of 4-ethylphenylsulfate, a bacterial-derived metabolite, is sufficient to induce anxiety-like behaviour in mice (Hsiao et al., 2013).

Moreover, the administration of large amounts of propionic acid, a SCFA, has been associated with an autistic-like phenotype in rodents (MacFabe, 2012). Interestingly, propionic acid is a fermentation product produced by Clostridia, *Bacteroidetes* and *Desulfovibrio* (MacFabe, 2012). In line with these findings, the presence of certain gut microbial communities that promote a pro-inflammatory status, particularly excessive $T_H17$ cell stimulation, has been implicated with ASD in the Poly (I:C) animal model (Kim et al., 2017). Recently, a reduction in the relative abundance of *Bifidobacterium* and *Blautia* microbial species was associated with deficient bile acid and tryptophan metabolism in the intestine and marked gastrointestinal dysfunction in a BTBR $T^{+}Itpr3^{tf}$/J mouse model of ASD (Golubeva et al., 2017) (Figure 1.10).

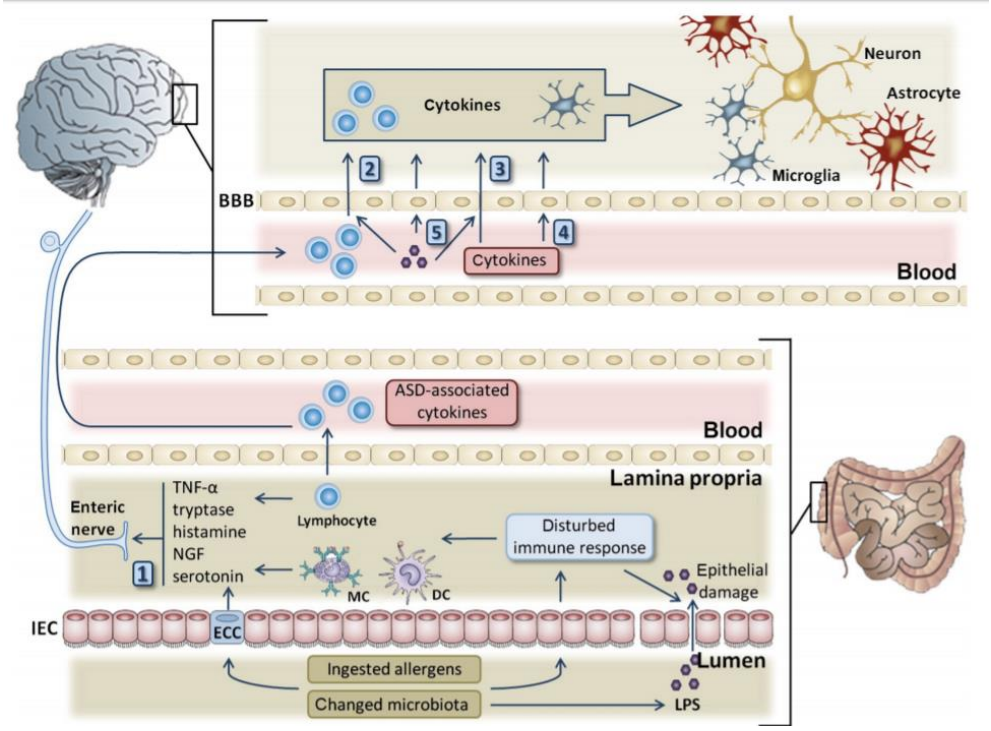



***Figure 1.10- Microbiota-gut brain axis and autism spectrum disorders.***

Potential mechanisms mediating the role of the microbiota-gut-brain axis in autism include neuroimmuno-endocrine interactions. BBB, blood brain barrier; LPS, lipopolysaccharide; EEC, enterochromaffin epithelial cell; IEC, intestinal epithelial cells; MC, mast cell; DC, dendritic cell; TNF, tumor necrosis factor; NGF, nerve growth factor. Figure taken from Kraneveld et al., 2016.

### 1.5.5 Maternal Immune Activation and the Risk for Neurodevelopmental Disorders

Over 50 years ago, the reported rates of ASDs and Schizophrenia (SZ) increased from less than 1% to approximately 13 and 20%, respectively, following congenital rubella infection (Meltzer and Van de Water, 2017; Patterson, 2002). Subsequent studies including prospective analyses reported an increase in SZ, ASD and mood disorders following bouts of flu, measles, mumps, chickenpox, and polio (Reisinger et al., 2015). Gestational exposure to other viruses such as the *Herpes simplex* virus and the parasites like *Toxoplasma gondii* have also been linked to development of SZ (Brown, 2006). More recently, the epidemic of Zika virus-induced microcephaly raised significant public health concerns as to the deleterious effects of maternal infection during pregnancy (Jurado et al., 2017).

Mounting evidence implicates dysfunctional immune responses, inflammation, cytokine dysregulation, and anti-brain autoantibodies in ASD. Given the influence of the immune system on neurodevelopment, cognitive function, and behaviour (Onore et al., 2012), an important hypothesis has emerged relating to the timing of maternal immune stimulation and the appearance of neurodevelopmental disorders in offspring (Estes and McAllister, 2016). Moreover, abnormal immune response to environmental stimuli are also known to strongly impact ASD (Meltzer and Van de Water, 2017; Patrich et al., 2016). These associations are supported by evidence that activation of the maternal immune system is a common factor in various other neurodevelopmental disorders, including SZ and depression. Therefore, whether maternal immune activation (MIA) has a causal role in neurodevelopmental disorders in being investigated in animal models (Estes and McAllister, 2016; Meyer et al., 2007; Shi et al., 2003).

#### 1.5.5.1 *Animal Models of Maternal Immune Activation*

Maternal Immune Activation (MIA) has been studied in animal models by exposing the dam to a variety of immunogens during a specific periods of gestation and then characterising the alterations in brain and behaviour of offspring (Careaga et al., 2017).

One of the very first models of MIA used live influenza virus (H1N1) to recapitulate some of the epidemiological observations obtained following the influenza epidemic of 1957 (Fatemi et al., 2002). MIA on gestational day 9 in mice was demonstrated to induce both short- and long-term effects on offspring brain structure and development, including abnormal corticogenesis and alterations in pyramidal and non-pyramidal cell (Fatemi et al., 2002). Currently, the most commonly used models are based on the administration of immunogenic substances such as the bacterial endotoxin LPS which targets Toll-like receptor 4 (TLR-4) ,and poly- (I:C), the double-stranded RNA (dsRNA) analog, which acts on TLR-3 (Reisinger et al., 2015; Shi et al., 2003; Smith et al., 2007; Spencer and Meyer, 2017; Straley et al., 2017). These substances robustly stimulate the maternal immune system, and the production and release of many pro-inflammatory mediators, including Interferon (IFN)-α and IFN-β, IL-1β, IL-6, and TNF-α (Meyer and Feldon, 2012). Although both substances produce these effects via different mechanisms, they both reproduce various aspects of psychiatric disorders, such as ASD, SZ and depression, in offspring (Reisinger et al., 2015). Models based on the administration of both LPS and poly (I:C) meet the three major criteria of validity for an appropriate animal model: they mimic a known disease-related risk factor (construct validity), they exhibit a wide range of disease-related symptoms (face validity), and they can be used to predict the efficacy of treatments (predictive validity) (Estes and McAllister, 2016). Given the limitations of current treatments and interventions for most neurodevelopmental conditions, the MIA model is an important tool for investigating novel therapies (Careaga et al., 2017) (Figure 1.11).

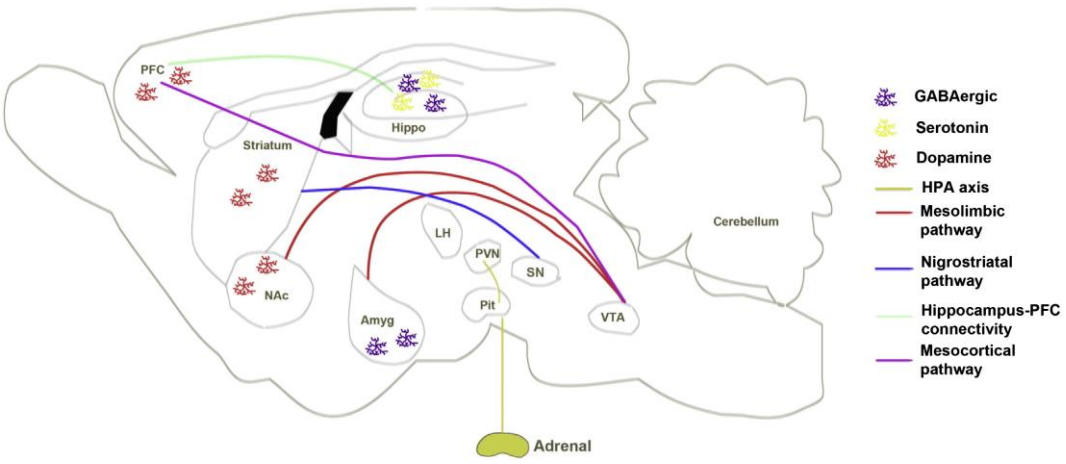



***Figure 1.11. Structure and function of brain regions impacted by poly(I:C)-induced maternal immune activation.***

Abnormalities in the HPA axis commonly associated with depression-like behaviour in offspring after poly(I:C) MIA have been documented (Babri et al., 2013). Alterations in the mesolimbic pathway, projecting from the VTA to the NAc and the Amyg, related to schizophrenia, have been identified in offspring exposed to poly(I:C) MIA (Zuckerman et al., 2003). Prenatal poly(I:C) is reported to compromise the function of the SN and striatum (Deleidi et al., 2010*). Abbreviations*: HPA: hypothalamic pituitary adrenal, VTA: ventral tegmental area, NAc: nucleus accumbens, Amyg: amygdala, SN: substantia nigra, Hipp: hippocampus, PFC: prefrontal cortex, GABA: γ-aminobutyric acid, Poly(I:C): polyinosinic:polycytidylic acid, MIA: maternal immune activation. Figure and legend modified from Reisinger et al., 2015.

## 1.6 Microbiota-gut Brain Axis and Social Behaviour

### 1.6.1 Social Behaviour

Social interaction is essential for reproduction and survival for humans. However, the need for interaction is not exclusive to humans and it is manifested early in evolution of both invertebrates and vertebrates (Carter, 2014). In fact, different species use social behavior strategies in response to specific selective forces driven by their environments (Johnson and Young, 2017). Even prokaryotes use advanced cooperative motility behavior, as observed in *Paenibacillus vortex*, which confers adaptive value to the species by increasing its survival and replication (Ingham and Ben Jacob, 2008). Although common among many forms of life, social behaviour is likely to have multiple genetic and physiological origins and many neuronal substrates. Whether social tendency is governed by a common genetic core remains to be determined (Carter, 2014).

The term “social neuroscience” was coined in the early 1990s to describe a research movement to understand the neural basis of social behaviour (Cacioppo et al., 2010). To study this connection, behavioural neuroscientists created a comprehensive set of behavioural assays for studying the neuronal basis of social behaviour and social-related disorders. Although many of the behavioural tests were originally designed for rats, they have been successfully adapted to mouse models reproducing robust features of social-related disorders (Silverman et al., 2010a). The use of mice for studying social behaviour is of scientific value because they manifest many types of social behaviour such as social investigation of an unfamiliar conspecific, communal nesting, sleeping in group huddles, aggression directed towards intruders, sexual approach and

mating behavior patterns, parental care of the pups and juvenile play (Crawley, 2007; Kaidanovich-Beilin et al., 2011). Specific assays applied in this thesis are described below and in the **Figure 1.12**.

## 1.6.2 Social Behavioural Tests

### 1.6.2.1 *Pup Ultrasonic Vocalization (USV)*

Mounting evidence suggests that disruptions in brain development in early life can contribute to the prevalence and severity of ASD and other neurodevelopmental disorders. Thus, it is important that animal models that are used for studying neurodevelopmental disorders are behaviourally phenotyped during the developmental period. In this regard, USV is being used as a measure of early communicative behaviour and as an aversive affective measure of separation stress (Scattoni et al., 2009). Pup vocalizations are important cues for maternal orientation, approach and retrieval, thus influencing the quality of maternal care overall and can be quantitatively measured with minimum manipulation of the offspring (Nagasawa et al., 2012). The range frequencies of isolation-induced USVs in pups vary from 30 kHz to 90 kHz, varying across strains and developmental age (Branchi et al., 2001). Usually, outbred pups elicit a higher number of USV calls around P8 and decreases to zero by two weeks. In contrast, C57Bl/6 strain pups exhibit lower calling rate and earlier profile peak around P3 (Scattoni et al., 2009). Although in this thesis we only focus on pup USVs, it is important to note that adult USVs can also be determined during the behavioural phenotyping of mouse models of human neurodevelopmental and neuropsychiatric disorders.

### 1.6.2.2 *Homing Test (attachment)*

Attachment is a psychological construct reflecting a strong emotional bond between two individuals, such as mother and offspring (Porges, 2003). The homing test paradigm is an additional test to access social behavioural deficits in early-life. It is a measurement of neonatal social recognition, maternal attachment, and early motivation towards a relevant social stimulus (2011). Pups are given the choice between a familiar odour (their own mother's nest) and a neutral odour (neutral

bedding or other mother nest). The test is conducted at the point of locomotive behavioural development when pups are able to coordinate their body movements towards the olfactory stimulus. As their eyes are still closed, their orientation towards the maternal nest occurs through olfactory system.

#### 1.6.2.3 *Three-chamber Test and Reciprocal Social Interaction Test*

The three-chamber test was originally proposed to evaluate social approach behaviors relevant to social disorders such as ASDs, social phobias and social anxiety, and schizophrenia (Moy et al., 2004; Nadler et al., 2004). It consists of a three-chambered apparatus where mice are given the choice between spending time in a chamber containing conspecific mice or an object, or in an empty compartment (Crawley et al 2007). Rodent tendency to spend more time with another rodent as compared with an object or an empty compartment represents sociability. An important aspect of social behaviour that can also be investigated in the three-chamber test is the preference for social novelty. Normally, when rodents are given the choice between a novel and a familiar rodent, they would prefer to spend more time with a novel rodent than a familiar one. In addition, this part of the test incorporates aspects of cognition in the form of interest in social novelty (Kaidanovich-Beilin et al., 2011). Thus, animal models that intent to mimic social deficits present in ASD and other social-related disorders usually demonstrate a deficit in sociability and/or decreased preference for social novelty (Crawley, 2012).

Sociability can also be investigated throughout other paradigm: the reciprocal social interaction test. This test was shown to be more sensitive for detecting deficits in social interaction in some peculiar cases where social approach is intact in animals that are expected to have sociability deficits (Kaidanovich-Beilin et al., 2011). The social interaction test offers the possibility to quantify different aspects of social behavioural traits such as nose-to-nose sniffing, anogenital sniffing, following the other mouse, initiating physical contact, and emitting ultrasonic vocalizations. Social interaction in juvenile mice has also been used as a readout of social withdrawal (Wilson et al., 2009) and social reward (Panksepp et al., 1984).

### 1.6.2.4 *Social Transmission of Food Preference*

The social transmission of food preference (STFP) is a social behaviour task that allows the investigation of social cognition components based on olfactory memory (Wrenn, 2004). In addition, the test is based on the principle that dietary information can be communicated between rodents during social interaction (Galef and Kennett, 1987). Smelling a novel food odour on another rodent could indicate that this food is safe to eat. The test consists in letting the "observer" mice interact with a "demonstrator" mouse that has recently been exposed to a novel food. When observer mice are given the choice between the food eaten by the demonstrator and the novel food, observer mice prefer the food eaten by the demonstrator. Social transmission of food preference depends on the observer mice detecting olfactory cues during social interaction with the demonstrator. The preference for the cued food serves as a measure of social memory (Wrenn, 2004).

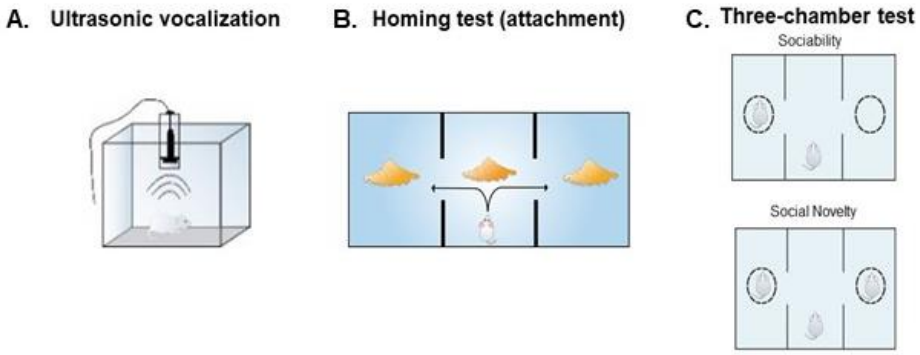



***Figure 1.12- Social behavioural paradigms applied in this thesis.***
**a)** Ultrasonic vocalization. **b)** Homing test (attachment). **c)** Three-chamber test.

## 1.6.3 Oxytocin and Vasopressin: Social Hormones

Oxytocin (OXT) and its analogue, vasopressin (AVP), are nonapeptides that are present in all placental mammals (Van Kesteren et al., 1995). OXT was the first neurophypophysial hormone to have its structure described, and the first to be chemically synthetized in a biologically active form (Vigneaud et al., 1953). Its genomic structure was elucidated in 1984 (Ivell et al., 1986), and its sequence was

described in 1992 (Kimura et al., 1992). During evolution, OXT, AVP, and their homologues remained highly conserved across the animal kingdom and are present in mammals (as OXT and AVP), bony fish (as isotoicin and vasotocin), other non-mammalian vertebrates (as mesotocin and vasotocin) and several invertebrates (as echinotocin, cephaloptocin, annetocin, annepressin, conopressin and inotocin) (Knobloch and Grinevich, 2014). Within the mammalian lineage these 2 peptides differ by a single amino acid, and both OXT and AVP genes are localized adjacent to one another on the same chromosome, but are oppositely transcribed (Feldman et al., 2016).

Interestingly, across species, OXT/AVP and their homologues play a critical role in adapting organisms to changes (Manning et al., 2012) that affect both behaviour and physiology (Feldman et al., 2016). OXT and AVP can modulate a wide range of biological functions, including neuroendocrine function, vocalization, regulation of emotional and stress responses, social bonding, affiliative behaviours, mating, parturition, milk ejection, ejaculation and cardiovascular tone (Gimpl and Fahrenholz 2001, Knobloch and Grinevich 2014, Dumais and Veenema 2016). Due to their sexual dimorphic actions, some of their roles differ in males and feels (Dumais and Veenema 2016). However, in general, OXT and AVP facilitate reproduction and social behaviour in all vertebrates, which is critical to survival and fitness of the species (Gimpl and Fahrenholz 2001).

#### 1.6.3.1 *Oxytocin and Vasopressin: Production and Release*

In vertebrates, OXT and AVP are predominantly synthesized in parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) and the supraoptic nucleus of the hypothalamus (SON) (Lee et al., 2009). However, some OXT synthesizing cells are also present in the accessory magnocellular nucleus of the hypothalamus in rodents and humans (Dumais and Veenema, 2016). OXT-producing neurons of the PVN, SON and magnocellular nucleus project to the posterior pituitary gland where OXT can be stored and/or released into the bloodstream (Lee et al., 2009). Moreover, OXT-synthesising neurons project centrally, where OXT and/or AVP can bind to receptors (Knobloch and Grinevich, 2014) In addition, OXT can also be synthesised

peripherally, in a number of tissues including the uterus, placenta, amnion, corpus luteum, testis and heart (Gimpl and Fahrenholz, 2001).

OXT and AVP are stored in large vesicles spread throughout the soma, dendrites and axons of OXT and AVP neurons. These vesicles can be exocytosed in response to increased concentrations of $Ca^{2+}$ and to a variety of osmotic, reproductive, stress, social and other behavioural and physiological stimuli (Lee et al., 2009). Release of OXT can occur via a number of routes, which include classical synaptic transmission, dendritic release and global diffusion (Knobloch and Grinevich 2014). For example, OXT secretion can occur by somatodendritic exocytosis, in which the peptide is diffused to hypothalamic and extra-hypothalamic areas and directly into the ventricular circulation. Moreover, axonal release of OXT/AVP from neurons innervating extrahypothalamic brain regions may underlie behavioural responses to peptide signaling. It has also been proposed that non-synaptic release of OXT can occur via periaxonal exocytosis or *en passant* OXT release of the vesicles (Johnson and Young 2017).

In rodents, OXT/AVP neurons of the hypothalamus mainly express vesicular glutamate transporter 2, and the depolarization of these neurons may involve synaptic glutamate release. In addition, OXT-OXT and AVP-AVP monocellular neurons are connected by intrahypothalamic glutamatergic synapses. However, axonal and somatodendric release of OXT can occur without depolarization, through the activation of melanocortin-4 receptors (MC4R4) and the transmembrane protein CD38 which are expressed in OXT neurons. Both pathways play an important role in triggering OXT secretion and modulating social behaviour (Bartz and McInnes 2007).

#### 1.6.3.2 *Oxytocin and Vasopressin Receptors*

AVP and OXT can act through three distinct AVP receptors, V1a, V1b, and V2, and one OXT receptor. Unlike the conserved nature of OXT and AVP and their analogue peptides and genes, receptor expression varies and is species-specific (Johnson and Young 2017). Moreover, genetic variations in the OXT/AVP gene region and polymorphisms in non-coding regions of OXTR and AVPR, predict individual

variations in behaviour that may be relevant to neuropathologies relating to OXT/AVP signalling in humans (Chen et al., 2011).

The biological effects of OXT and AVP can exert their effects at very low concentrations through binding to their specific receptors (OXTR and AVPR) for which they have great affinity and (Johnson and Young 2017). OXTRs and AVPRs belong to the G-protein coupled receptor (GPCR) family, having seven putative transmembrane helices. OXTR is a 389 amino acid polypeptide that is located at 3p25-3p26.2 (Manning et al., 2012).

Though agonists and antagonists of OXT and AVP are important in elucidating key mechanisms in their biological function, there are a number of caveats to note: their lack of receptor selectivity (Johnson and Young 2017), species-specificity (Manning et al., 2012), complementarity between OXTR and AVPR expression in the brain, and sex-specific expression (Dumais and Veenema 2016) (**Figure 1.13**).

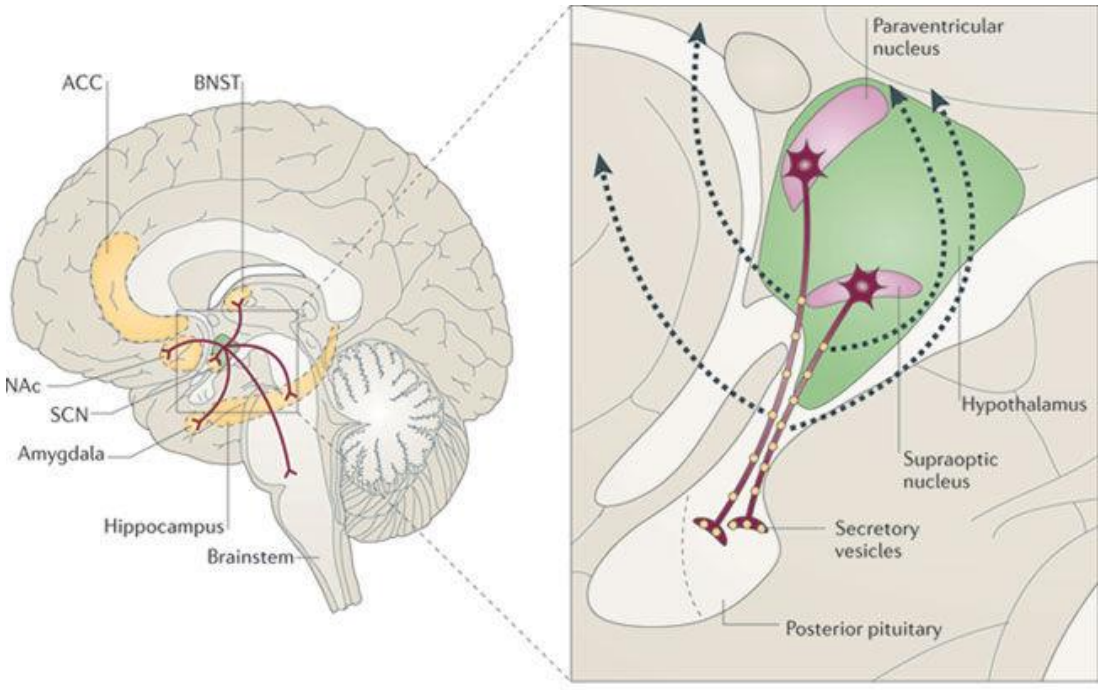



***Figure 1.13- Oxytocin and vasopressin production and release sites in the brain.***

Oxytocin (OXT) and arginine vasopressin (AVP) are synthesized in magnocellular in the paraventricular neurons and supraoptic nuclei of the hypothalamus, and are stored in the posterior lobe of the pituitary, and released into peripheral circulation or brain. OXT and AVP receptors are expressed

in different brain areas including the amygdala, hippocampus, striatum, suprachiasmatic nucleus, bed nucleus of stria terminalis and brainstem — where they act as neuromodulators or neurotransmitters, and thereby influence neurotransmission in these areas. ACC, anterior cingulate cortex; BNST, bed nucleus of the stria terminalis; NAc, nucleus accumbens; SCN, suprachiasmatic nucleus. Figure from (Meyer-Lindenberg et al., 2011)

#### 1.6.3.2.1. Distribution of OXTR in the Brain

The ontogeny of OXTR varies within species and age (Johnson and Young 2017) and it has been investigated predominantly by two methods: expression of the mRNA transcript and ligand binding studies using autoradiography (Vaidyanathan and Hammock 2017). Receptor autoradiography had been described as the most commonly used and reliable method for localisation of OXTR in rodent brain tissue (Elands, Beetsma et al. 1988), but the OXTR radioligand is now known to bind to both human OXTR and AVPR1a with a high affinity (King 2016). More recently, studies have pharmacologically optimized OXTR receptor autoradiography, resulting in the first reliable technique for specifically identifying OXTR (and AVPR1a) in human and nonhuman primate brain tissue (Freeman et al., 2017).

Although analyses of OXTR in rodent brain have revealed some variations in the OXTR distribution in mice and in rats (King et al., 2016), they confirm that OXTR are strongly and widely expressed in brain during the postnatal period through weaning, and expression decreases into adulthood (Insel and Shapiro, 1992). In the rat brain, robust OXTR labeling has been observed in the posterior cingulate cortex, dorsal subiculum, lateral septum, and the CA1 subfield of hippocampus during the early postnatal period. However, of these regions, only the lateral septum expresses oxytocin receptors in adulthood. Conversely, OXTR binding in the bed nucleus of the *stria terminalis* and in the ventromedial nucleus of the hypothalamus are only obseved in adulthood (Shapiro and Insel, 1989) (Shapiro and Insel 1989). OXTR distribution in the brain is also regulated in a regional manner by gonadal steroids (Insel et al., 1997).

Detailed analyses performed by Hammock and Levitt (2013) revealed differences in OXTR distribution in the mouse brain when compared to that of the rat. In the mouse brain, OXTR ligand binding is evident in the lateral septum, diagonal band of broca,

piriform cortex, central and medial amygdala, and hypothalamus. However, OXTR binding has only been detected in the CA3 subregion of the hippocampus (Hammock and Levitt, 2013). OXTR binding is also expressed in the accessory and main olfactory bulbs, claustrum, endopiriform cortex, bed nucleus of the *stria terminalis*, ventral caudatoputamen, and the periventricular thalamus for the majority of the ages examined. Expression of OXTR binding in rats contrasts with the data from mice within in the neocortex; while there is a transient and limited OXTR ligand binding in the neocortex of rats (Shapiro and Insel, 1989), the data from mice demonstrate a much broader OXTR expression within this area (Hammock and Levitt, 2013). In addition, voles have a much wider neocortical expression of OXTR than mice and rats with peak binding in some brain areas during the 2nd and 3rd postnatal weeks (Wang and Young 1997).

*Oxtr* mRNA expression in rat (Chen et al., 2000) and mouse brain (Tamborski et al., 2016) has been detected at E12 by qPCR, and at E13 by *in situ* hybridization in rats (Yoshimura et al., 1996). By E13 *Oxtr* mRNA was also detected in the dorsal motor nucleus of the vagus nerve, with a progressive increase in expression in other regions over time. In this study, the authors noted two distinct expression patterns: transient and constant (Yoshimura et al., 1996). Both patterns were observed in brainstem, mesencephalon, diencephalon, and telencephalon. Transient *Oxtr* mRNA expression was detected in the caudate, putamen, cingulate cortex, anterior thalamic nuclei, ventral tegmental area, and the hypoglossal nucleus during the early postnatal period, whereas while constant expression, once detected, persisted into adulthood in the anterior olfactory nucleus, tenia tecta, some amygdaloid nuclei, piriform cortex, the ventromedial hypothalamic nucleus, subiculum, and the dorsal motor nucleus of the vagus (Yoshimura et al., 1996).

The expression patterns of OXTR in the human brain is sparse due to the lack of specificity in methods currently available (Freeman et al., 2017). Using autoradiography, OXTR binding in human brain was localised in the ventrolateral septum, and throughout the preoptic and hypothalamic area, similar to observations in rodents (Loup et al., 1989, 1991). In contrast to rodents, no binding in human amygdala or hippocampus was observed (Loup et al., 1989, 1991). However, the validity of these studies has been questioned since a subsequent study has

demonstrated that $^{125}$I-OTA ligand binds to AVP receptor 1a as strongly as OXTR in non-human primate brain (Toloczko et al., 1997).

Human OXTR expression has also been investigated using immunohistochemistry, but the reliability of this method has also been questioned (Yoshida et al., 2009). These studies demonstrated the presence of the OXTR in cortical, limbic and brainstem areas of female brain tissue. OXTRs were also visualized in discrete cell bodies and/or fibers in the central and basolateral regions of the amygdala, medial preoptic area (MPOA), anterior and ventromedial hypothalamus, olfactory nucleus, vertical limb of the diagonal band of broca, ventrolateral septum, anterior cingulate and hypoglossal and solitary nuclei. OXTR staining was not observed in the hippocampus (including CA2 and CA3 subregions), parietal cortex, raphe nucleus, nucleus ambiguus or pons (Boccia et al., 2013).

### 1.6.4 Functions of Oxytocin

OXT regulates a wide range of behavioural and physiological responses. The most well-studied of OXT functions pertain to parturition, lactation and pair bonding (Lee et al., 2009, Waldherr and Neumann, 2007). However, OXT has wide-ranging effects and can also influence interpersonal trust, anxiety, HPA axis and stress responses, immune system development and responses, memory formation and information processing (Buisman et al., 2015). Some aspects of OXT function will be discussed extensively in this chapter.

#### 1.6.4.1 *Developmental Effects of Oxytocin*

The OXT system starts to develop *in utero* and continues developing throughout early-life and adolescence in both rodents and humans (Buisman-Pijlman et al., 2014). Despite variations across species, the peak of OXTR binding occurs prior to weaning and coincides with critical periods for brain wiring and development (Hammock and Levitt 2013). This overlapping time-window leads to increased vulnerability of the OXTR to adverse early-life experiences and external factors (Bales and Perkeybile, 2012). The majority of studies demonstrate that early-life stress and social environment can have lasting consequences on OXT system development and function

(Buisman-Pijlman et al., 2014). For example, in rats, OXTR density is affected by maternal separation during the early postnatal period (Lukas et al., 2011). The OXTR system of offspring is also sensitive to stress exposure during gestation; exposing pregnant rat dams to unpredictable stressors during the last week of gestation alters social behavior of male offspring with concomitant reductions in OXTR mRNA expression in the PVN and increased OXTR binding in the central amygdala (Lee et al., 2007).

In this context, early-life pharmacological manipulations of the OXTR system and subsequent behaviour and physiological changes are of great interest (Carter, 2003). The magnitude of effects of OXTR manipulation in early life can project far beyond the OXT system itself, having possible organisational effects and influencing other neurotransmitter systems, estrogen and AVP receptor systems, and affecting a variety of behaviours (Miller and Caldwell, 2015). Some of these effects are summarized below.

One of the first reports of early-life organisational effects of OXT dates back to 1989 (Noonan et al., 1989). In this study, treatment of rats with OXT at postnatal day three (PND3) resulted in exaggerated novelty-induced grooming responses to stress in adult (Noonan et al., 1989). The developmental effects of OXT have been extensively studied in voles (Bales and Perkeybile 2012). In voles, a single intraperitoneal administration of OXT on PND1 decreases AVP 1a labeling in many brain areas including the stria terminalis, cingulate cortex, mediodorsal thalamus, medial preoptic area of the hypothalamus, and lateral septum, with differential effects in males and females (Bales et al., 2007). In a separate study, the same treatment protocol at PND1 indicated that oxytocin can have organisational effects on the expression of estrogen receptor alpha in a sexually dimorphic manner (Yamamoto et al., 2006). In mice, a single subcutaneous injection of OXT, 3-5h after birth, rescued a lethal feeding defect in melanoma antigen gene expression L2 (*Magel2*) deficient mice-a gene associated with Prader-Willi syndrome and ASDs (Schaller et al., 2010). It is interesting to note that, whereas the administration of OXT is capable of rescuing these abnormal phenotypes, a single administration of AVP to newborns is lethal to mutant pups (Schaller et al., 2010). Moreover, daily administration of OXT in the first week of life prevents impairments in social behaviour and cognitive deficits observed in adult

*Magel2* deficient mice at the same time as it corrected OXT alterations in the their brains (Meziane et al., 2015). Postnatal OXT injections has also been demonstrated to ameliorate abnormal social behaviours associated with ASD in contactin-associated protein-like 2 (*Cntnap2*) knockout mice (Peñagarikano et al., 2015). Intranasal delivery of OXT from P21-51 was not as successful in reversing ASD-related behaviours in the BTBR model of ASD (Bales et al., 2014).

These studies are relevant in the context of human neuropsychiatric disorders, as there are growing numbers of clinical trials investigating the efficacy of single and prolonged-dose infusions of OXT for ameliorating symptoms in ASD individuals (Andari et al., 2010; Guastella et al., 2010; Hollander et al., 2003, 2007; Kosaka et al., 2012; Tachibana et al., 2013). However, systematic reviews and meta-analyses have also reported adverse side-effects associated with OXT treatment in clinical trials (Cai et al., 2017).

It is also important to note that, at birth, OXT plays an important role in the switch of GABAergic activity from excitatory to inhibitory, which is a common feauture among several neurodevelopmental disorders (Ben-Ari et al., 1989; Khazipov et al., 2008). In fact, alterations in the timing of the GABA switch have been previously reported in mice lacking the OXTR (*Oxtr*$^{-/-}$), a model of ASD, and in Fragile X Mental Retardation 1 knockout (*Fmr1*$^{-/-}$) mice, a model of fragile X syndrome (Leonzino et al., 2016; Tyzio et al., 2014). OXT-mediated effects in GABA switching are also associated with protective effects, preparing the fetal brain for delivery by increasing protection against hypoxia and preventing an autistic-like phenotype in mice (Khazipov et al., 2008; Tyzio et al., 2014). Perinatal administration of synthetic OXT (pitocin) is a common obstetric practice to accelerate childbirth, and OXTR antagonists are often administered in order to prevent premature labor. Given the multitude of effects that OXT can have on health and neurodevelopment, it is important to better characterise the effects of these early-life manipulations.

#### 1.6.4.2 *Oxytocin and Social Behaviour*

Among its various functions, OXT is a key regulator of social bond formation, from early-life parent-infant bonds to adult pair-bond relationships (Pohl et al., 2018). Thus,

it regulates social behaviour, attachment and affiliative behaviour in human and non-human mammals (Lim and Young, 2006). Understanding the role of OXT in social behaviour is relevant to the field of neuroscience, brain evolution, and disorders such ASD, SZ and anxiety (Churchland et al., 2012). The first demonstrations of OXT's modulatory effect on social recognition dates back approximately 30 years (Dantzer et al., 1987). Subsequent studies have demonstrated that mice lacking the OXT gene exhibit disruptions in social recognition of familiar conspecifics (Choleris et al., 2003; Ferguson et al., 2001). However, this deficit was temporarily reversed following central administration of OXT prior to, but not after the initial encounter with the familiar mouse (Ferguson et al., 2001). Likewise, AVP has been implicated in social recognition for many decades. Peripheral or intracererebroventricular (i.c.v.) injection of AVP receptor 1a in rats as well as null mutations of this receptor in mice has been demonstrated to inhibit social recognition (Dantzer et al., 1987), whereas activation of AVP receptor 1a in the lateral septum via viral vector administration (AAV-V1a) restored the phenotype (Bielsky et al., 2005). Additional examples of OXT's role in social behaviour and social recognition come from studies of sheep and the monogamous prairie vole. In the monogamous prairie vole, OXT regulates mate social recognition, determining the monogamous status of the species (Young and Wang, 2004). Overall, OXT seems to facilitate approach behaviour by decreasing the fear of social interaction in laboratory animals (Neumann, 2008).

OXT also modulates social behaviours in many other vertebrate species. In humans, intranasal OXT administration is shown to increase trust and may have an essential role in the biological basis of prosocial approach behaviour. For example, in an economic game involving allocating money to a stranger, OXT increases individual's willingness to accept social risks arising through interpersonal interactions (Kosfeld et al., 2005). Furthermore, OXT was demonstrated to facilitate positive trait judgements of strangers (Theodoridou et al., 2009). However, there are relatively few studies of OXT on social behavior of humans, warranting further investigation (Campbell, 2010).

Early work on OXT and social behaviour focused mainly on its role in supporting maternal behaviours (Insel, 2010). Although the role of OXT in mouse maternal behaviour was questioned by initial observations from studies performed in mice

lacking OXT (Nishimori et al., 1996), subsequent experiments demonstrated that OXT knockout mice display impaired maternal behaviours under semi-naturalistic conditions (Ragnauth et al., 2005). Moreover, OXT is critical for the expression of spontaneous maternal behaviours in both adult and juvenile prairie voles (Olazabal and Young, 2006). Regulation of maternal behaviours by OXT occurs through activation of the receptor in several brain areas including the MPOA, VTA, BNST, medial amygdala and olfactory bulb). The bond between mother-offspring is essential and it is utmost importance for offspring survival, as this bond influences cognitive, social, ,emotional and physiological development, with implications for mental health later in life (Pohl et al., 2018).

#### 1.6.4.3 *Oxytocin and Vasopressin Systems and the Neuroendocrine–Immune Network*

As mentioned before, OXT and AVP are mainly produced by the magnocellular neurons of the hypothalamus, stored in the pituitary gland and secreted into the circulation. In addition, oxytocin is produced in small amounts outside the CNS in immune cells, the GI tract, pancreas, skin, testis, ovary, uterus, placenta and kidney (Knobloch and Grinevich, 2014). OXT and AVP can also bind to widespread peripheral OXT receptors influencing several physiological functions, such as GI motility, metabolism, cardiovascular function, thermoregulation (Gimpl and Fahrenholz, 2001; Wang et al., 2015). At the same time, oxytocin has a fundamental role in the immune and neuroendocrine system (Wang et al., 2015).

##### 1.6.4.3.1. The Influence of Oxytocin and Vasopressin on Immune System Maturation

Data obtained from preclinical research indicates that several peptide hormones (as well as nonpeptide hormones) act on the immune cells, mediating their proliferation, differentiation, migration and apoptosis (Savino et al., 2016). For instance, OXT and AVP can influence the thymus and bone marrow, two major immune organs (Wang et al., 2015). OXT and AVP can also be produced by thymic epithelial cells and bind through both OXT and AVP 1b thymic receptors (Savino et al., 2016). OXT

mediates T cell maturation in a cryptocrine (hidden secretion) manner, transducing signaling pathways between thymic epithelial cells and pre T cells (Li et al., 2016). In line with this, it has been reported that inhibition of the OXT receptor in foetal thymic cell cultures results in early apoptosis of $CD8^+$ mature T cells, while inhibition of AVP receptor 1b receptor abolishes T-cell differentiation and favours the development of $CD8^+$ T cells (Hansenne et al., 2005). Additionally, OXT/AVP can also participate in the transport of hematopoietic progenitor cells from the bone marrow to blood and peripheral lymphoid tissues (Elabd et al., 2014). OXT/AVP receptors are also present in mouse spleen eosinophils as detected by *in situ* hybridization, and a mechanism of synthesis and storage for these neuropeptides in eosinophils has been proposed (Kumamoto et al., 1995). However, the role for these peptides in the spleen is less not well understood or represented in literature.

In general, OXT-mediated effects on inflammatory processes are regulatory and suppressive (Wang et al., 2015). The hormone mainly suppresses neutrophils, oxidative stress and proinflammatory cytokines including TNF-α, IL-4, IL-6, macrophage inflammatory protein-1α and 1β, monocyte chemoattractant protein 1, and IL-1β. Importantly, it also contributes to the efficient regulatory T-cell functions (Li et al., 2016).

#### 1.6.4.3.2. Oxytocin, the HPA Axis and Anxiety

The PVN integrates neuroendocrine and autonomic functions, and contains neurons that release ACTH, PVN and OXT. Interestingly, within the PVN, both OXT receptor mRNA and CRH are co-expressed in OXT neurons (Neumann et al., 2000). Therefore, it is not surprising that brain OXT can also regulate the activity of the main neuroendocrine stress system, the HPA axis. Moreover, local release of OXT within the PVN has been proposed to regulate stress responses through the modulation of CRH neuron excitability (Jamieson et al., 2017). Clear evidence for this relationship is evidenced by research demonstrating that the cortisol response in infants is attenuated by breastfeeding immediately before exposure to a social stressor (Heinrichs et al., 2001) Additionally, both OXT and social support blunt HPA axis activation (Heinrichs et al., 2003). However, the direction of the relationship between OXT and HPA axis is not straightforward (Neumann et al., 2000).

Besides playing a role in stress response, OXT has been demonstrated to mediate anxiety-related behaviours (Neumann, 2008). Interestingly, acute or chronic central administration of synthetic OXT was found to reduce anxiety in both female and male rats and mice (Ring et al., 2006; Windle et al., 1997). However, a subsequent study demonstrated that in females, the anxiolytic action of OXT, specifically when applied to the central amygdala, occurs solely in pregnant and lactating rats (Neumann, 2002). The brain regions where OXT acts in order to modulate anxiety, have yet to be fully elucidated. However, brain substrates whereby OXT may act include the PVN and amygdala (Blume et al., 2008), and are likely to be part of a widespread network that may also include the medial prefrontal cortex (Sabihi et al., 2014).

### 1.6.5 Oxytocin: a Possible Mediator of Microbiota-gut Brain Axis

As described in previous sections, the gut microbiome and host animal interact via immune–endocrine–brain signalling networks. Interestingly, the administration of *L. reuteri* was demonstrated to increase plasma levels of OXT in mice at the same time as it accelerated skin wound healing, increased resistance to obesity and increased maternal care (Erdman and Poutahidis, 2014; Poutahidis et al., 2013; Varian et al., 2017). Moreover, the beneficial effects induced by *L. reuteri* were found to be dependent upon OXT, as they were not observed in OXT knockout mice (Varian et al., 2017). These findings opened a venue for investigation into microbial regulation of the oxytocinergic system with implications for promoting general well-being and mental health (Erdman and Poutahidis, 2014) .

A recent study in mice supports this hypothesis and demonstrates that a gut microbiota with reduced *L. reuteri* is associated with deficits in social behaviour in a MHFD model of ASD (Buffington et al., 2016). Conversely, supplementation with *L. reuteri* increased oxytocin positive neurons in the PVN and tegmental-ventral areas and ameliorated social behavioural deficits (Buffington et al., 2016). Although the mechanisms of interaction between OXT system and the gut microbiota are yet to be fully elucidated, microbial regulation of OXT systems may occur through the immune system (Erdman and Poutahidis, 2014; Varian et al., 2017). Mice consuming *L. reuteri* retain a sizable thymus in adulthood and this effect is mediated by probiotic-induced upregulation of Forkhead Box N1 (FoxN1) in thymic epithelial cells and IL-

10-mediated induction of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg lymphocytes (Poutahidis et al., 2013). In a more recent study, treatment with *L. reuteri* was shown to increase thymus weight, increase blood levels of OXT, lower circulating neutrophil counts, and decrease circulating corticosterone (Varian et al., 2017), thus, supporting a role for OXT as key microbiota-gut brain axis mediator **Figure 1.14**.

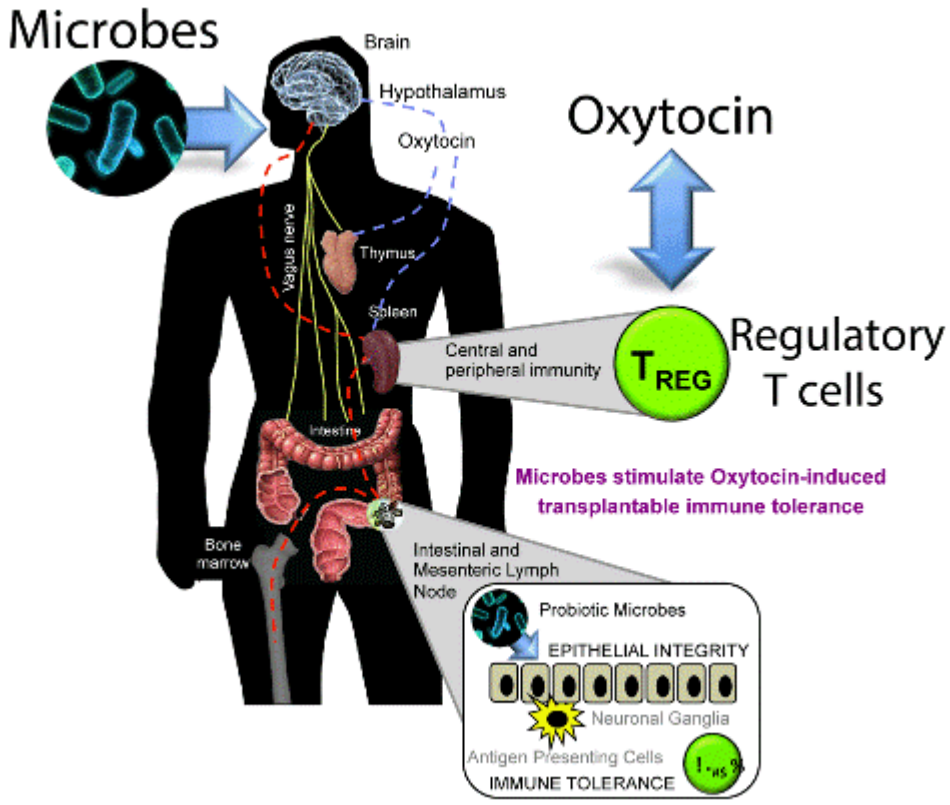



***Figure 1.14- Microbiota and oxytocin interactions.***

Possible pathways mediating the cross-talk between gut microbiota and oxytocin system may invovle neuro-immuno interactions including the vagus nerve and activation of T-regulatory cells (TREG) Figure taken from (E Erdman, 2014).

## 1.7 Goals and Aims of this Thesis

The overall goal of this thesis was to investigate the impact of gut-brain axis disruptions in early-life and their associated neurobehavioural consequences. Moreover, we investigated whether targeting the gut-brain axis early in life could potentially represent a viable strategy to overcome long-term deficits induced by microbial perturbations during this critical period. To this end, we utilised two animal

models of microbiota-gut-brain axis perturbations: the C-section animal model and the well-established model of autism induced by gestational poly (I:C) administration. Given the importance of the initial colonisation of the gut microbiota on brain development and behaviour, we developed a mouse model to assess the long-term consequences of birth by C-section. Moreover, we investigated the impact of microbiome-based interventions (prebiotics, probiotics and faecal microbiota transfer) and of pharmacological treatment with OXT on such effects. To further assess the translational potential of our model of C-section, we investigated whether mode of birth could impact stress in young adults. Lastly, we investigated whether genetic background influences susceptibility to gut-brain axis dysregulation, following gestational poly (I:C) injection.

- **Aim 1:** To investigate whether the mode of delivery at birth could alter microbiota-gut-brain axis function, with neurobehavioural and physiological consequences *(Chapter 2).*
- **Aim 2:** To determine whether microbiome-based interventions (prebiotics, probiotics and microbiota transfer) could reverse effects associated with C-section *(Chapter 2).*
- **Aim 3:** To assess the translational value of our animal model of C-section, by determining whether the mode of birth could impact stress responses of young adults *(Chapter 2).*
- **Aim 4:** To investigate whether pharmacological treatment with OXT during early-life was able to reverse behavioural and physiological effects associated with C-section *(Chapter 3).*
- **Aim 5:** To investigate whether genetic background of mice could influence the effects of MIA on behaviour and function of the gut-brain axis *(Chapter 4).*

# Chapter 2. Targeting the Microbiota-Gut-Brain Axis Reverses the Enduring Behavioural Effects Induced by Birth by Caesarean Section

**Livia H. Morais**[1,4,†], Paul J. Kennedy[1,2,†], Angela Moya-Pérez[1], Gerard Moloney[1,4], Anna V. Golubeva[1], Orla O'Sullivan[1,3], Ana Paula Ventura-Silva[1], Silvia Arboleya[1,3#a], Kieran Rea[1], Yuliya Borre[1], Karen Scott[1], Elaine Patterson[1,3, #b], Roman Stilling[1#c], Alan E. Hoban[1,4,#d], Sahar El Aidy[1,#e], Ana Marta Sequeira[1], Sasja Beers[1], Julia Marr[1], Rachel D. Moloney[1,#f], David Mullins[1], Ingrid B. Renes[5,6], Shugui Wang, Jan Knol[5,8], R. Paul Ross[1,3], Paul W. O'Toole[1,3], Paul D. Cotter[1,3], Catherine Stanton[1,2,3], Timothy G. Dinan[1,2~] & John F. Cryan[1,4~*]

[1] APC Microbiome Institute, University College Cork, Cork, Ireland

[2] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

[3] Teagasc Food Research Centre, Moorepark, Fermoy, Cork, Ireland

[4] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[5] Nutricia Research, Utrecht, The Netherlands

[6] Department of Pediatrics, AMC, Amsterdam, The Netherlands

[7] Nutricia Research, Singapore, Singapore

[8] Laboratory of Microbiology, Wageningen University, Wageningen, the Nederlands

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*Correspondence to: j.cryan@ucc.ie

†Authors contributed equally to the completion of the manuscript

~ Joint Senior Authors.

**Current addresses:**

[#a] Department of Microbiology and Biochemistry of Dairy Products, Instituto de Productos Lácteos de Asturias. Consejo Superior de Investigaciones Científicas (IPLA-CSIC), Villaviciosa, Asturias, Spain

[#b] Global Health and Nutrition Science, DuPont Nutrition & Health, FI-02460 Kantvik, Finland.

[#c] German Primate Center, Goettingen, Germany

[#d] Dept. Anatomy and Neuroscience, University College Cork

[#e] Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands

[#f] Dept. Psychiatry and Behavioral Neuroscience University of Cincinnati, Ohio, USA

## 2.1 Abstract

Birth by Caesarean (C)-section impacts early gut microbiota colonization, bypassing stress and immune priming, and is associated with an increased relative risk of developing immune & metabolic disorders. The long-term effects of C-section on neurobehavioral processes remain unknown. Here, we demonstrate that birth by C-section in the mouse leads to enduring social and cognitive deficits in addition to increased anxiety and stress response. These behavioral alterations are associated with marked but transient changes in the gut microbiota composition and diversity in early-life and in adolescence, particularly affecting *Bifidobacterium spp*. Next, we find that specific behavioral deficits induced by C-section are corrected by co-housing with vaginally born mice. Moreover, supplementation with dietary prebiotics which stimulate the growth of Bifidobacteria, or colonization from birth with a *Bifidobacterium breve* strain, prevented both the neurobehavioral and microbiota alterations in C-section mice. Finally, to assess the translatability of the findings we demonstrate that healthy young adults initially delivered by C-section also have increased psychological vulnerability to either acute or prolonged stress. Given the global rise in C-section rates, our findings have significant implications for public health policy and for determining vulnerabilities to stress-related mental health problems.

## 2.2 Introduction

The number of infants delivered by Caesarean (C)-section worldwide has rapidly increased over recent years, in many jurisdictions far exceeding the World Health Organization guidelines of between 10-15% (Dominguez-Bello et al., 2016). This trend is occurring despite growing evidence that birth by C-section is associated with an increased likelihood of developing immune and metabolic disorders in childhood, including specific allergies and asthma (Decker et al., 2010; Mårild et al., 2012), hypertension in young adults (Horta et al., 2013), type 1 diabetes (Algert et al., 2009) and obesity in both children and adults (Darmasseelane et al., 2014). The mechanisms underlying such changes remain elusive but there is increasing focus on alterations in the composition of the gut microbiota (the trillions of microorganisms inhabiting the

human gastrointestinal tract) in infants born by C-section (Dominguez-Bello et al., 2010; Penders et al., 2006). Indeed, alterations in abundance of *Bifidobacterium* species, which are associated with appropriate immune and gastrointestinal development in infancy, are among the first microorganism to colonize the human gut by vertical transmission from mother-to-infant during birth (Dominguez-Bello et al., 2010; Hill et al., 2017; Makino et al., 2013).

Given that the gut microbiota has a significant influence on the programming of multiple body systems in early-life (Bäckhed et al., 2015; Borre et al., 2014), it is perhaps not surprising that C-section-induced perturbations of the early microbial environment has such widespread effects on host physiology (Cho and Norman, 2013; Hansen et al., 2014). However, the relationship between C-section delivery and brain health is less clear (Brander et al., 2016; Curran et al., 2015a, 2015b, 2016a, 2016b; Al Khalaf et al., 2015). This is despite the growing realisation that the gut microbiota, especially in early-life, can influence many aspects of neurodevelopment and behavior. Preclinical studies using germ-free mice have highlighted the long-lasting effects of the disruption of the normal acquisition and maturation of gut microbiota on social behaviour (Desbonnet et al., 2014), cognition (Gareau, 2014), hypothalamic-pituitary (HPA) axis development (Clarke et al., 2013; Sudo et al., 2004), stress-related behavior (Bercik et al., 2011b; Diaz Heijtz et al., 2011; Dinan and Cryan, 2017a; Foster and McVey Neufeld, 2013) and brain development (Dinan and Cryan, 2017a; Sampson and Mazmanian, 2015). However, germ free studies are specialized model systems and it is unclear if more medically relevant alterations in microbiome composition in early-life can have enduring psychological, neurobehavioral and physiological effects.

Similarly in healthy humans, a growing body of data indicates that the gut microbiota composition can influence aspects of cognitive function (Chung et al., 2014; Schmidt et al., 2015), brain morphology (Labus et al., 2014, 2015, 2017), sociability, mood and anxiety (Allen et al., 2014; Benton et al., 2007; Messaoudi et al., 2011; Steenbergen et al., 2015), the neuroendocrine response to stress (Allen et al., 2016), and brain activity under baseline conditions (Allen et al., 2016) or in response to an emotional attention task (Labus et al., 2014, 2015, 2017; Pinto-Sanchez et al., 2017). In addition,

emerging evidence has identified gut microbiota composition alterations in stress-related psychiatric cohorts (Kelly et al., 2016; Zheng et al., 2016).

Given the importance of the initial colonization of the gut microbiota on brain development and behavior, we developed a mouse model to assess the long-term consequences of birth by C-section compared with vaginal delivery on physiology and behavior. Moreover, we investigated the impact of targeting the microbiome on such effects. Finally, the translatability of the findings to humans was assessed in a sample of healthy young adults undergoing acute or chronic stress.

## 2.3 Results

### 2.3.1 C-section-induced Neurobehavioral Changes in a Preclinical Mouse Model

Following birth by C-section, pups were given to foster dams (CS). To control for the potential effects of fostering itself, pups born per *vaginum* were also given to foster dams (CF). Additional pregnant females were allowed to deliver spontaneously, and the litters were used as a full-term vaginally-born control group (VB) (**Figure 2. 1a**). We compared the consequences of mode of delivery in early-life and in adulthood by using a sequence of behavioral tests relevant to stress-related disorders (**Figure 2. 1b**). We found that, in early-life (P9), CS pups exhibited increased ultrasonic vocalizations when isolated from their littermates and mother at a frequency far greater than that of CF or VB animals (**Figure 2. 1c**). It has been previously demonstrated that by P10 pups are normally able to discriminate relevant social stimuli and move towards the mother's nest when physically separated (Macrì et al., 2010). Unlike VB or CF animals, CS failed to display preference for their home bedding (**Figure 2. 1d**) indicating early social recognition and maternal attachment deficits. One caveat to the mouse studies is the fact that CS animals are born prematurely (max 1 day), and thus this is a potential confounder for some of these early measures.

In adulthood, CS offspring develop exaggerated anxiety-like behavior as observed by increased number of buried marbles when compared with VB offspring (**Figure 2. 1e**), reduced locomotion and time spent in the central zone of an aversive open-field arena

(**Figure 2. 1f**), and reduced number of entries in the open arms of the elevated plus maze (EPM) in comparison with both VB and CF offspring (**Figure 2. 1g**). In order to verify if an anxiety-like behavioral phenotype is accompanied by a disrupted HPA axis, we examined the acute stress response in CS offspring by measuring plasma corticosterone (CORT) levels at various time-points. C-section delivery did not affect baseline CORT secretion (**Figure 2. 1h**). However, it induced an exaggerated HPA axis response 15 minutes following an acute stressor in comparison with VB and CF offspring (**Figure 2. 1h**). This differential maturation of the HPA axis in CS mice was coincident with increased corticotropin-releasing factor receptor 1 (*Crhr1*) expression in the pituitary gland in comparison with the CF group (**Figure 2. 1i**). However, no differences were found between the groups for glucocorticoid and mineralocorticoid receptors (**Supplementary figure 2.1 a-b**). Since stress has been demonstrated to reduce social interactions in a variety of social paradigms (Sandi and Haller, 2015), and microbiota-deficient mice have social deficits (Desbonnet et al., 2014), we investigated if mice born by C-section exhibit deficits in social behavior. Although CS mice have normal sociability in the three-chamber test (**Figure 2. 1j**), a specific deficit in social-novelty preference was revealed compared with VB and CF mice (**Figure 2. 1j**). Interestingly, this phenotype is similar to that of germ-free mice that have been colonized post-weaning (Desbonnet et al., 2015). Furthermore, working memory is susceptible to the effects of stress and here we demonstrate that CS-born offspring exhibit deficits in a novel object recognition working memory task (**Figure 2. 3j**). Although both stress and the microbiota have been strongly associated with pain sensitivity, when we tested the mice in the hot-plate test, no group differences were identified (See **Supplementary figure 2.2a**). In addition, we investigated if the observed behavioral changes are accompanied by alterations in gene expression in the brain. To this end, we performed deep sequencing of cDNA (RNAseq) extracted from the ventral hippocampus, paraventricular nucleus of the hypothalamus (PVN), and the amygdala. Analysis of the ventral hippocampus but not amygdala or PVN revealed differential expression with 48 (up) and 31 (down) partially-overlapping genes with the CS group compared to VB and CF group, respectively (**Figure 2. 1k, see online supporting material**). Functional enrichment analysis was used to search for over-representation of genes involved in the proteinacious extracellular matrix and cell adhesion processes in CS offspring compared with VB, with no significant differences between VB and CF groups.

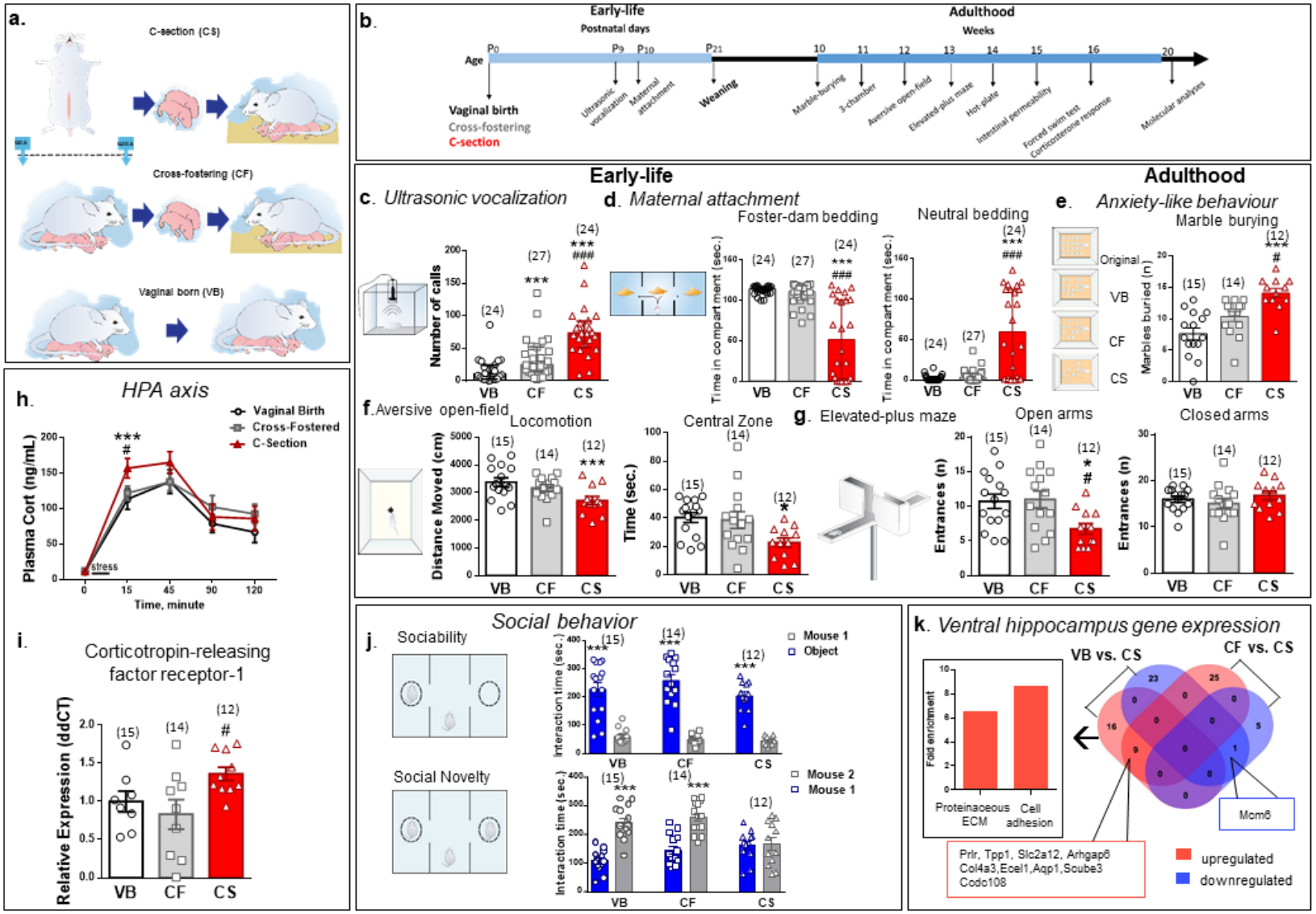

a.
C-section (CS)
Cross-fostering (CF)
Vaginal born (VB)
b.
Early-life
Postnatal days
Adulthood
Weeks
Age
P0
P9
P10
P21
10
11
12
13
14
15
16
20
Vaginal birth
Cross-fostering
C-section
Ultrasonic vocalization
Maternal attachment
Weaning
Marble-burying
3-chamber
Aversive open-field
Elevated-plus maze
Hot-plate
Intestinal permeability
Forced swim test
Corticosterone response
Molecular analyses
Early-life
Adulthood
c. Ultrasonic vocalization
Number of calls
d. Maternal attachment
Foster-dam bedding
Neutral bedding
Time in compartment (sec.)
e. Anxiety-like behaviour
Marble burying
Original
VB
CF
CS
Marbles buried (n)
f. Aversive open-field
Locomotion
Distance Moved (cm)
Central Zone
Time (sec.)
g. Elevated-plus maze
Open arms
Closed arms
Entrances (n)
h.
HPA axis
Vaginal Birth
Cross-Fostered
C-Section
Plasma Cort (ng/mL)
stress
Time, minute
i.
Corticotropin-releasing factor receptor-1
Relative Expression (ddCT)
Social behavior
j.
Sociability
Social Novelty
Mouse 1
Object
Mouse 2
Mouse 1
Interaction time (sec.)
k. Ventral hippocampus gene expression
VB vs. CS
CF vs. CS
Fold enrichment
Proteinaceous ECM
Cell adhesion
Mcm6
Prlr, Tpp1, Slc2a12, Arhgap6
Col4a3,Ecel1,Aqp1,Scube3
Codc108
upregulated
downregulated

***Figure 2. 1- Behavioral and physiological changes in CS offspring across lifespan.***

**a-b**) CS animal model and experimental design. **c**) CS-born offspring exhibit a higher number USV calls ($x^2$=33.303, p<0.001) ([Kruskal-Wallis, followed by U-Mann Whitney test]). **d**) CS-born offspring failed to exhibit preference for their home bedding ($x^2$=26.106, p<0.0001) but displayed increased preference for a neutral bedding ($x^2$=20.577, p<0.0001) ([Kruskal-Wallis, followed by U-Mann Whitney test]). Scatter dot-plots represent Median ± interquartile range. **e-g**) In adulthood, mice delivered by CS displayed enhanced anxiety–like behaviour; **e**) increased the number of buried marbles (F (2,38)= 14.73, p<0.0001); **f)** in the open-field arena; reduced time spent in the central zone (F(2,38) = 4.43, p= 0.019) and reduced total distance travelled (F(2,38) = 5.22, p= 0.01); and **g**) decreased number of entrances in the open arm of the elevated plus maze (F (2,38)= 4.74, p=0.015). No differences between groups for the number of entrances in the closed arms (F (2, 38) =0.614, p=0.4047) ([One-way ANOVA, followed by Tukey post hoc]). **h**) Corticosterone (CORT) release in response to an acute stress. Time effect (F (4, 112) = 108.869, p<0.001); group effect (F (2,28)= 3.8, p= 0.034); time x group (F(8,112)= 2.751, p=0.008 ([Two-way ANOVA]). A one-way ANOVA and Tukey post hoc for each time-point revealed significant increased CORT in CS at T15 (F (2,34) = 10.435, p<0.0001) ([VB n= 13, CF n=13, CS n=11]). T0 (F (2,37) =0.001, p=0.999) ([VB n= 15, CF n=13, CS n=12]); T45 (F (2,34) =2.689, p=0.082) ([VB n= 12, CF n=13, CS n=12]); T90 (F (2,34) =0.409, p=0.667) ([VB n= 14, CF n=12, CS n=11]); T120 (F (2, 32) =2.060, p=0.144) ([VB n= 13, CF n=11, CS n=11]). **i**) Increased Corticotropin-releasing factor receptor 1 expression in the pituitary gland of CS born offspring (F (2,23) = 3.829, p=0.037) ([One-way ANOVA, followed by Tukey post hoc]). **j**) Three-chamber test. C-section does not impact sociability. VB t (14) = 6.341, p<0.0001, CF t (13) =9.776, p<0.0001, CS t (11) =9.811, p<0.0001. Reduced social novelty preference in CS. VB t (14) = 7.8, p<0.001, CF t (13) = 5.1, p<0.0002, CS t (11) =-0.167, p= 0.8707 ([Paired-Student T-test]). **j**) RNA-sequencing of the adult hippocampal transcriptome ([Raw data online; VB n= 5, CF n= 4, CS n= 6]). **c-j**) Animal numbers per group are specified above each bar. **c-j**) Scatter dot-plots represent Mean ± Stard Error of the Mean (S.E.M.) Behavioral results shown representative of 2-4 experimental replicates. Offspring in each cohort derived from three independent litters/ group. *p< 0.05 and ***p<0.0001 CS vs. VB; #p<0.05 and ###p<0.0001 CS vs. CF. VB, vaginal birth; CF, cross fostered; CS, C-section.

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### 2.3.2 Impaired Intestinal Barrier Function Mediates Immune Activation in C-section Model

Given the importance of gut health for brain function, we next assessed whether gastrointestinal function including intestinal motility and permeability and, in turn, systemic endotoxemia (Hsiao et al., 2013; Kelly et al., 2015), were modified by C-section. Using carmine red as an *in vivo* marker of motility, we determined that CS born mice have faster gastrointestinal transit than VB mice (See **Supplementary figure2.4d**). In addition, we measured small-intestinal permeability in adolescent and adult mice. The presence of fluorescein isothiocyanate (FITC) in plasma of mice reflects the level of paracellular permeability in the small intestine. In adolescence (P23), small intestine permeability was significantly reduced in CS mice compared with VB mice reflecting a more effective barrier (**Figure 2. 2a**). At P23 intestinal development is incomplete and the digestive system is still immature. In contrast, there was no significant difference in intestinal permeability between CS mice and VB mice in adulthood, suggesting that when the intestinal barrier is fully-formed, paracellular permeability increases in CS mice (**Figure 2. 2b**). Next, we examined if mode of delivery affected systemic endotoxemia in mice at different stages of life. Lipopolysaccharide (LPS) levels were measured in the plasma of male mice born by VB, CF and CS at early-life (P9), adolescence (P25) and in adulthood. At P25, plasma LPS concentrations in the plasma were higher in CS compared with VB and CF mice. By P9 and adulthood, there was a trend towards higher levels in CS mice (**Figure 2. 2c**), but differences failed to reach statistical significance.

Having observed changes in intestinal permeability and plasma LPS concentration in CS compared with VB mice in early-life, we investigated changes in gene expression in the colon and ileum of male mice at P9, P25 and adulthood for Toll-like receptors 4 (*Tlr4*), a marker of host-response, and Tight-junction protein 1 (*Tjp1*) and occludin, markers of colonic barrier integrity. However, no difference in gene expression was observed in *Tlr4* (See **Supplementary figure 2.3a**), *Tjp1* (See **Supplementary figure 2.3b** and **d**) or Occludin (See **Supplementary figure 2.3c** and **e**) in CS compared with VB mice,

suggesting that although paracellular permeability is altered due to CS birth mode, transcellular permeability is unaffected by mode of delivery. The increased levels of plasma LPS in CS compared with VB and CF mice suggests subtle changes in the immune response due to C-section birth mode. To further probe this phenomenon, cells isolated from the thymus and spleen were stimulated with Concanavalin A (ConA) and LPS, activators of T lymphocytes and TLR4 receptors respectively. Cytokines were measured in supernatants of these isolated cells 24 hours after the immune challenge. Stimulated thymocytes from CS mice when challenged with ConA produced significantly more IL-10 and TNFα, than VB mice (**Figure 2. 2d).** There were no differences in splenocyte responses between CS and VB stimulated with ConA **(**Error! Reference source not found.**)**. Similarly, there was a significant increase in IL-12p70 production in the CS group as compared with CF mice in LPS-stimulated thymocytes (Error! Reference source not found.). However, there was no significantly different increase in LPS-stimulated splenocyte (**Supplementary Table 2. 1**).

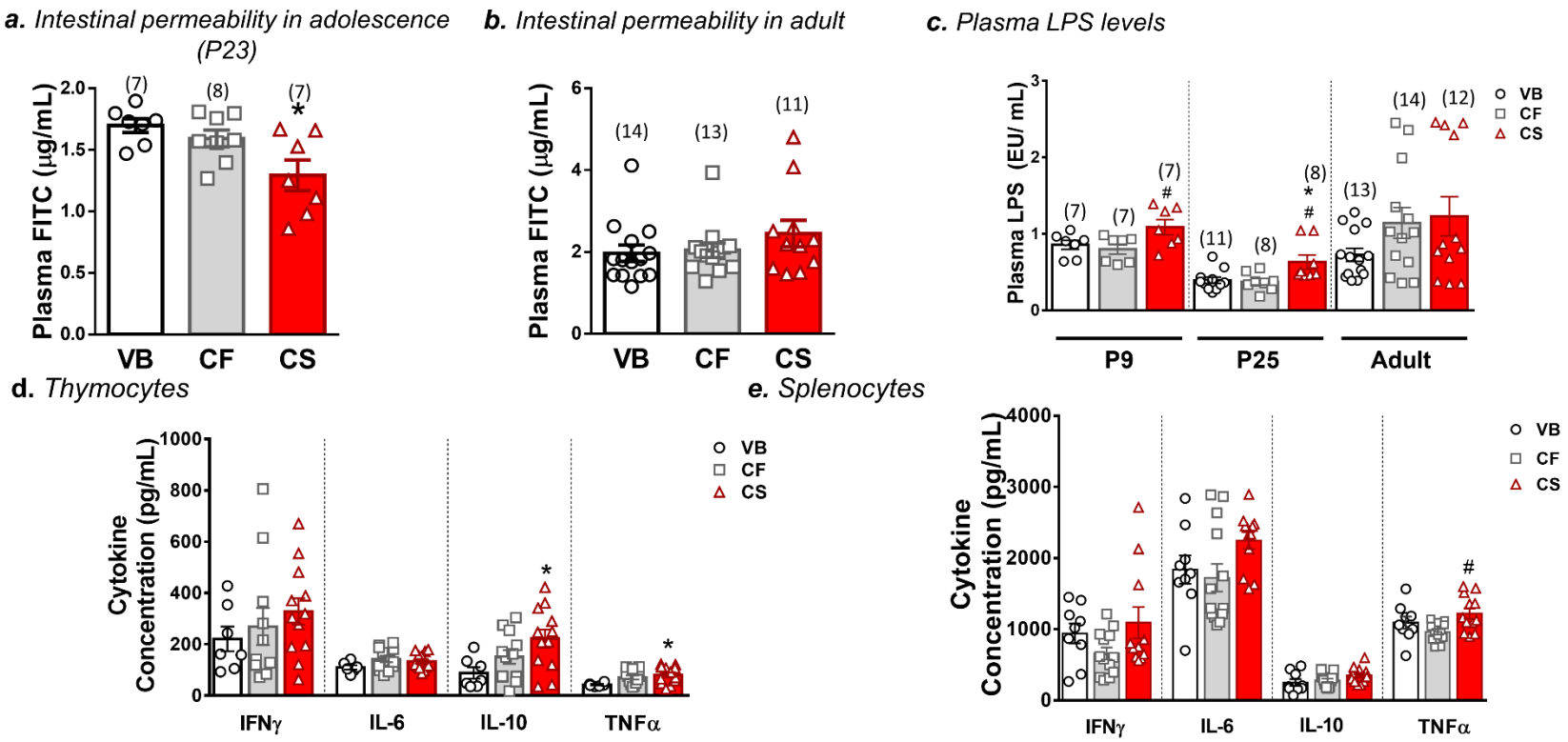

a. Intestinal permeability in adolescence (P23)
Plasma FITC (µg/mL)
VB
CF
CS
b. Intestinal permeability in adult
Plasma FITC (µg/mL)
VB
CF
CS
c. Plasma LPS levels
Plasma LPS (EU/ mL)
P9
P25
Adult
VB
CF
CS
d. Thymocytes
Cytokine Concentration (pg/mL)
IFNγ
IL-6
IL-10
TNFα
e. Splenocytes
Cytokine Concentration (pg/mL)
IFNγ
IL-6
IL-10
TNFα

***Figure 2. 2- Mode of Delivery has modest effect on intestinal permeability and immune function***

**a,b**) Intestinal permeability as measured by plasma fluorescein isothiocyanate (FITC)-dextran concentration (μg/mL): **a**) decreased intestinal permeability to FITC-dextran in adolescence ($F(2,19)=5.633$, $p=0.012$); **b**) no differences in the intestinal permeability between groups in adulthood ($F(2,35)=2.583$, $p= 0.090$) ([One-way ANOVA, followed by Tukey post hoc]). **c**) Plasma lipopolysaccharide concentration (enzyme units (EU)/mL of plasma) at P9 ($F(2,18) = 3.988$, $p=0.037$); at P25 ($F(2,24)=5.57$, $p=0.01$) and in adulthood ($F(2,36)= 2.140$, $p=0.132$) ([One-way ANOVA, followed by Tukey post hoc showed an increase in LPS in the CS group at P25]). **a-c)** Animal numbers per group are specified above each bar. **d,e)** Immune response: **d**) thymocytes and **e**) splenocytes were isolated from male adult mice and stimulated with Concanavalin A (2μg/mL). Concentration of IFNγ, Interleukin (IL)-6, IL-10 and TNFα were measured (pg/mL). Thymocytes: IFNγ ($F(2,27)=0.707$, $p= 0.502$) ([VB n= 7, CF n=11, CS n=12]); IL-6 ($F(2,27)=1.594$, $p= 0.222$) ([VB n= 6, CF n=12, CS n=12]); IL-10 ($F(2,28)=4.099$, $p=0.027$) ([VB n= 7, CF n=12, CS n=12]); TNFα ($F(2,27)=3.660$, $p= 0.039$) ([VB n= 6, CF n=12, CS n=12]). Splenocytes: IFNγ ($F(2,30)=2.225$, $p= 0.126$) ([VB n= 9, CF n=13, CS n=11]); IL-6 ($F(2,31)=2.642$, $p= 0.087$) ([VB n= 9, CF n=13, CS n=12]); IL -10 ($F(2,30)=2.046$, $p= 0.147$) ([VB n= 9, CF n=13, CS n=11]); TNFα ($F(2,31)=1.5.091$, $p= 0.012$) ([VB n= 9, CF n=13, CS n=12]). One-way ANOVA, followed by Tukey post hoc. **a-e)** Scatter dot-plots represent mean ± S.E.M. Offspring in each cohort derived from three independent litters/group. *$p< 0.05$ CS *vs*. VB; #$p<0.05$ CS *vs*. CF. VB, vaginal birth; CF, cross fostered; CS, C-section.

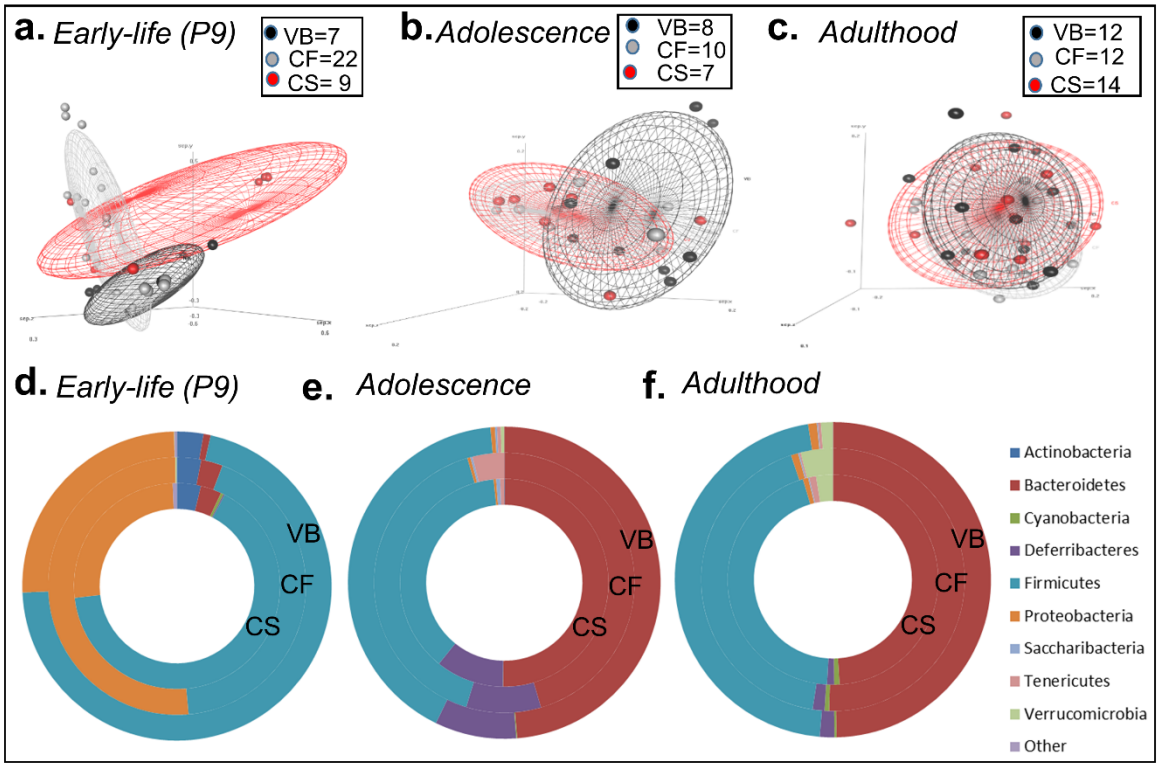
### 2.3.3 Early-life Microbiome Changes Are Evident in C-section Offspring

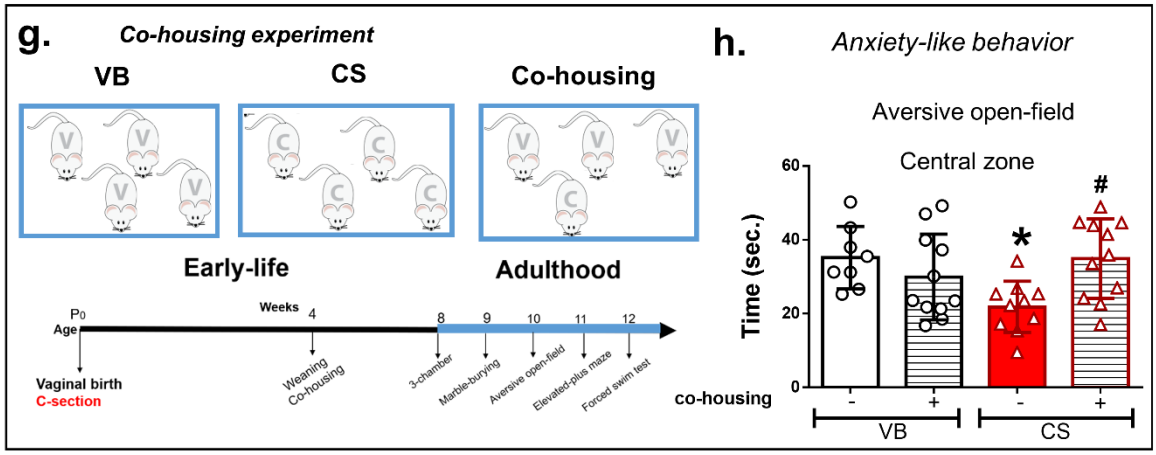
Given the role of the microbiome in early-life in programming body systems and how its composition is altered by C-section in humans (Hill et al., 2017), we investigated the composition of the microbiota in the mouse model. Principal coordinate analysis of unweighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset showed that CS samples clustered separately from VB in early-life (**Figure 2. 3a**). In adolescence, a trend towards a separation still persists (**Figure 2. 3b**) (p=0.056), whereas by adulthood no statistically significant separation between the groups was found (**Figure 2. 3c**).

Taxonomy-based analysis of the assigned sequences showed that at phylum, family and genus level the intestinal microbial signature is distinct between CS and VB groups at all ages (**Supplementary Table 2. 2**). The main differences in the microbiota composition between VB and CS-born offspring in microbiota composition are found in early-life in the phyla Firmicutes and Proteobacteria (**Figure 2. 3d).** During adolescence and through adulthood, the microbiota separation based on mode of delivery at birth was still statistically significantly, although at a lower magnitude than during early-life, possibly due to the former group receiving increased microbial exposure from the laboratory environment compared with the VB group (**Figure 2. 3e-f**). Although there are marked differences in the microbiota composition and diversity, the alpha-diversity index (reflecting net microbiota phylogenetic diversity) did not differ between CS and VB mice (**Supplementary Table 2. 3**). The observed differences in microbiota between treatment groups (**Figure 2. 3a-f)** were associated with alterations in the short chain fatty acid (SCFA) profile, whereby cecal levels of acetate were lower in adolescence and butyrate levels in adulthood in CS compared with CF mice (**Supplementary Table 2. 4**).

To investigate a causal role of microbiota in mediating the observed effects, we tested whether transferring microbiota from VB to CS mice at P21 could prevent CS-mediated effects on behavior. We exploited the copraphagic nature of mice and performed fecal transfer by co-housing one CS mice with three VB mice in adolescence (based on the strategy utilized by (Buffington et al., 2016). Behavior was assessed in adulthood (**Figure**

**2. 3g**). as previously described. Strikingly, co-housing CS with VB mice selectively reversed the CS-induced deficits on preference for social novelty (**Figure 2. 3i),** working memory (**Figure 2. 3j**) and time spent in the central zone of the aversive open-field (**Figure 2. 3h**), but not the entire behavioral phenotype with some aspects of anxiety-like behaviour being resistant to reversal (See **Supplementary figure 2.4a**)**.** Thus, as previously described in germ free animals (Diaz Heijtz et al., 2011; Neufeld et al., 2011), there are critical windows post-weaning in which the restoration of microbiota can reverse some but not all of the responses observed due to the CS-associated altered microbiota.


a. Early-life (P9)
VB=7
CF=22
CS= 9
b. Adolescence
VB=8
CF=10
CS=7
c. Adulthood
VB=12
CF=12
CS=14
d. Early-life (P9)
e. Adolescence
f. Adulthood
VB
CF
CS
Actinobacteria
Bacteroidetes
Cyanobacteria
Deferribacteres
Firmicutes
Proteobacteria
Saccharibacteria
Tenericutes
Verrucomicrobia
Other


g. Co-housing experiment
VB
CS
Co-housing
Early-life
Adulthood
P0
Age
Weeks
4
8
9
10
11
12
Vaginal birth
C-section
Weaning
Co-housing
3-chamber
Marble-burying
Aversive open-field
Elevated-plus maze
Forced swim test
h. Anxiety-like behavior
Aversive open-field
Central zone
Time (sec.)
co-housing
VB
CS

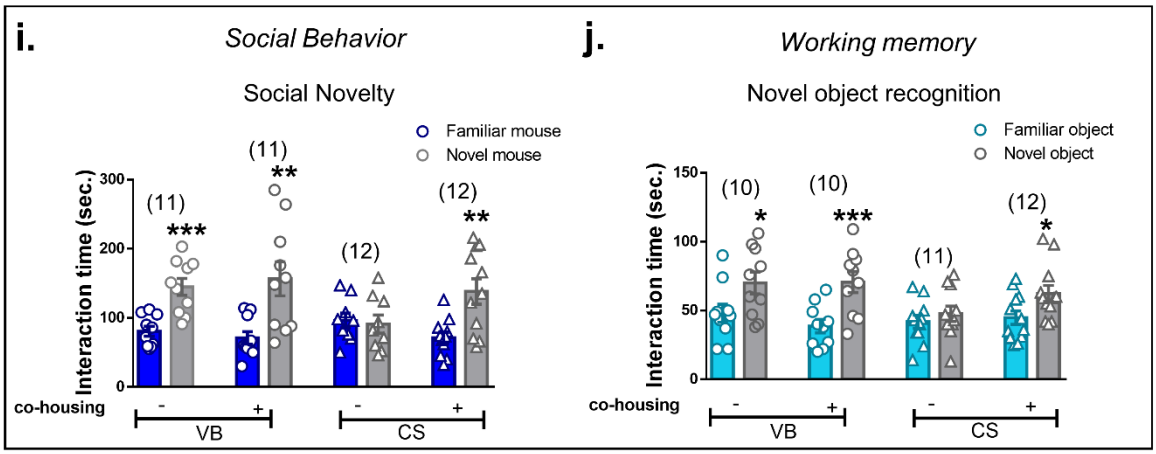

i. Social Behavior
Social Novelty
Familiar mouse
Novel mouse
Interaction time (sec.)
co-housing
VB
CS
j. Working memory
Novel object recognition
Familiar object
Novel object
Interaction time (sec.)
co-housing
VB
CS

***Figure 2. 3- Mode of delivery affects microbial composition throughout the lifespan.***

DNA was extracted from colonic contents in early-life (P9) and cecal contents in adolescence (P25) and adulthood (week 20): (**a-c**) Principal coordinate analysis (PCoA) plots, based on bray-curtis distances: **a**) Early-life (p=0.003); **b**) Adolescence (p=0.056); **c**) Adulthood (p=0.128). (**d-f**) Relative abundance of phylum in vaginal birth (VB); cross-fostered (CF) and C-section (CS) mice. The dominant phyla are labelled in the legend on the right. **g**) Co-housing experimental design. **h**) Aversive open-field. Time spent in the central zone. Group effect (F(1, 36)=1.566, p=0.219); mode of delivery effect (F(1, 36)=1.790, p=0.189); group x mode of delivery effect (F(1, 36)=8.648, p=0.0.006) ([Two-way ANOVA, followed by Tukey post hoc]). **i**) Three-chamber social novelty preference. VB (t (9) =-4.566, p=0.001), VB co-housed (t (9) =-3.199, p=0.0011), CS (t (9) =0.21, p=0.830), CS co-housed (t (10) =-4133, p=0.002) ([Paired-Student T-test]). *p< 0.05, **p<0.01, ***p<0.001 Novel *vs*. Familiar mouse. **j**) Novel object recognition. VB (t (9) =2.678, p=0.0025), VB co-housed (t (9) =4.146, p=0.002), CS (t (10) =1.909, p=0.085), CS co-housed (t (12) =2.445, p=0.035) ([Paired-Student T-test]). *p< 0.05, ***p<0.001 Novel *vs*. Familiar object. **h-j)** Scatter dot-plots represent mean ± S.E.M. Animal numbers per group are specified above each bar. **a-f)** Offspring in each cohort derived from three independent litters/ group. **h-i)** VB group, 9 litters**;** CS group, 6 litters; VB co-housed group, 10 litters; and CS co-housed, 7 litters. VB, vaginal birth; CF, cross fostered; CS, C-section.

### 2.3.4 *Bifidobacterium* spp. Contribute to C-section Induced Neurobehavioral Changes.

Knowing that mode of delivery is an important factor in shaping Bifidobacteria community structure in infants (Rodríguez et al., 2015), we sought to investigate if C-section could alter these specific bacteria taxa in the animal model. The levels of *Bifidobacterium* genus members was quantified by q-PCR specific for this microbial group in the feces of VB and CS mice at weaning (P21) and in adulthood (wk 8). We identified a transient decrease in the total Bifidobacteria number in CS offspring at weaning (***Figure 2. 4*****a**) which was rectified by adulthood (***Figure 2. 4*****b**). *Bifidobacterium spp*. are critical in shaping neonatal intestinal and immune system development (Bakker-Zierikzee et al., 2005). Thus, we hypothesized that a reduction in its abundance during early neurodevelopmental windows could contribute significantly to the specific behavioral effects induced by C-section. To test this, we supplemented a group of CS pups

with a dietary intervention of specific prebiotics (a combination of short-chain galacto-oligosaccharides and long-chain fructo-oligossaccharides -scGOS/lcFOS- in a ratio of 9:1), that are known to stimulate Bifidobacterium growth (or with specific human commensal *Bifidobacterium breve* M16-V (*B.breve*) (Wopereis et al., 2014), by giving it to dams during the lactation period. By P21, CS and VB offspring were weaned to their maternal diet. Indeed CS offspring have a significant reduction in *Bifidobacterium spp.* levels, whereas treatment with scGOS/lcFOS and *B.breve* corrects these CS-associated changes in *Bifidobacterium spp.* levels (***Figure 2. 4*i**).

Notably, even as early as P9, treatment with prebiotic prevented anxiety-like behavior by reducing the number of calls emitted by the CS pups when they were isolated from their nest (***Figure 2. 4*d**). Moreover, at P10, treatment with both interventions had marked effects on restoring neonatal recognition abilities and maternal attachment deficits in the CS pups (***Figure 2. 4*e**).

Since we determined that social and object recognition memory deficits present in adult CS mice were at least in part driven by gut microbiota alterations (**Figure 2. 3i-j**), we next assessed if the selective decrease of *Bifidobacterium spp.* abundance in CS offspring could functionally contribute to these deficits. Indeed, treatment with *B.breve* ameliorated CS-induced social and working memory deficits while treatment with GOS/FOS completely reversed this effect (***Figure 2. 4*f-g**). Collectively, this supports the concept of a causal role of *Bifidobacterium* early in life for recognition memory programming.

Oxytocin is one of the key neurochemical mediators of attachment, social memory and anxiety (Ross and Young, 2009). Moreover, alterations in the oxytocin receptor (OXTR) have been implicated to the aetiology of a wide range of psychopathologies (Johnson and Young, 2017). Moreover, gut microbiota can stimulate the oxytocin system in the brain leading to beneficial effects to behaviour and stress and improving general health of the host (Varian et al., 2017). Here we demonstrate that CS mice had elevated levels of oxytocin receptor mRNA in the amygdala, an effect which was reversed by both the prebiotic and *B.breve* treatments **(*Figure 2. 4*h)**.

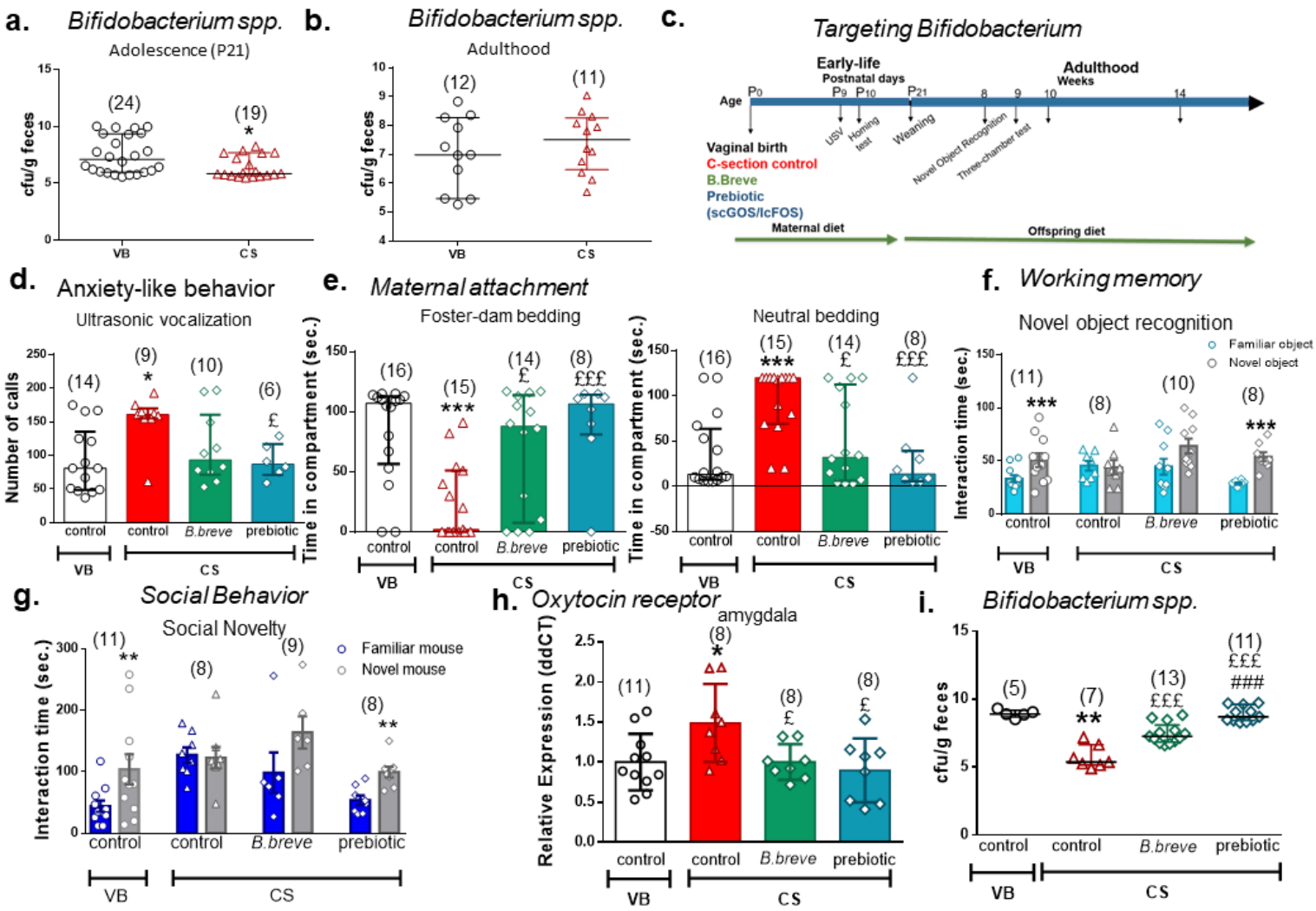

a. Bifidobacterium spp.
Adolescence (P21)
cfu/g feces
VB
CS
b. Bifidobacterium spp.
Adulthood
c. Targeting Bifidobacterium
Early-life
Postnatal days
Adulthood
Weeks
Age
Vaginal birth
C-section control
B.Breve
Prebiotic
(scGOS/lcFOS)
USV
Homing test
Weaning
Novel Object Recognition
Three-chamber test
Maternal diet
Offspring diet
d. Anxiety-like behavior
Ultrasonic vocalization
Number of calls
control
B.breve
prebiotic
e. Maternal attachment
Foster-dam bedding
Neutral bedding
Time in compartment (sec.)
f. Working memory
Novel object recognition
Familiar object
Novel object
Interaction time (sec.)
g. Social Behavior
Social Novelty
Familiar mouse
Novel mouse
h. Oxytocin receptor
amygdala
Relative Expression (ddCT)
i. Bifidobacterium spp.

***Figure 2. 4- Reduction of Bifidobacterium spp. abundance mediates behavior and microbiota changes in C-section born mice.***

**a**) *Bifidobacterium spp.* is reduced in CS adolescent microbiome (colony forming unit (cfu)/g feces) (U=129.00, p=0.015; Mann Whitney test). **b**) *Bifidobacterium spp.* concentration is normalized by adulthood in the CS offspring ($x^2$=2.327, p=0.507; Kruskal-Wallis test). **c**) *B.breve* and scGOS/lcFOS administration and experimental design. **d**) scGOS/lcFOS administration reversed CS increase in number of ultrasonic vocalization calls (VB *vs* CS, U=27.500, p=0.025; CS *vs* CS + treatment; $x^2$=6.203, p=0.045, Kruskal-Wallis test followed by U-Mann Whitney test). **e**) CS reduced preference for the foster dam bedding is reversed by *B.breve* and scGOS/lcFOS administration (VB *vs* CS, $x^2$=35.000, p=0.001, Mann-Whitney test; CS *vs* CS + treatment; $x^2$=10.484, p=0.005, Kruskal-Wallis test followed by multiple comparisons). CS increased preference for neutral bedding is normalised by treatment with *B.breve* and GOS/FOS (VB *vs* CS, $x^2$=35.000, p=0.001, Mann-Whitney test; CS *vs* CS + treatment; $x^2$=10.484, p=0.005, Kruskal-Wallis test followed by multiple comparisons test).**f**) Novel object recognition. VB (t (10)=3.935, p=0.003), CS (t (7)=-0.211, p=0.839), CS+*B.breve* (t (9)=1.876, p=0.093), CS+scGOSlc/FOS (t(7)=5.772, p=0.001) ([Paired Student T-test]).Novel *vs*. Familiar object. **g**) Social novelty.VB (t (10)=-2.974, p=0.014), CS (t (7)=0.180, p=0.863), CS+*B.breve* (t (8)=-2.227, p=0.057), CS+scGOS/lcFOS (t(7)=-3776, p=0.007) ([Paired Student T-test]).Novel *vs*. Familiar mouse. **h**) Oxytocin receptor (*Oxtr*) relative expression (VB *vs* CS, t (17)=-2.543, p=0.021, Student t- test; CS *vs* CS + treatment (F(2,21)= 5.402, p=0.013, One-way ANOVA, followed by Tukey post hoc. **i**) (VB *vs* CS, U=0.000, p=0.004, Mann-Whitney test; CS *vs* CS + treatment; $x^2$=20.472, p<0.0001, Kruskal-Wallis test followed by multiple comparisons). **i**) Treatment with *B.breve* and scGOS/lcFOS restores *Bifidobacterium spp.* concentration in the CS offspring (VB *vs* CS, $x^2$=0.000, p=0.004, Mann-Whitney test; CS *vs* CS + treatment; $x^2$=20.472, p<0.0001, Kruskal-Wallis test followed by multiple comparisons. Exact n numbers of offspring used are specified at the graphs. **a-b)** Offspring in each cohort derived from VB group, 9 litters**;** CS group, 6 litters. **c-i**) Offspring in each cohort derived from VB group, 4 litters**;** CS group, 3 litters; CS+ *B.breve*, 3 litters; and CS scGOS/lcFOS, 3 litters.*p< 0.05 CS *vs* VB; £p=0.05 and £££p<0.001 CS *vs* CS+ treatment groups, ###p<0.001 CS prebiotic *vs.* CS *B.breve*. VB, vaginal birth; CS, C-section.

### 2.3.5 C-section Increases Stress Vulnerability in Healthy Young Adults

Although microbiome changes have been well documented in C-section born individuals (Dominguez-Bello et al., 2010; Hill et al., 2017; Penders et al., 2006), there has been limited investigation on the long-term effects of this dysbiosis on psychological and physiological responses to stress. To fill this knowledge gap and to test the translatability of our mouse model findings, adult human volunteers of known birth modality underwent a Trier Social Stress Test (TSST) to investigate the HPA axis, inflammatory and psychological response to acute psychosocial stress (Allen et al., 2014). Although C-section participants did not exhibit a differential salivary cortisol response to the TSST (**Figure 2. 5a**), concentrations of IL-1β (**Figure 2. 5b**.) and IL-10 (**Figure 2. 5c**), but not IL-6, IL-8 or TNF-α **(Supplementary figure 2.5a-c**), were significantly higher in C-Section subjects compared with vaginally born participants throughout the testing period, although this was due to elevated baseline levels and not acute stress. Measures of positive affect were significantly lower throughout the procedure (See **Supplementary figure 2.6a**), although negative affect was not (See **Supplementary figure 2.6**b). Participants born by C-section reported greater psychological stress in response to the TSST when compared to vaginally born participants (**Figure 2. 5c**).

To further examine the effect of stress on individuals born by C-section, a subset of participants attended two experimental days, one of which took place during their end-of-term university examination period (Exam Stress; after their 1$^{st}$ but prior to their last exam, and not on the same day as an exam) and one took place during term-time, but at least 4-6 weeks before or after their end-of-term examination period (Non-Stress). HPA axis function, as measured by the cortisol awakening response during the Non-Stress and Exam-Stress periods, was not significantly different between C-section and vaginally born participants (See **Supplementary figure 2.7a**). However, the anti-inflammatory cytokine IL-10 was significantly elevated during the Exam-Stress period in C-Section participants (See **Supplementary figure 2.7a**). There was no group difference in IL-1β,

IL-6 or IL-8 (See **Supplementary figure 2.7b-d**). Marginally elevated levels of TNF- α were observed in the C-section participants during the Non-Stress and Exam-Stress periods compared with vaginally born participants (**Figure 2. 5d**). When comparing psychological distress levels during the Non-Stress and Exam-Stress periods, participants born by C-section reported significantly greater levels of trait anxiety (**Figure 2. 5e**), perceived stress (**Figure 2. 5f**), but not depression (See **Supplementary figure 2.6c**) when compared to vaginally born participants, during the Exam Stress period but not during the Non-Stress period. Interestingly, we identified no difference in cognitive performance on tests of visuospatial memory (See **Supplementary figure 2.8a**), response inhibition (See **Supplementary figure 2.8b**) attentional flexibility (See **Supplementary figure 2.8c**) or reversal learning (See **Supplementary figure 2.8d**) were detected between C-section and vaginally born participants during the Non-Stress or Exam Stress period. With regard to the microbiome composition unweighted UniFrac distances from the averaged rarefied 16S rRNA gene dataset determined that as in the adult mouse study, the microbiota data from C-section delivered subjects did not separate from vaginally born young adult participants. Likewise, there were no differences in alpha diversity and relative phylum abundance in vaginally-born and C-section born adults (See **Supplementary figure 2.11, Supplementary Table 2. 7** and **Supplementary Table 2. 8**).

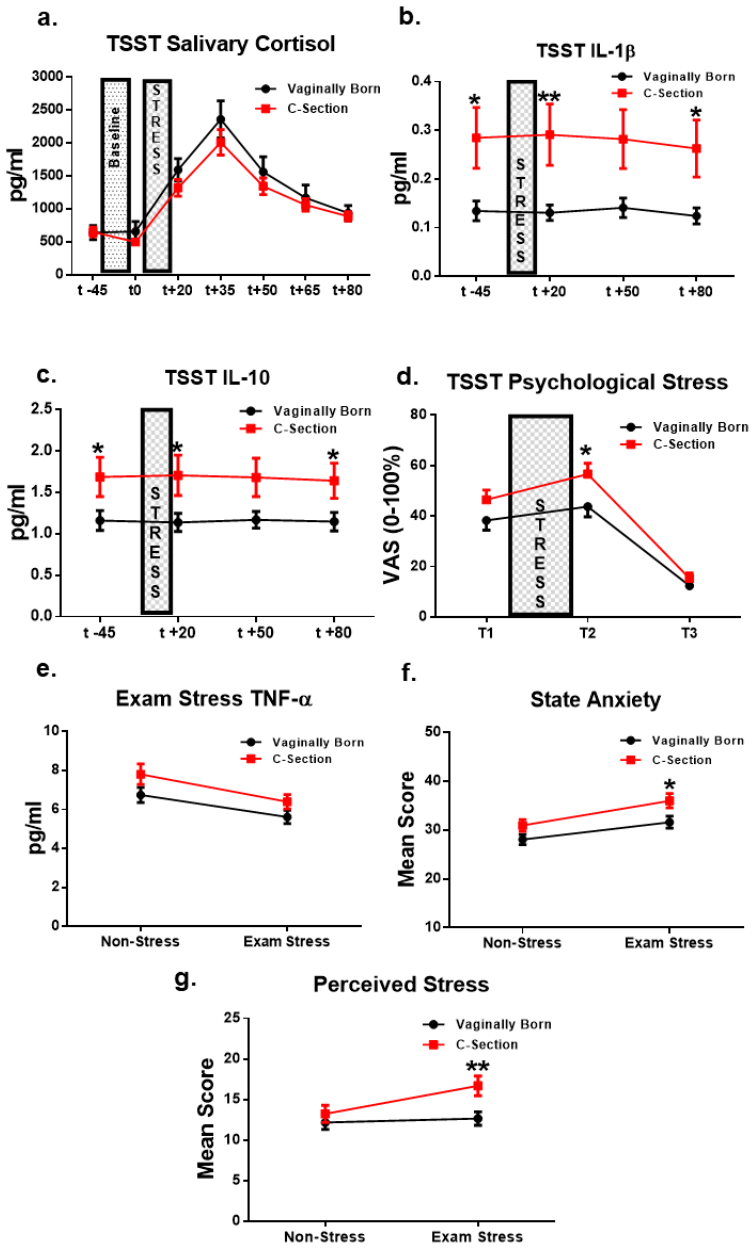



***Figure 2. 5- Increased psychological response to acute psychosocial stress and exam stress, and elevated inflammatory markers in C-section participants.***

**a**) Salivary cortisol response (Stress ($F(3.19, 220.01)= 73.26$, $p=0.001$, $\eta_p^2 = 0.52$); Group ($F(1, 69)= 0.14$, $p=0.7$, $\eta_p^2 = 0.002$); Stress x Group ($F(3.19, 220.01)= 0.36$, $p=0.79$, $\eta_p^2 = 0.005$; n= 3 [VB], n= 32 [C-section]). **b**) IL-1β response (Stress ($F(3, 120)= 3.143$, $p=0.026$, $\eta_p^2 = 0.043$); Group ($F(1, 70)= 6.584$,

p=0.012, $\eta_p^2$ = 0.086); Stress x Group (F(3,120)= 5.92, p=0.62, $\eta_p^2$ = 0.008; n= 39 [VB], n= 33 [C-section]). **c)** IL-10 are elevated in C-Section participants from baseline and across time-points (Stress (F(2.492, 176.96)= 0.521, p=0.634, $\eta_p^2$ = 0.007); Group (F(1, 71)= 4.24, p=0.043, $\eta_p^2$ = 0.056); Stress x Group (F(2.492, 176.96)= 0.615, p=0.58, $\eta_p^2$ = 0.009; n= 39 [VB], n= 34 [C-section]).**d**) Self-reported psychological stress response (Stress (F(2, 146)= 92.53, p<0.001, $\eta_p^2$ = 0.56); Group (F(1, 73)= 4.72, p=0.033, $\eta_p^2$ = 0.061); Stress x Group (F(2, 146)= 1.17, p=0.31, $\eta_p^2$ = 0.016; n= 40 [VB], n= 35 [C-section]), to the TSST procedure. **e**)TNF-α levels (Stress (F(1, 65)= 30.448, p<0.001, $\eta_p^2$ = 0.319); Group (F(1, 65)= 4.513, p=0.037, $\eta_p^2$ = 0.065); Stress x Group (F(1, 65)= 0.006, p=0.936, $\eta_p^2$ < 0.001; n= 38 [VB], n= 29 [C-section]), post-hoc comparisons at Non-Stress period [ p= 0.069] and Exam-Stress period [ p=0.058]). **f)** State anxiety levels as measured using the State Trait Anxiety Inventory (Stress (F(1, 67)= 35.48, p<0.001, $\eta_p^2$ = 0.35); Group (F(1, 67)= 5.21, p=0.026, $\eta_p^2$ = 0.072); Stress x Group (F(1, 67)= 0.59, p=0.59, $\eta_p^2$ = 0.45; n= 38 [VB], n= 31 [C-section]). **g)** Psychological stress as measured using the Perceived Stress Scale (Stress (F(1, 68)= 5.12, p=0.027, $\eta_p^2$ = 0.07); Group (F(1, 68)= 5.45, p=0.023, $\eta_p^2$ = 0.074); Stress x Group (F(1, 68)= 2.98, p=0.089, $\eta_p^2$ = 0.07; n= 38 [Vaginally born], n= 32 [C-section]), during a Non-Stress and Exam Stress period. Post hoc comparisons using Bonferroni correction: *p> 0.05; **p< 0.01. Data are presented as mean ± S.E.M.

## 2.4 Discussion

Mode of delivery at birth is one of the key factors regulating microbiota composition of infants (Brestoff and Artis, 2013; Makino et al., 2013). Disturbances in the appropriate establishment of the microbiota composition at the beginning of life have been implicated in long-term programming of health and in increasing susceptibility of immune and metabolic disorders (Brestoff and Artis, 2013). Here, for what is to our knowledge the first time, we extend the known negative outcomes of C-section to include enduring changes in behavior and stress sensitivity. In the mouse model we describe that birth by C-section leads to elevated stress, anxiety-like behavior, working memory, social recognition deficits, immunological and physiological changes. The translational relevance of these preclinical findings is demonstrated by the increased psychological distress and anxiety in healthy young adults born by C-section.

Use of an animal model allowed us to interrogate a number of potential mechanisms whereby C-section delivery may lead to changes in behavior and physiology. Of interest,

a recent study demonstrated that birth by C-section results in metabolic changes in a similar animal model. Here we demonstrate that neurobehavioral differences in mice are associated with gut microbiota changes in early-life and adolescence whereas, by adulthood, all differences in gut microbiota are resolved both in animals and humans (**Figure 2. 2 and Supplementary figure 2.9**). Altered microbiome composition at critical stages of early-life, during which rapid central nervous system development occurs, has been implicated in a variety of behavioral alterations in animals (O'Mahony et al., 2017) and humans (Carlson et al., 2018; Christian et al., 2015). Interestingly, although the microbiota composition changes are fully recovered by adulthood the negative behavioral effects of C-section endure. The finding that co-housing can reverse the cognitive, anxiety and social deficits, which occur due to C-section, further implicates the microbiota in mediating some of these deficits. Moreover, co-housing VB with CS mice induced intestinal motility deficits but not behavioral deficits. Previous human studies have demonstrated that C-section reduces bifidobacterium abundance (Dominguez-Bello et al., 2010) and given the importance of this species to early priming of gut and immune health we investigated whether alterations in Bifidobacteria levels contribute to the changes observed. Indeed, C-section resulted in a transient decrease in *Bifidobacterium spp.* abundance in the fecal microbiota of adolescent mice. Further promotion of growth of *Bifidobacterium spp.* from birth with either prebiotics (scGOS/lcFOS) or a specific *B. breve* strain both prevented CS offspring from developing anxiety-like, working memory and social cognition deficits. These data, coupled with that emanating from germ free animals (Diaz Heijtz et al., 2011; Foster and McVey Neufeld, 2013) and other models of disrupted microbiota (Diaz Heijtz et al., 2011; Foster and McVey Neufeld, 2013; Kelly et al., 2016), confirm that there are critical windows in which the restoration of microbiota post-weaning can reverse some but not all of the responses observed due to loss of microbiota. Moreover, they support the concept of microbiota-targeting interventions to reverse C-section-induced changes across the microbiota-gut-brain axis (Moya-Pérez et al., 2017).

Alterations in early-life gut microbiota are also implicated in social behavior development (Sandi and Haller, 2015) and here we show that CS mice have maternal attachment deficits in

early-life. Although these animals present normal social preference, they have a specific deficit in social-novelty preference which is similar to the social behavior of germ-free mice that have been colonized post-weaning (Desbonnet et al., 2015). Oxytocin signaling in the amygdala is essential for processing of relevant social cues and modulating social recognition (Ferguson et al., 2001). In addition, alterations in the oxytocin receptor (OXTR) have been implicated to the aetiology of a wide range of psychopathologies (Johnson and Young, 2017). In accordance with this, CS mice have elevated gene expression levels of the oxytocin receptor in the amygdala. There is growing evidence that microbiota could influence oxytocin levels improving social behaviour, stress and general health in the host (Buffington et al., 2016; Poutahidis et al., 2013). Interestingly, in this study promotion of growth of *Bifidobacterium spp.* from birth also corrected C-section mediated changes in the OXT receptor system. Moreover, as in germ free mice, CS-born offspring exhibit deficits in hippocampal-dependent working memory tasks (Desbonnet et al., 2014). In agreement with this, gene expression analysis of the hippocampus revealed differential expression of genes involved in the proteinacious extracellular matrix (ECM) and cell adhesion processes in the hippocampus of CS offspring, both of which have been implicated in memory processes (Tsien, 2013). Indeed, during brain maturation, the ECM is crucial for plasticity and synapse formation underpinning cognitive flexibility and memory (Happel et al., 2014). Further studies on the relative contribution of the up-regulation of proteinaceous ECM genes in the hippocampus of CS mice to their social and cognitive deficits are now warranted.

Gastrointestinal dysfunction may be a potential mechanism mediating the C-section-induced behavioral and HPA axis alterations observed (Hsiao et al., 2013; Kelly et al., 2015). Indeed CS born mice have faster gastrointestinal motility compared with VB mice. Moreover, in adolescence (P23), small intestine permeability was significantly reduced in CS mice compared with VB mice reflecting a more resilient small intestine barrier whereas in adulthood this had resolved. Conversely, circulating LPS levels were higher in the plasma of adolescent mice which would result in elevated inflammatory processes at this critical period. Interestingly, immune changes persist into adulthood whereby CS mice have more IL-10, TNFα and IL-12p70, produced from stimulated thymocytes challenged with ConA than VB mice.

The data from healthy human volunteers, although preliminary, implies that mode of delivery at birth has an enduring effect on host immune system and behavioral response to stress. Indeed, as in our animal studies (**Figure 2. 3c**), the microbiota of both groups is indistinguishable in adulthood (See **Supplementary figure 2.9**). The higher levels of IL-1β and 1L-10 in C-section when compared to vaginally-born participants support a dysregulation of immune-brain signaling in regulating behavior (Sternberg, 2006) and parallels the enduring immune changes in the animal model. Some early gut bacteria colonizers (i.e. Bacteroides; Bifidobacteria and Lactobacillus species) promote regulatory T cells (Foxp3(+) regulatory T cells (Tregs) and induce IL-10 production (Johansson et al., 2012; Round and Mazmanian, 2010). Likewise, these early bacterial colonizers are important for the activation of regulatory B cells that can drive IL-10 and IL1-ß production (Rosser et al., 2014). The association between the mode of delivery and the production of IL-10 and IL1- ß has been previously described in early-life in humans (Malamitsi-Puchner et al., 2005) and mice (Hansen et al., 2014).

Measures of positive affect in the human cohort were significantly lower throughout the acute stress procedure and these individuals reported greater psychological stress in response to the TSST. Perhaps surprisingly, there is a dissociation between self-reported stress measures and cortisol output in the TSST. However, there are a large number of studies which have not reported any relationship between self-reported stress and cortisol output (See (Campbell and Ehlert, 2012) for review). To probe this dysregulation in stress sensitivity we took advantage of a naturalistic stressor, University examination stress, and found that the anti-inflammatory cytokine IL-10 was significantly elevated during the Exam-Stress period in C-section participants and levels of TNF-α were increased. Individuals born by C-section also reported significantly greater levels of trait anxiety and perceived stress during the Exam Stress period but not during the Non-Stress period. Given the importance of mode of delivery in microbiota composition and subsequent immune and HPA axis priming (Foster and McVey Neufeld, 2013), it is tempting to speculate that it may be causally related to the changes observed. However, the nature of the current study design does not allow us to investigate factors that are responsible for such changes in this cohort as they occurred >20 years prior to testing. Moreover, although both mice and men show increased stress vulnerability, the behavioral

and physiological manifestations of these is qualitatively different in both species which may reflect their very different life and nutritional experiences. Future cohort studies, which have temporal microbiota analysis coupled with different mode of delivery stratifications, across the lifespan, are now warranted to investigate such mechanisms (Dominguez-Bello et al., 2010). Such studies should take into account the rationale underpinning the C-section (e.g. complicated pregnancy) and could also include prenatal maternal stress measures which is known to affect microbiome composition and offspring stress responses (Jašarević et al., 2015a). Moreover, the role of sex, birth-order and season of testing should be explored in future studies. Until recently there has been limited epidemiological data examining behavioral and psychiatric outcomes in individuals born by C-section. Indeed, where associations have been made in autism and psychosis they disappear when familial confounding is taken into place (Curran et al., 2015a; O'Neill et al., 2016). However, associations between mode of delivery and attention deficit disorder (Curran et al., 2016b) and school performance in Swedish adolescents have been described (Curran et al., 2017), however direct measures of stress and cognitive performance have not been investigated to date. Thus, our human data are, to our knowledge, the first attempt at stratification of stress response by mode of delivery.

Together these findings raise significant concerns regarding the increased use of C-section deliveries because of likely consequential stress, social and cognitive effects. The animal model data implicate the microbiome in mediating such effects. However, it is clear that C-section induces many other physiological changes (Golubeva et al., 2015; Jašarević et al., 2015b; Zijlmans et al., 2015) such as stress and immune priming during the birthing process that may also contribute to the phenotype (Lagercrantz and Slotkin, 1986a). Finally, since C-section deliveries when medically indicated are unavoidable lifesaving interventions, our data point to the possibility of developing adjunctive microbiota-targeted therapies (Dominguez-Bello et al., 2016; Moya-Pérez et al., 2017) which may help to avert any long-term negative consequences on stress responsivity, physiology and behavior.

## 2.5 Online Methods

### 2.5.1 Animals

The experiments were performed out in male Swiss mice of different ages. 8 week old female and male breeders were obtained from Harlan laboratories, UK. Breeding began after 1-2 weeks of acclimatization to the animal holding room. The animals were kept under a strict 12:12-h dark-light cycle and temperature (20±1 $^{o}$C, 55.5%), with food and water *ad libitum* unless specified otherwise. Male offspring were weaned at P21 and group-housed in 3-4 mice per cage. Groups consisted of offspring from 3-10 independent litters. Moreover, 10 weeks old Swiss mice, purchased from Harlan laboratories, UK, were used as conspecifics on the three-chamber test. All the procedures used in the present study were conducted in accordance with the Directive 2010/63/EU for the protection of animals used for scientific purposed and were approved by the Animal Experimentation Ethics Committee of University College Cork # 2012/036.

### 2.5.2 C-section Surgery

Mice were time-mated, and the presence of a vaginal plug was marked as gestational day 0.5 (G0.5). Males were removed from the cage and pregnant females were not disturbed unless for cage cleaning. At full term (G20.5) female mice was euthanized by cervical dislocation. To reduce bacterial contamination of the abdominal cavity, the abdominal skin was prepped by application of 70 % ethyl alcohol and the abdominal skin was retracted. The abdomen was incised and the uterus was removed and placed on sterile gauze. To prevent hypothermia of the fetus in the uterus, a heating pad was placed beneath to provide thermal support. The pups were then removed by gentle pressure with a sterile swab and the umbilical cord was cut. Sterile cotton swabs were used to tear the amniotic membrane and massage each pup until spontaneous breathing was noted. The pups were given to a foster dam that gave birth on the same day. Additional pregnant females were allowed to deliver spontaneously and the litters were used as full-term vaginal delivery

controls. The pups were dried by smearing them with the bedding material in the cage of foster dam. To control for the effects of fostering an additional cross-fostered vaginally born group was included in the design.

### 2.5.3 Cross-fostering

Cross-fostering was performed on pups born from breeding pairs within 12 hours of each other. On the day of the birth the litters were removed and put with their nurse mothers. The pups were nursed by their respective foster mothers until weaning. Given that CF and VB animals had the same phenotype across a variety of domains (anxiety, HPA axis response, and social behavior and immune system), for later experiments (**Figure 2. 3** and **Figure 2. 4**) we focused on just one control group to minimize animals usage.

### 2.5.4 Co-housing Procedure

At three weeks of age, male offspring born by vaginal birth or C-section were weaned and mice were divided into different housing conditions: vaginal birth group, where each cage consisted of three to four vaginal delivered mice housed together (from 9 litters); C-section, where each cage consisted of four C-section born mice housed together (from 6 different litters); co-housing, where for each cage one C-section born mice was housed together with three vaginal born mice (CS co-housed, from 7 litters and VB co-housed: n=36, from 10 litters) (**Figure 2. 3**). The co-housing system was based on Buffington et al., (2016) (Buffington et al., 2016). In the vaginal birth co-housed group, one animal per cage was randomly selected to pass through behavioral and for *post-mortem* analysis.

### 2.5.5 Probiotic and Prebiotic Administration

Following birth by C-section pups were exposed to probiotic *Bifidobacterium breve M16V* (*B.breve*) suspended in MediDrop clear $H_2O$ (75-02-1001) at a concentration of $10^9$ c.f.u./ml or a combination of scGOS and lcFOS in a 9:1 ratio at a final concentration

of 1% into the custom-made AIN93G rodent diet. Both interventions were given to the dams from birth during the lactation period. Male offspring were weaned at P21 on to the same corresponding interventions throughout behavioral testing. Foster dams of VB control mice received MediDrop clear $H_2O$ (75-02-1001) and AIN93G diet at libitum during lactation. At P21 the VB mice were weaned on the same corresponding diet as their foster dams.

### *2.5.6* Behavioral Testing

The short and long-term effects of C-section were evaluated in male mice both during early-life and (P9/P10) adulthood (week 8-16). An independent cohort of animals of mice was used for the behavioral tests conducted in adolescence starting at P21. Mice were habituated to the room 30 minutes prior to each test. The experimental procedures are described below and illustrated in **Figure 2. 1b.** The order of selected behavioral test and intervals was chosen to minimize the potential confounding carryover effects from the previous behavioral test.

#### *2.5.6.1 Isolation-induced Ultrasonic Vocalizations Test (USV)*

At P9, the USV was performed accordingly with the protocol described previously (Robertson et al., 2017).

#### *2.5.6.2 Homing Test*

The homing test was performed at P10 in order to evaluate the tendency of pups to recognize their mother and siblings nest as previously described (Macrì et al., 2010).

#### *2.5.6.3 Forced-swim Test*

Forced swim test is widely used experimental paradigm to assess depression-like behaviour in rodents as described (Cryan et al., 2001; Mombereau et al., 2004).

#### *2.5.6.4 Three-chamber Test*

Sociability and preference for social novelty were assessed in a three-chamber apparatus, as described previously by (Desbonnet et al., 2014).

#### *2.5.6.5 Defensive Marble Burying*

The defensive marble burying test was performed accordingly to (Sweeney et al., 2014).

#### *2.5.6.6 Elevated Plus Maze*

The elevated plus maze was conducted as described (Jacobson et al., 2007).

#### *2.5.6.7 Aversive Open Field*

The test was performed accordingly to (Savignac et al., 2011) with the lights set as 1000 lux.

#### *2.5.6.8 Hot Plate*

The hot plate test was performed as described by (Tramullas et al., 2012).

#### *2.5.6.9 Novel Object Recognition Task*

Working memory was assessed through the novel task as described by (Robertson et al., 2017).

See **Supplementary Methods** for detailed description of behavioral tests.

### 2.5.7 *In Vivo* Intestinal Motility

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

### 2.5.8 Murine HPA Axis Response

#### *2.5.8.1 Corticosterone Response to FST*

Blood samples were taken to assess the HPA axis response to a mild acute stress (FST) in adolescence (P25) and adulthood (week 16) accordingly to (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.

### 2.5.9 Intestinal Permeability Assays

#### *2.5.9.1* In Vivo *Intestinal Permeability to* Fluorescein *Isothiocyanate–* dextran *(FITC-dextran)*

For this procedure mice were fasted overnight. Next day early in the morning FITC-dextran, MW = 4 kDa (FD4, Sigma) was given orally by gavage, (600 mg/kg, 80 mg/ml) in phosphate buffered saline (PBS). A blood sample (100 µl in heparin-coated glass capillary) is taken from tail vein 2 hours after oral gavage. Samples were kept on ice, centrifuged at ~ 3500 g for 15 min, plasma was aspirated, transferred to amber tubes, kept on ice and analysed on the day of sampling. Plasma was measured undiluted, 25 µl in 384 well plate. FITC was measured with a Victor spectrometer. The excitation maximum is 490 nm, the emission maximum was 520 nm (measured at 535 nm). For a standard curve, serial dilutions of FITC were prepared in PBS.

#### *2.5.9.2 Detection of Gram-negative Bacterial Endotoxin*

Endotoxin levels in the murine plasma were estimated using the Limulus Amebocyte Lysate (LAL) QCL-1000 assay kit (Lonza) according to manufacture's instructions.

### 2.5.10 RNA Isolation and Synthesis of cDNA

Total RNA was isolated from the ventral hippocampus (HIP), paraventricular nucleus of the hypothalamus (PVN), amygdala (AMY), hypothalamus (HYP), pituitary gland, colon and ileum using the mirVana™ miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific). 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 °C until time of assay.

### 2.5.11 qRT-PCR

Gene expression was analyzed using Gene Expression Assays on an AB7300 system (Applied Biosystems, Thermo Fisher 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).

### 2.5.12 RNA Sequencing Brain Samples

Library preparation and sequencing as well as Fastq-file generation was done by Beckman Coulter Genomics service (Danvers, MA, USA). Paired-end reads of 2×100 bp were produced on an Illumina HiSeq2500 sequencer.

#### *2.5.12.1 Bioinformatic Analysis Pipeline*

Fastq-format reads were quality filtered and trimmed using Trimmomatic (v0.32) (Bolger et al., 2014) with the following non-default parameters: *AVGQUAL*: 20; *SLIDINGWINDOW*: 4:20; *LEADING*: 10; *TRAILING*: 10; *MINLEN*: 60. Alignment to the mouse reference genome (GRCm38.p3) was achieved using the STAR aligner (v2.4.0f1) Dobin (Dobin et al., 2013) with default options and an index compiled with gene models retrieved from the Ensembl database (release 78). These gene models were also used for read counting for each gene using HTSeq-Count (v0.6.0) (Anders et al., 2015) with the following non-default parameters: -s: no; -r: pos; -q –f bam –m intersection-nonempty. Differential gene expression was determined using the DESeq2 R-package (v1.6.2) (Love et al., 2014) with default parameters on pairwise comparisons of all possible group combinations. An adjusted p-value ≤ 0.1 (Benjamini-Hochberg method) was considered significantly differentially regulated. Raw and processed original data will be deposited in NCBI's Gene Expression Omnibus (Barrett et al., 2013) and made accessible through a unique GEO accession number upon publication.

### 2.5.13 Murine Splenocyte and Thymocyte Stimulation

Spleens and thymus were collected immediately following sacrifice and cultured as described (Robertson et al., 2017).

### 2.5.14 Study Participants

The study protocol (APC050 2014) and all procedures were approved by the Cork Teaching Hospitals Ethics Committee and conducted in accordance with the ICH Guidelines on Good Clinical Practice, and the Declaration of Helsinki. Study participants were all male between 18-25 years of age, recruited via advertisement from the student population of University College Cork. Forty vaginally born participants and by 36 participants born by C-section (18-planned C-section; 15-emergency C-section; 3-

unknown. Self-reported race and ethnicity were: for vaginally born participants (38 Caucasian and 2 Asian) and C-section participants (34 Caucasian and 2 Asian) matched on the basis of age, BMI and average units of alcohol consumed per week. Exclusion criteria included having a BMI ≥30, formal psychiatric diagnosis of major depression, anxiety disorder, bipolar spectrum disorder, schizophrenia or other DSM-IV Axis-I disorder, use of psychoactive medication(s) (anxiolytics, antipsychotics, antidepressants, corticosteroids and opioid pain relievers), regular use of non-steroidal anti-inflammatory medications or antibiotic use in the previous 4 weeks. In addition, healthy control participants were excluded if they reported a history of chronic physical illness

### 2.5.15 Human Study Procedures

Participants were screened to check suitability for study inclusion and were subsequently scheduled to attend one or two study visits at the Mercy Hospital, Clinical Research Facility, Cork City. All participants provided full written informed consent before any experimental procedures commenced. All participants attended one visit during term-time (Non-Stress/TSST visit), but not within ± 6 weeks of a formal end of term examination period, and a subset of 38 vaginally born and 32 C-section participants completed another visit carried out during the participants University Examination period (Exam Stress visit) (See **Supplementary Supplementary Table 2. 5** for detailed description of participants demographic). Order of visits was counterbalanced so that roughly half of each group completed the Non-Stress visit first and the other half completed the Exam Stress visit first. At the Exam Stress and Non-Stress visit, participants completed a battery of cognitive tests and completed self-report measures assessing mood, anxiety and perceived stress (see **Self-Report Measures**). In addition, during the Non-Stress visit participants completed the Trier Social Stress Test (TSST; See **Supplementary Methods** for detailed description of TSST methodology).

#### *2.5.15.1 Self-Report Measures*

See Supplementary Methods for detailed description of Self-Report Measures.

### 2.5.16 Human Proinflammatory Cytokine Sampling & Analysis

To determine the immune response to acute psychosocial stress 5-10ml of whole blood was collected in EDTA tubes at 4 time-points throughout the TSST; t -45, t+20, t+50, t+80. To determine the effect of exam-stress on immune function 10-15ml of whole blood was collected in EDTA tubes during each study visit (Non-Stress/ Exam Stress). Samples were centrifuged immediately at 1,500 x g for 10 minutes and aliquoted plasma samples were frozen at -80°C until analysis. Plasma levels of IL-10, IL-1β, IL-6, IL-8, and TNF-α were assayed in duplicate using high sensitivity commercially available electrochemiluminescence MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are; IL-6- 0.06 pg/ml, IL-8- 0.04 pg/ml, TNF-α- 0.04 pg/ml and IFN-γ- 0.2 pg/ml.

### *2.5.17* Human Hypothalamic-pituitary-adrenal (HPA) axis response

#### *2.5.17.1 Salivary Free Cortisol Sampling & Analysis*

Saliva samples were obtained using Salivette® devices (Sarstedt, Ireland). Participants were instructed to roll the synthetic bud around their mouths while chewing lightly for 1.5 minutes. To determine the HPA axis response to acute psychosocial stress, saliva samples were collected at seven time-points throughout the TSST; t -45, t0, t+20, t+35, t+50, t+65, t+80. Samples were kept chilled at ~4°C until the end of the experimental protocol. To determine the effect of exam stress on HPA axis function, the salivary cortisol awakening response (CAR) was measured at each time point. On the morning prior to each study visit (Non-Stress/ Exam Stress) participants were instructed to collect

4 saliva samples (upon wakening, 30 minutes post-wakening (t+30), 45 minutes post-wakening (t+45), 60 minutes post-wakening (t+60).

Saliva samples were centrifuged for 5 minutes at 1000g to extract saliva and samples were stored at -80°C until analysis. Cortisol concentrations were determined using the Cortisol Enzyme Immunoassay Kit as per manufacturers' instruction (Enzo® Life Sciences, Exeter, UK). Assay detection limit was 0.16 nmol/L. Inter and intra-assay % C.Vs were 11.24 % and 8.2% respectively.

### 2.5.18 Cognitive assessment: Tests from the CANTAB battery (http://www.cambridgecognition.com/)

See Supplementary information for detailed description of cognitive tests.

### 2.5.19 16S rRNA Gene Amplicon Sequencing of Intestinal Microbiota Animal and Human Samples

Raw sequences were merged using Flash (with a minimum overlap of 30 bp and minimum read length of 460bp) and quality checked using the split libraries script (with default parameters) from the QIIME package version 1.8.1. Reads were clustered into OTUs with 97% sequence identity threshold and chimeras and singletons removed with the 64-bit version of USEARCH (version 7). Subsequently, OTUs were aligned to the SILVA rRNA specific database (version 123) to assign taxonomy and a phylogenetic tree was generated within QIIME (for animal samples). Taxonomy to genus level was assigned using the Mothur program (version 11.4) using the RDP database (version 11.4). Species level classification was performed using SPINGO (version 1.3) using the RDP database (version 11.2) (for human samples). Alpha diversity analyzes were also implemented within QIIME. Principal coordinate analysis plots, based on bray-curtis distances, were generated using the vegan package in R.

### 2.5.20 DNA Isolation from Fecal Samples for Bifidobacteria Determination

Total bacterial DNA was extracted from the fecal pellets using the QIAamp DNA Stool Mini Kit (Qiagen, Sussex, UK) according to manufacturer's instructions, coupled with an initial bead-beating step.

### 2.5.21 Quantitative Determination of Bifidobacteria in Fecal Pellets

Absolute quantification of *Bifidobacterium spp.* in fecal pellets DNA was determined by q-PCR using the Roche LightCycler 480 platform (Roche Diagnostics, West Sussex, UK) and KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal (KAPA Biosystems, Massachusetts, US) as per the manufacturers recommended protocol. Previously described genus-specific primers were used for the quantification (Matsuki et al., 2002). Standard curves were created using bacterial DNA extracted from a pure culture of *Bifidobacterium longum* NCBIM8809 as previously reported (Arboleya et al., 2012).

### 2.5.22 Statistical Analysis

See Supplementary Methods for detailed description of statistical methodology.

## 2.6 Supplementary Methods

### 2.6.1 Preclinal Study

#### 2.6.1.1 *Behavioral Tests*

Behavioral tests were performed and analyzed by two independent experimenters blinded to group and intervention.

#### 2.6.1.1.1. Isolation-induced Ultrasonic Vocalizations Test

Isolation-induced ultrasonic vocalizations (USV) are produced by mice pups during the first two weeks of life when separated from mother and littermates (Winslow et al., 2000). Pups were isolated from their mother and littermates and placed in a clean plastic container into a sound attenuating chamber. Emission of ultrasonic vocalizations are monitored by an ultrasound sensitive microphone – a bat detector (US Mini-2 bat detector, Summit, Birmingham, USA) tuned in the range of 60-80 kHz – suspended above the isolated pup for 3 minutes and the number of calls were noted.

#### 2.6.1.1.2. Homing Test

The homing test evaluates the tendency of pups to recognize their mother and siblings nest (Macrì et al., 2010). At P10 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 with clean bedding material. Individual pups were placed in the middle section for 1 minute, the dividers were then removed and the pups were allowed to freely move around for 2 minutes. Total time spent in each area was noted.

#### 2.6.1.1.3. Forced-swim Test

In this test, animals were placed into a cylinder of tepid water (23-25 $^{o}$C) to a depth of 17 cm. Behavior was recorded by a camera positioned from above the swim tank. The immobility time was scored during the last 4 min of the 6 min test. Animals, after removal from the cylinder, are placed into a separate cage for recovery.

#### 2.6.1.1.4. Three-chamber Test

Animals were placed in a rectangular apparatus divided into three chambers (left and right and a smaller center chamber) by transparent partitions with small circular openings allowing easy access to all compartments. The test is composed of three sequential 10 min trials: (1) habituation (the test animal were allowed to explore the three empty chambers); (2) sociability (an unfamiliar animal was placed in an inner mesh wire cage in either the left or right chambers); (3) social novelty preference (a novel animal was placed into the previously empty inner cage in the chamber, opposite the now familiar animal). All animals were age- and sex-matched, with each chamber cleaned and lined with fresh bedding between trials. For each of the three stages, behaviors were recorded by a video camera mounted above the apparatus and time of active interaction was measured.

#### 2.6.1.1.5. Defensive Marble Burying

The defensive marble burying (DMB) test measures repetitive and anxious behavior, indicating higher levels of anxiety at high number of marbles buried. Clean cages were filled with a 4-cm layer of chipped cedar wood bedding. Animals were habituated to the room for 30 minutes prior to the test. Twenty glass marbles (15 mm diameter) were gently laid on top of the bedding, equidistant from each other in a 4×5 arrangement. During the testing phase, each mouse was placed in the cage and allowed to explore it for 30 min. At the end of the test, animals were placed back into the home and the number of marbles buried (> 2/3 marble covered by bedding material) in 30 min was recorded.

#### 2.6.1.1.6. Elevated Plus Maze

The elevated plus maze (EPM) is one of the most widely-used behavioral tests to screen anxiety-related behaviors (Holmes, 2001; Rodgers, 1997a). The plexiglas maze consists of a plus-shaped apparatus with two open and two enclosed arms (50 cm × 5 cm × 15 cm walls) elevated from the floor by 1m. The animal was placed in the center of the EPM

apparatus facing an open arm and was allowed to explore it for a total period of 5 minutes. The apparatus was cleaned with 10% (vol/vol) ethanol after each subject to prevent olfactory cues from the previous mouse. Main classic parameters considered were the number of entries into open and closed arms and the total entries (open and closed arms). Entries into the open and closed arms were considered when mice placed all four paws on the arm.

#### 2.6.1.1.7. Aversive Open Field (OF)

The open field (OF) test is used to assess the locomotor activity and the response to a novel stressful environment. Light was set as 1000 lux. Mice were placed in the center of an open field arena (Perspex box with white base: 30 x 30 x 20 cm) and were allowed to explore the arena for a 10 minute period. The distance moved and velocity of movement in the open field were recorded using Ethovision videotracking system (Noldus Information Technology). Using this technology, a center zone and outer zone were demarcated. The time spent in and frequency of entry into each zone was also measured for each mouse. Mice were placed back into the home cage after testing. The box was cleaned with 10% alcohol and allowed to dry prior to next test.

#### 2.6.1.1.8. Hot Plate

Mice were placed on a hot plate (Panlab SL, Barcelona, Spain) with the temperature adjusted to $55 \pm 1$ °C. The time to the first sign of nociception, paw licking, flinching or jump response was recorded and the animal immediately removed from the hot plate. A cut-off period of 30 seconds was maintained to avoid damage to the paws.

#### 2.6.1.1.9. Novel Object Recognition

On day 1 animals are habituated to a square open field box (Perspex sides and base: 34.5cm x 42.7 cm for mice; in a dimly lit room, by individually placing animals into the

apparatus for 10 min exploration periods. On day 2, two identical objects are positioned on adjacent corners approximately 5 cm from each wall of the open field and each animal was introduced for a 10 min exploration period. Animals were then placed directly back into their home cages. After a 24hr inter-trial interval, one familiar object was replaced with a novel and each animal was introduced for a further 10 minutes exploration period. On each day, animals are acclimatized to the testing room for approximately 1 hr. Object exploration is defined as when the animal's nose comes within a 2cm radius of the object. In between trials, objects and testing arenas are cleaned with alcohol wipes and rinsed with water.

### *2.6.1.2 Murine Splenocyte/ thymocyte Stimulation and Cytokines Measurement*

Spleens were collected immediately following culling and were cultured. To culture spleen cells, first the spleens were dissociated in media [RPMI (with l-glutamine and sodium bicarbonate, R8758 Sigma +FBS) (F7524, Sigma)+Pen/Strep P4333, Sigma)]. The homogenate was then filtered over a 70µm strainer, centrifuged at 200g for 5min and resuspended in media. Cells were counted and seeded ($4x10^6$ cells /ml of media). After 2.5 hours cells were stimulated with lipopolysaccharide (LPS-2 µg/ml) or Concanavalin A (ConA-2.5 µg/ml) for 24 hours. Similarly, thymocytes were isolated as above and seeded at a concentration of $2x10^6$ cells /mL of media.

Following stimulation, the supernatants were harvested to assess $IFN_\gamma$, IL1β, IL-2, IL-4, IL-5, IL-6, KCGRO, IL-10, IL-12p70 concentration (pg/mL) using the Proinflammatory Panel 1 (mouse) V-PLEX MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are; $IFN_\gamma$ 0.04 pg/mL, IL1β 0.11 pg/mL, IL-2 0.22 pg/mL, IL-4 0.14 pg/mL, IL-5 0.07 pg/mL, IL-6 0.61 pg/mL, KCGRO 0.24 pg/mL, IL-10 0.95 pg/mL, IL-12p70 0.13 pg/mL, TNFα 0.13 pg/mL.

### *2.6.1.3 Qualitative Determination of* Bifidobacterium Breve *in Fecal Pellets*

The occurrence of *Bifidobacterium breve* species was determined by qualitative PCR (Applied Biosystems 2720 Thermal Cycler, Fisher Scientific Ireland Ltd, Ireland) using species-specific primers F (5'-AATGCCGGATGCTCCATCACAC-3'), R (5'-GCCTTGCTCCCTAACAAAAGAGG-3') and conditions as previously described (Gueimonde et al., 2006). The total volume of each reaction mixture was 25 µl, employing 1µl of DNA extract as a template, 12.5 µl of BioMix$^{TM}$ Red Master Mix (2x) (Bioline, UK) and 0.4 µM each primer. The thermal cycle program consisted of an initial cycle of 95°C for 10 min for denaturation and polymerase activation, 30 cycles of 95°C for 15 s, 62°C for 1 min, and 72°C for 45 s, and a final extension step of 10 min at 72°C. *Bifidobacterium breve* NCBIM2257 was used as positive control. Amplified products were subjected to gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining. Samples were analysed in two independent PCR runs.

### *2.6.1.4 Short Chain Fatty Acid Analysis*

Briefly, cecal content (30- 40mg) from adolescent and adult mice was vortex-mixed with 1ml Milli-Q water and incubated at room temperature for 10 min and subsequently centrifuged at 10,000g for 5mins to pellet bacteria and other solids. The supernatant was filtered, transferred to a clear gas chromatography (GC) vial and 2-ethylbutyric acid (Sigma-Aldrich, Ireland) was added as an internal standard. Standard solutions of 10.0 m mol/L, 8.0m mol/L, 6.0m mol/L, 4.0m mol/L, 1.0m mol/L and 0.5m mol/L of acetic acid, propionic acid, isobutyric acid and butyric acid (Sigma-Aldrich), respectively were used for calibration. The concentrations of SCFA were measured using a Varian 3800 GC-flame-ionization system fitted with a ZB-FFAP column (30 m X 0.32 mm X 0.25 µm; Phenomenex, Macclesfield, Cheshire, UK). Initial oven temperature was set at 100ºC for 30 secs and raised to 180ºC at 8ºC per min and subsequently held for 1 min, then increased to 200ºC at 20ºC per min and finally held at 200ºC for 5 min.

Helium was used as the carrier gas at a flow rate of 1.3ml/min. The temperature of injector and the detector were set at 240ºC and 250ºC respectively. A standard curve was constructed with different concentrations of a standard mix containing acetic acid, propionic acid, isobutyric acid and N-butyric acid (Sigma-Aldrich). Peaks were integrated using the Varian Star Chromatography Workstation v6.0 software.

#### *2.6.1.5 Statistical Analysis for Animal 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 Tukey 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 figure legends and **Supplementary tables**. Statistical significance was accepted at the level of $p< 0.05$. All parametric data is expressed as the means ± S.E.M. Non-parametric data is expressed by median and interquartile range.

### 2.6.2 Human Study Procedures

#### *2.6.2.2 TSST Methodology*

The TSST procedure began between 13.00-14.30h for each participant to control for diurnal fluctuations in cortisol levels. Participants were instructed to abstain from alcohol and strenuous physical exercise for 24 hours prior to visits. In addition, they were asked not to consume any caffeine containing products on the day of their Non-Stress study visit, and to consume only water for 2 hours prior to the TSST procedure. After collection of the first saliva sample for measuring salivary cortisol, participants rested for a 45

minute baseline period. Participants were then given standardized written instructions, introducing the TSST which was carried out as previously described (Allen et al., 2014). Participants were then led to a separate room, equipped with a video camera and microphone and two desks. After reiterating the task instructions, participants were given a 3 minute speech preparation period, after which, participants were required to perform a 5 minute speech outlining their suitability for an ideal job of their choice. This was followed by a 5 minute mental arithmetic task in which they serially subtract 17 from 2023. Task were performed in front of two committee members (one male and one female), wearing white laboratory coats and introduced as being experts in identifying non-verbal aspects of behavior. Participants were also informed that their speech would be both audio and video recorded for later behavioral analysis. Saliva samples and self-report measures of mood and stress were collected at a number of time-points pre and post the TSST procedure (see below). Participants were fully debriefed following collection of the final sample.

#### *2.6.2.3 Self-Report Measures*

Mood was assessed using the Positive and Negative Affect Schedule (PANAS) (Watson et al., 1988), and psychological stress was measured using a visual analogue scale (VAS), ranging from 0 (*'not stressed at all'*) to 100 ('*As stressed as I could possibly imagine'*).

### State Trait Anxiety Inventory (STAI)

The STAI is a self-report measure consisting of two subscales each with 20 items, one measuring trait anxiety and the other measuring state anxiety (Spielberger et al., 1983). For the current study we only measured state anxiety. Participants rate how they feel either right now (state) or generally (trait), in response to each item on a 4-point scale from 'not at all' to 'very much.' The range of scores for each sub-scale is 20-80 with higher scores indicating greater anxiety.

## Perceived Stress Scale (PSS)

The PSS is a self-report measure in which participants rate, on a 5 point scale ranging from 0 (never) to 4 (very often), how often they have particular thoughts or feelings described by each of the 10 items (Cohen et al., 1983). Scores range from 0-40 with higher scores indicating greater stress over the previous month.

## Beck Depression Inventory (BDI)-II

The BDI-II is a self-report measure consisting of 21 items rated on a 4-point scale from 0 (absence of symptom) to 3 (severe manifestation of symptom) (Beck et al., 1996). Scores range from 0-63. Cut-off scores indicating clinically relevant levels of depression have been determined as 0-13 (minimal); 14-19 (mild); 20-28 (moderate); 29-63 (severe).

## Cognitive Assessment: Tests from the CANTAB Battery

(http://www.cambridgecognition.com/)

CANTAB tests were presented on a high-resolution touch-screen monitor under computer control. Participants interact with the system by touching the touch screen whilst a test administrator provides verbal instructions from a standardised script, as well as specific verbal prompts and encouragement when needed. The test administrator has full control of a keyboard used to start, pause or abort each test. Participants were assessed on the following tests from the battery:

## IED: Attentional Flexibility and Reversal Learning

The IED is a test of executive function and assesses rule acquisition and reversal, attentional set formation, maintenance and shifting. The outcome measures assessed to determine reversal learning performance were the errors made on stage 2, 5, 7 and 9, and to determine attentional flexibility performance the errors made on stage 6 and 8.

### Stop Signal Task (SST): Response Inhibition

The SST assesses participant's ability to inhibit a prepotent response. The outcome measure assessed was the stop signal reaction time (SSRT; calculated for last 20 sub-blocks).

### Paired Associates Learning (PAL): Visuospatial Memory

The PAL assess conditional learning of pattern-location associations and gives an index of visuospatial memory. The outcome measure assessed to determine visuospatial memory was the PAL total errors adjusted.

#### *2.6.2.4 Statistical Analysis for Human Data*

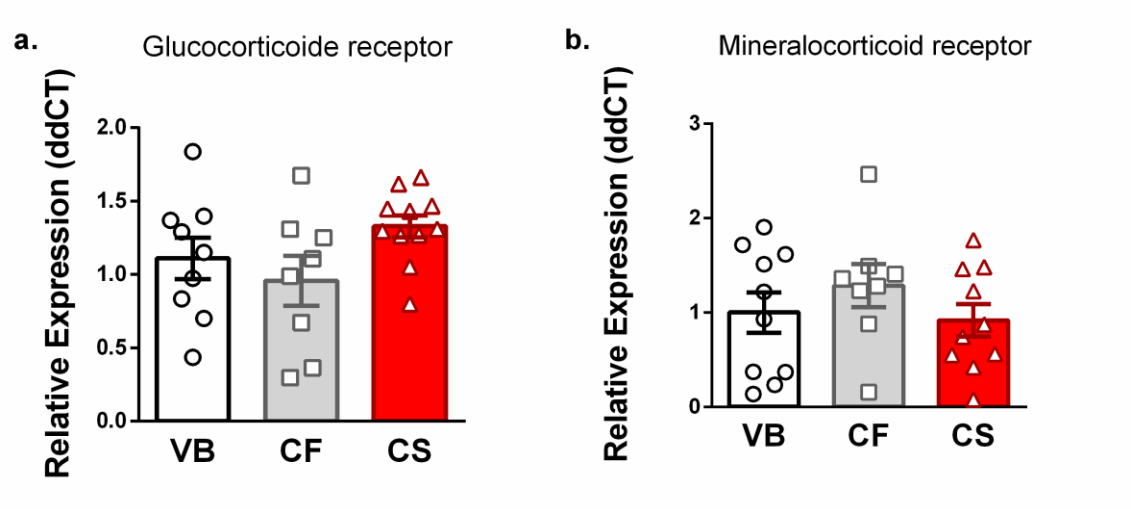
Independent sample t-tests were used to explore differences in group characteristics (age, BMI, years of education and units of alcohol per week). To allow for repeated measures analysis and to avoid bias that may be introduced by using list-wise deletion of incomplete cases (Graham, 2009), missing data analysis was performed on physiological, psychological and cognitive variables subject to repeated measures analysis. In total 5.95% of data was missing and determined to be missing completely at random (MCAR) using Littles MCAR test (Little, 1988); $\chi 2$ (688) = 703.356, p=0.334). Missing values were inputted by assigning the group mean for that variable except for cytokine and cortisol awakening response data. All analyzes were performed with missing data excluded (data not shown) and missing data included, which showed that inputing values using this method did not significantly change the nature of our results. Participants with more four or more missing data points for salivary cortisol during the TSST were excluded from this analysis. If a participant had missing data at all time-points for a given variable (during the TSST or Exam Stress/ Non Stress visit) missing values were not imputed and the participant was excluded from this analysis. Following missing data insertion, normality checks were performed using the Shapiro-Wilk test and visual inspection of

histograms. Outliers were checked using box and whisker plots and only extreme outliers were considered for exclusion from analysis. Salivary cortisol (TSST), IL-10 (TSST), IL-1β (TSST), IL-6 (TSST), IL-8 (TSST), TNF-α (TSST), IL-8 (Non-Stress/Exam Stress), TNF-α (Non-Stress/Exam Stress), PANAS, STAI, BDI and CANTAB PAL (Visuospatial Memory) data was not normally distributed and transformed using a natural log transformation (ln); VAS psychological stress data was transformed using a square-root transformation; IL-10 (Non-Stress/Exam Stress), IL-1β (Non-Stress/Exam Stress), IL-6 (Non-Stress/Exam Stress), CANTAB IED (Attentional Flexibility/ Reversal Learning) data was not normally distributed, but no transformations improved normality, so we proceeded with parametric analysis but with caution in interpreting the analysis. Salivary cortisol awakening response values at each time-point were converted to area under the curve with respect to ground (AUCg) values (Pruessner et al., 2003). AUCg cortisol data was not normally distributed and no transformations improved normality, so again we proceeded with parametric analysis but with caution in interpreting the results. PSS and CANTAB SST data (stop signal response time last 20) were normally distributed and no transformations were performed. Following data imputation and transformation (if needed) to improve normality, repeated measures analysis of variance (ANOVA) with *group* as between-subjects factor and change across time points due to *stress* in each variable (salivary cortisol (TSST), IL-10 (TSST), IL-1β (TSST), IL-6 (TSST), IL-8 (TSST), TNF-α (TSST), cortisol awakening response AUCg ((Non-Stress/Exam Stress), IL-10 (Non-Stress/Exam Stress), IL-1β (Non-Stress/Exam Stress), IL-6 (Non-Stress/Exam Stress), IL-8 (Non-Stress/Exam Stress), TNF-α (Non-Stress/Exam Stress), PANAS Positive/Negative, VAS psychological stress, STAI State, BDI, PSS, PAL Total Errors Adj., SSRT last 20, IED Attentional Flexibility, IED Reversal Learning) as the within-subjects factor. Significant main effects were followed by *post-hoc* comparisons using a Bonferroni correction for multiple comparisons as appropriate. Where Mauchly's test of sphericity was significant, the Greenhouse-Geisser or Huynh-Feldt correction was applied. Our primary outcome variable was salivary cortisol output in response to the TSST. Based on previous findings from out laboratory (Kennedy et al., 2014) we powered our study to detect between group differences in the salivary cortisol response with a

medium effect size (f= 0.25). At an alpha of 0.05 and obtaining a power of 0.08, a total sample size of 74 was required. This calculation is based on a repeated measures ANOVA, between factors, using G*Power software. Non-transformed data are presented as mean ± S.E.M. Effect sizes are reported as partial Eta squared ($\eta_p^2$). All statistical analyzes were carried out using IBM SPSS Statistics 22.0 for Windows software package.

### 2.6.3 Microbiota Statistical Analysis (Mice and Human Samples)

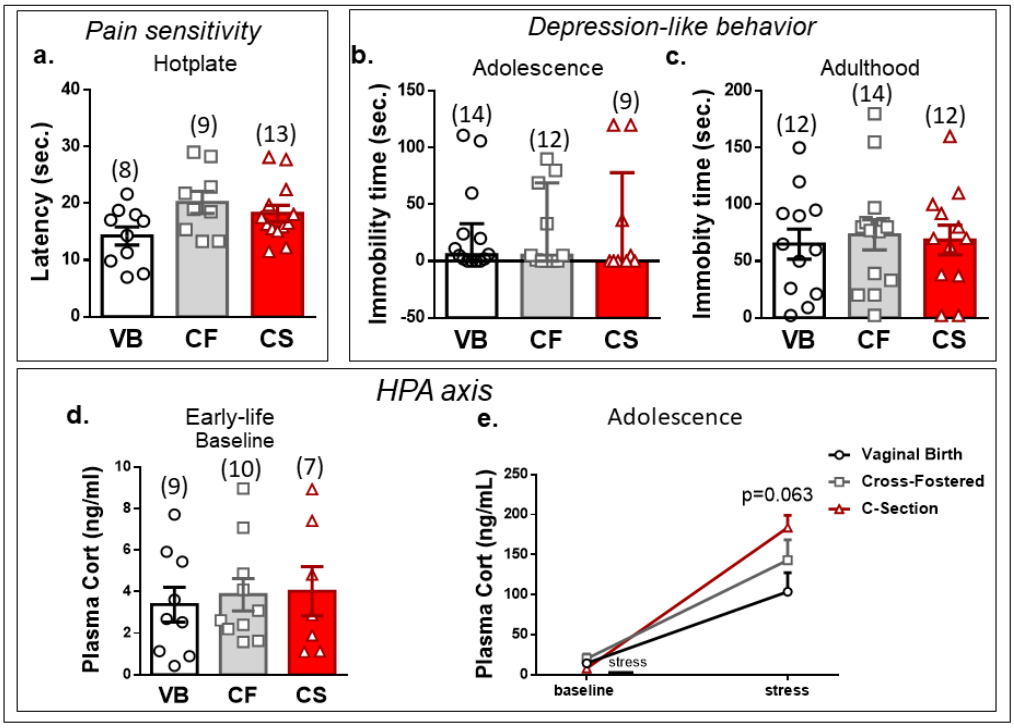
Statistical differences for microbiota composition based on alpha diversity and for the abundance of taxa were calculated using the compare Groups package in R. P-values were adjusted using the Benjamini-Hochberg method. Three dimensional principal coordinate analysis plots, based on Bray-Curtis beta diversity, were created using the made4 and car packages in R. Supplementary figures




***Supplementary figure 2.1- Mode of delivery does not alter expression of glucocorticoid and mineralocorticoid receptors in the pituitary gland.***

RNA was isolated from the pituitary gland and gene expression for glucocorticoid receptor (*Nr3c1*) and mineralocorticoid receptor (*Nr3c2)* relative to *Actb* expression was examined. Fold changes were calculated using the $^{\Delta\Delta}$Ct method. a) Glucocorticoid receptor expression. No differences were found between groups for *Nr3c1* expression (F (2,25)= 2.259, p= 0.1253). b) Mineralocorticoid receptor expression. No differences were found between groups for *Nr3c2* expression (F(2,25)= 0.8219, p= 0. 4511). One-way

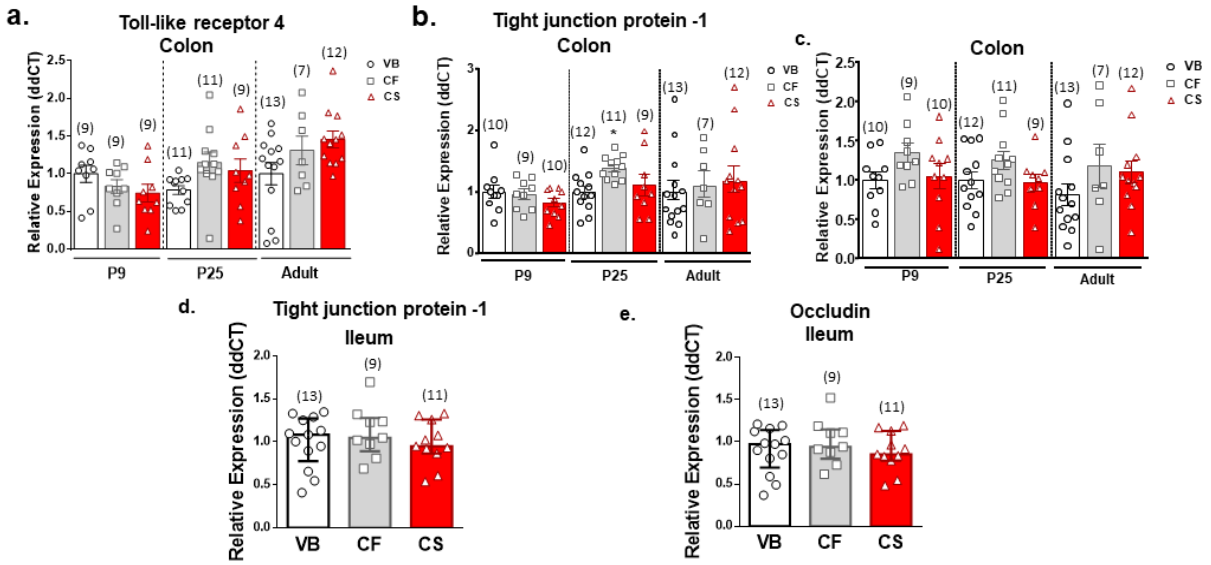
ANOVA followed by Tukey post hoc. Scatter dot-plots represent mean ± S.E.M. Behavioral results shown representative of 2 experimental replicates. Offspring in each cohort derived from three independent litters/ group. Animal numbers per group are specified above each bar. VB, vaginal birth; CF, cross fostered; CS, C-section




***Supplementary figure 2.2- Effects of the mode of delivery on nociception, depression-like behaviour and HPA axis response in early-life (P9) and adolescence (P23).***

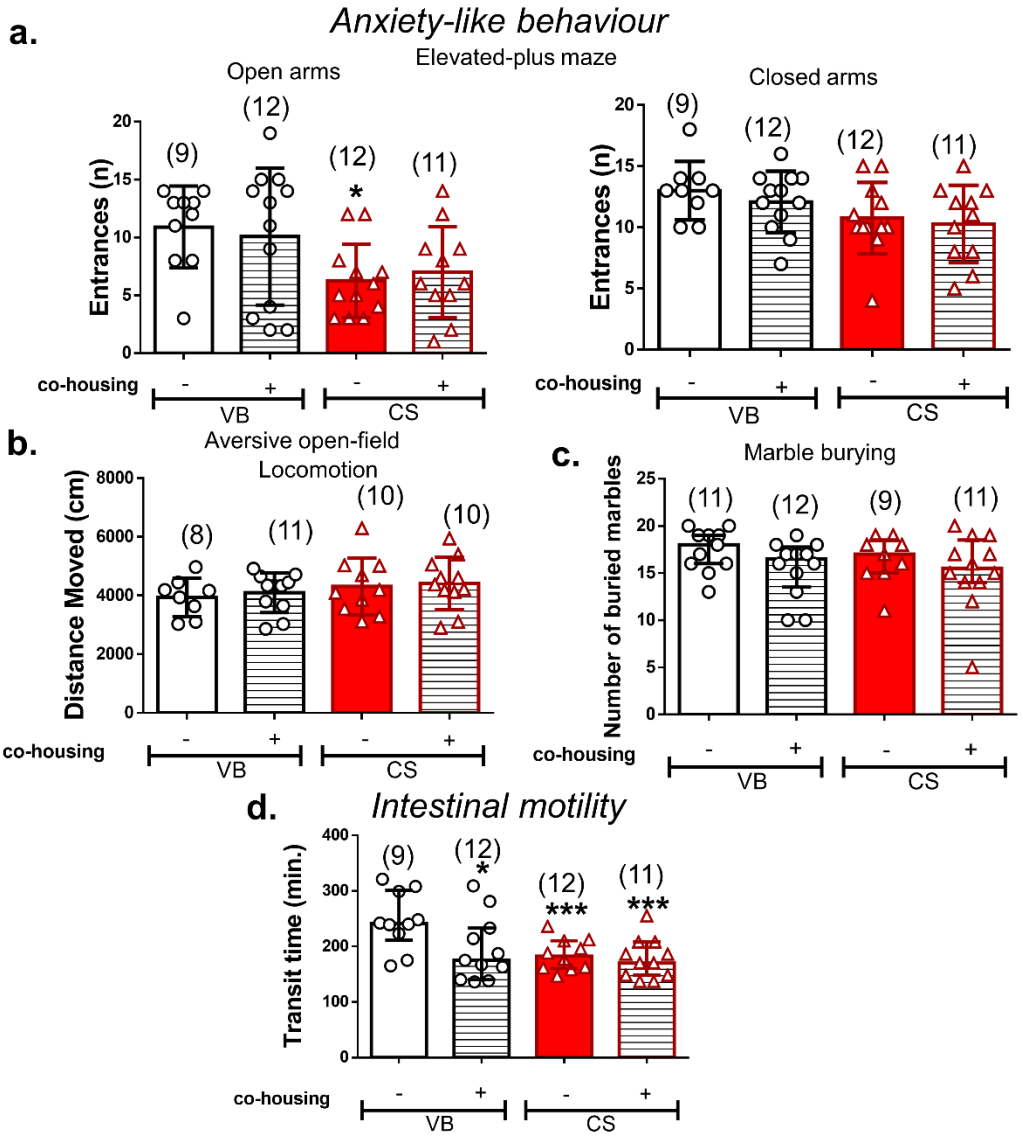
**a)** Pain sensitivity**.** No significant differences were found in the latency of nociception (sec.) ($F(2,27)= 3.084$, $p= 0.064$ [One-way ANOVA]). **b-c)** Depression-like behavior. **b)** Forced swim test in adolescence. No differences were found between the groups in the immobility time (sec.) in adolescence ($x^2(34)= 0.2654$, $p= 0.87$ [Kruskal Wallis]). Scatter dot-plots represent Median and interquartile range **c)** Forced swim test in adulthood. No differences were found between groups ($F (2,35)= 0.1002$, $p= 0.9049$) [One-way ANOVA]). **d-e)** Hypothalamic-pituitary-adrenal (HPA) axis response in early-life and adolescence. **d)** No differences were found between the groups in the baseline corticosterone in early-life (P9) ($F (2,23)= 0.1312$, $p= 0.87$). **e)** Corticosterone response to an acute stress in adolescence (P25). Time effect ($F (1,22)= 85.821$, $p<0.0001$); group effect ($F (2,22)= 2.054$, $p=0.152$) and time x group effect ($F (2,22)=3.017$, $p=0.069$) ([Repeated measures ANOVA]). T0 ($F (2, 25) = 1.695$, $p=0.204$) ([VB n= 10, CF n=10, CS n=8]);

T45 ($F(2, 23) = 2.885$, $p = 0.076$) ([VB n= 10, CF n=9, CS n=7]). Data are represented by mean + S.E.M. **a, c, d)** Scatter dot-plots represent mean ± S.E.M. **a-e)** Offspring in each cohort derived from three independent litters/group. Animal numbers per group are specified



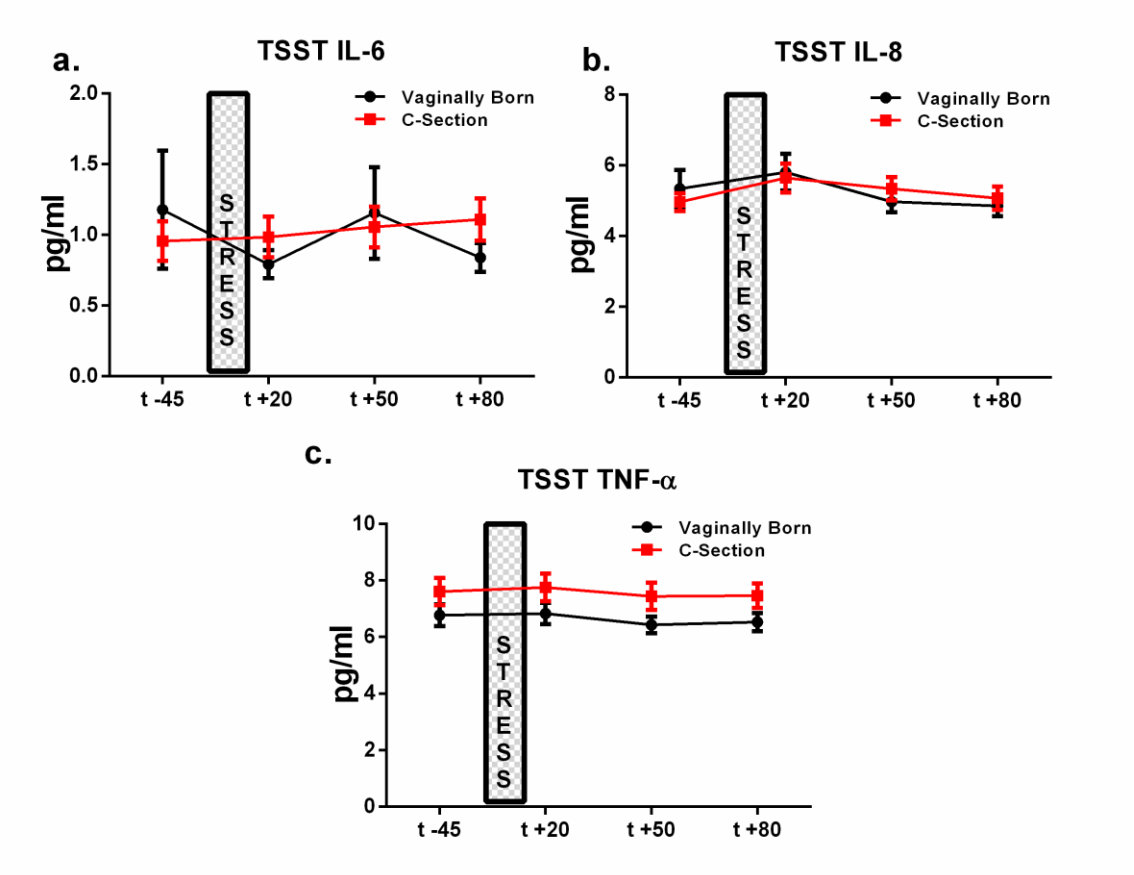

***Supplementary figure 2.3- Barrier function-related genes in the colon and ileum.***

RNA was extracted from the colon and ileum of mice. a) *Tlr4* expression in the colon of P9 ($F(2, 24)=1.485$, $p=0.247$), P25 ($F(2, 28)=2.560$, $p=0.095$), and adult mice ($F(2, 29)=2.975$, $p=0.067$). b) *Tjp1* expression in the colon of P9 ($F(2, 26)=1.166$, $p=0.327$), P25 ($F(2, 29)=3.808$, $p=0.034$), and adult mice ($F(2, 29)=0.775$, $p=0.470$). c) *Occludin* expression in colon of P9 ($F(2, 26)=1.935$, $p=0.165$), P25 ($F(2, 29)=1.926$, $p=0.164$) and adult mice ($F(2, 29)=0.1.286$, $p=0.292$). d) *Tjp1* expression in the Ileum of adult mice ($F(2, 30)=0.601$, $p=0.555$). e) *Occludin* expression in the ileum of adult mice ($F(2, 30)=0.601$, $p=0.555$). *Tlr4*, *Occludin* and *Tjp1* were quantified by qRT-PCR relative to *Actb*. Fold changes were calculated using the $^{\Delta\Delta}$Ct method. One-way ANOVA followed by Tukey post hoc. Scatter dot-plots represent mean ± S.E.M. Behavioral results shown representative of 2 experimental replicates. Offspring in each cohort derived from three independent litters/ group. Animal numbers per group are specified above each bar. VB, vaginal birth; CF, cross fostered; CS, C-section.



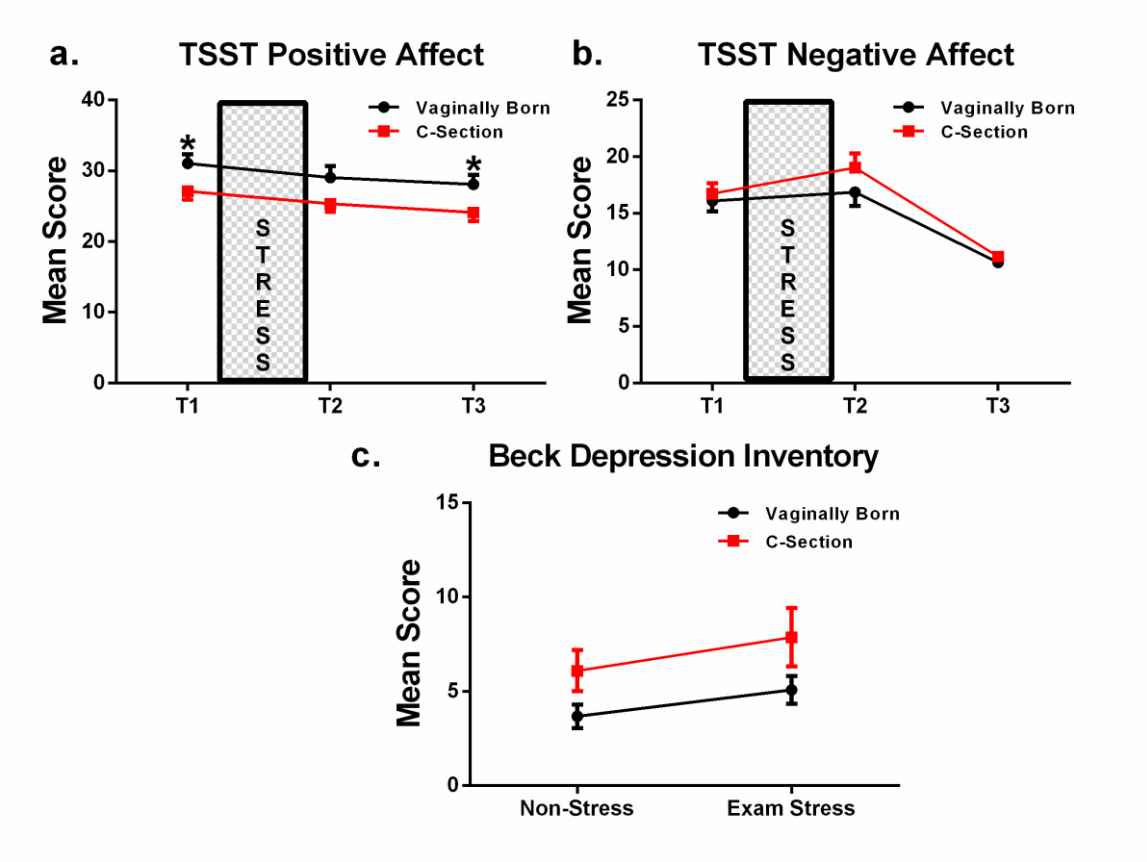

***Supplementary figure 2.4- Co-housing effects on anxiety-like behavior and on gastrointestinal motility.*** **a-b)** Anxiety-like behaviour: **a)**Time spent in the open arms of the elevated plus maze (EPM). Group effect ($F(1, 40)=0.139$, $p=0.711$); mode of delivery effect ($F(1, 40)=11.580$, $p=0.002$); group x mode of delivery ($F(1, 40)=0.933$, $p=0.340$).Time spent in the closed arms of the EPM. Group effect ($F(1, 40)=0.681$, $p=0.414$); mode of delivery effect ($F(1, 40)=5.783$, $p=0.021$); group x mode of delivery ($F(1, 40)=0.068$, $p=0.796$). ([Two-way ANOVA, followed by Tukey post hoc]). **b)** Aversive open-field. Total distance moved (cm). Group effect ($F(1, 36)=0.646$, $p=0.590$); mode of delivery effect ($F(1, 36)=1.758$, $p=0.193$); group x mode of delivery effect ($F(1, 36)=0.016$, $p=0.901$). **c)** Marble burying. Group effect ($F(1, 42)=0.181$, $p=0.672$); mode of delivery effect ($F(1, 42)=3.207$, $p=0.081$); group x mode of delivery effect

(F(1, 42)=2.611, p=0.114). Two-way ANOVA, followed by Tukey post hoc. Scatter dot-plots represent mean ± S.E.M. **d**). Intestinal motility time in ($x^2$(3)=9.931, p= 0.019 ([Kruskal-Wallis, followed by U-Mann Whitney test]). Scatter dot-plots represent median and interquartile range. **a-c)** Animal numbers per group are specified above each bar. VB group, 9 litters**;** CS group, 6 litters; VB co-housed group, 10 litters; and CS co-housed, 7 litters. Animal numbers per group are specified above each bar. Behavioral results shown representative of 2 experimental replicates. VB, vaginal birth; CS, C-section.



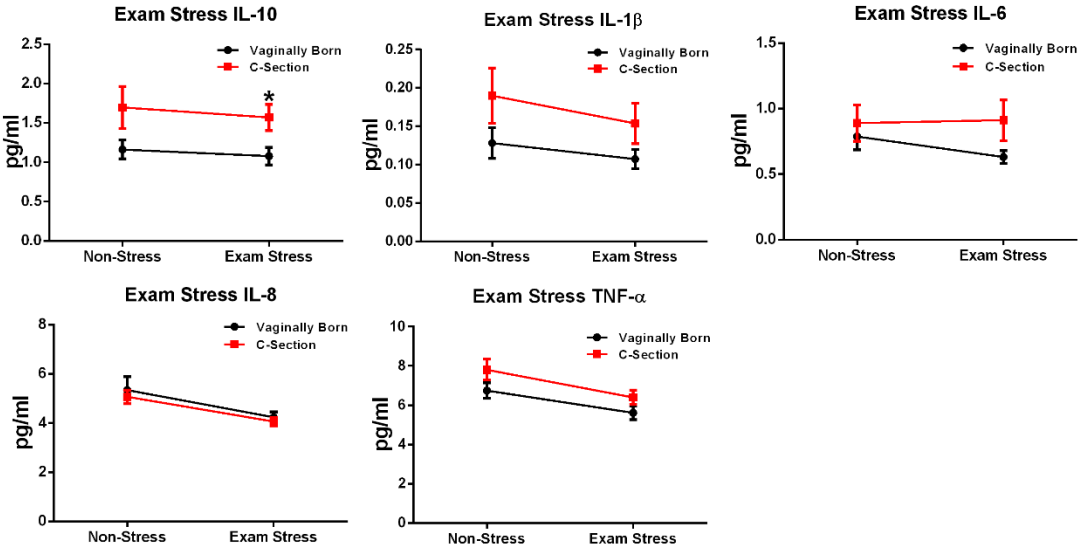

***Supplementary figure 2.5- The TSST had no differential effect on immune activity in C-section participants in response to the TSST procedure.***

**a)** IL-6; (Stress (F(1.491, 104.37)= 1.6, p=0.21, $\eta_p^2$= 0.022); Group (F(1, 70)= 0.71, p=0.4, $\eta_p^2$ = 0.01); Stress x Group (F(1.491, 104.37)= 1, p=0.35, $\eta_p^2$ = 0.014; n= 39 [VB], n= 33 [C-section]). **b)** IL-8; (Stress (F(3, 210)= 5.51, p=0.001, $\eta_p^2$= 0.073); Group (F(1, 70)= 0.278, p=0.6, $\eta_p^2$ = 0.004); Stress x Group (F(3, 210)= 0.552, p=0.65, $\eta_p^2$ = 0.008; n= 39 [VB], n= 33 [C-section]). **c)** TNF-α; (Stress (F(2.061, 144.283)= 7.02, p=0.001, $\eta_p^2$= 0.091); Group (F(1, 70)= 2.898, p=0.093, $\eta_p^2$ = 0.04); Stress x Group (F(2.061, 144.283)= 0.169, p=0.85, $\eta_p^2$ = 0.002; n= 39 [VB], n= 33 [C-section]) response to the TSST. (Post hoc comparisons using Bonferroni correction: *p> 0.05; **p< 0.01. Data are represented by mean + S.E.M.



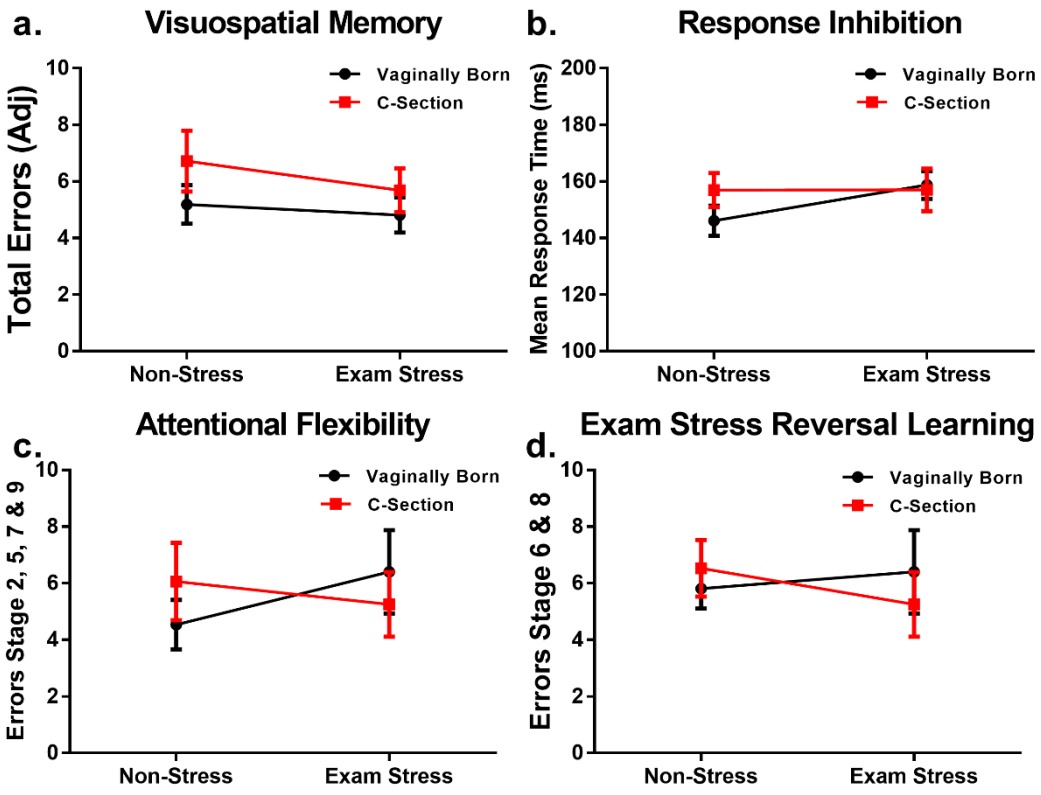

***Supplementary figure 2.6- Positive affect is lower in C-Section participants throughout the TSST procedure but there is no differential effect of stress between groups***

**a)** Positive Affect response as measured using the Positive and Negative Affect Schedule (Stress ($F(2, 144)= 6.74$, $p=0.02$, $\eta_p^2= 0.086$); Group ($F(1, 72)= 5.78$, $p=0.019$, $\eta_p^2 = 0.074$); Stress x Group ($F(2, 144)= 0.17$, $p=0.85$, $\eta_p^2 = 0.002$; n= 40 [VB], n= 34 [C-section]). **b)** Negative Affect response to the TSST procedure as measured using the Positive and Negative Affect Schedule; (Stress ($F(2, 144)= 101.12$, $p<0.001$, $\eta_p^2= 0.58$); Group ($F(1, 72)= 1.71$, $p=0.19$, $\eta_p^2 = 0.023$); Stress x Group ($F(2, 144)= 0.86$, $p=0.43$, $\eta_p^2 = 0.012$; n= 35 [VB], n= 31 [C-section]). **c)** Depression levels as measured using the Beck Depression Inventory-II during a Non-Stress and Exam Stress period (Stress ($F(1, 66)= 5.67$, $p=0.02$, $\eta_p^2= 0.079$); Group ($F(1, 66)= 2.53$, $p=0.12$, $\eta_p^2 = 0.037$); Stress x Group ($F(1, 66)= 0.776$, $p=0.38$, $\eta_p^2 = 0.012$; n= 38 [VB], n= 30 [C-section]). Data are presented as mean ± S.E.M.



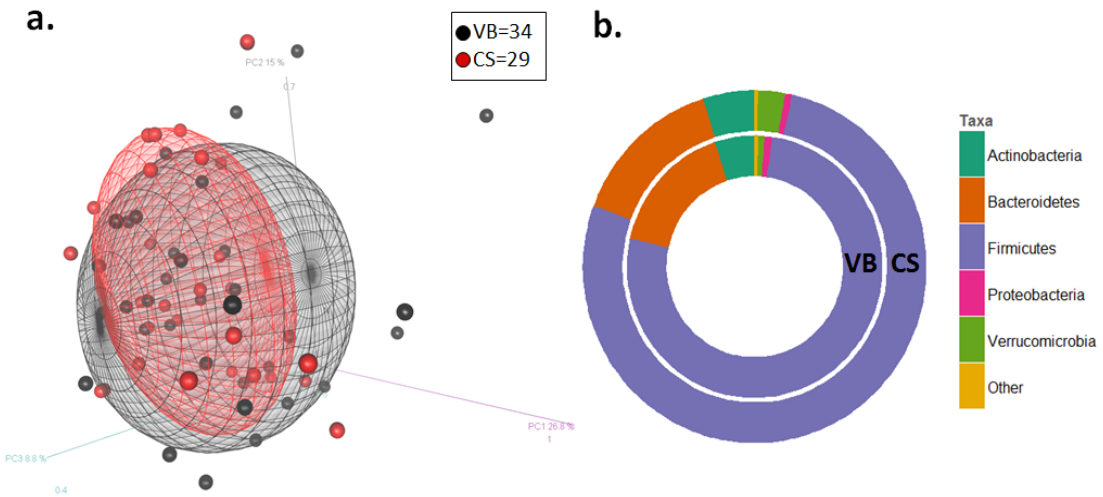

***Supplementary figure 2.7- Exam stress has no effect on HPA axis function as measured by the cortisol awakening response but causes elevated levels of the anti-inflammatory cytokine IL-10 in C-Section participants.***

Salivary cortisol awakening response (Stress ($F(1, 56)= 2.692$, $p=0.11$, $\eta_p^2= 0.046$); Group ($F(1, 56)= 0.146$, $p=0.704$, $\eta_p^2 = 0.003$); Stress x Group ($F(1, 56)= 3.711$, $p=0.059$, $\eta_p^2 = 0.062$; n= 33 [VB], n= 35 [C-section]). **b)** IL-10 (Stress ($F(1, 66)= 0.853$, $p=0.359$, $\eta_p^2= 0.013$); Group ($F(1, 66)= 6.044$, $p=0.017$, $\eta_p^2 = 0.084$); Stress x Group ($F(1, 66)= 0.22$, $p=0.884$, $\eta_p^2 < 0.001$; n= 38 [VB], n= 30 [C-section])**c)** IL-1β (Stress ($F(1, 61)= 7.878$, $p=0.007$, $\eta_p^2= 0.114$); Group ($F(1, 61)= 2.934$, $p=0.092$, $\eta_p^2 = 0.046$); Stress x Group ($F(1, 61)= 0.57$, $p=0.453$, $\eta_p^2 = 0.009$; n= 37 [VB], n= 28 [C-section]). **d)** IL-6 (Stress ($F(1, 64)= 0.434$, $p=0.512$, $\eta_p^2= 0.007$); Group ($F(1, 64)= 2.304$, $p=0.134$, $\eta_p^2 = 0.035$); Stress x Group ($F(1, 64)= 0.825$, $p=0.367$, $\eta_p^2 = 0.013$; n= 37 [VB], n= 29 [C-section]). **e)** IL-8 (Stress ($F(1, 65)= 11.557$, $p=0.001$, $\eta_p^2= 0.151$); Group ($F(1, 65)= 0.027$, $p=0.87$, $\eta_p^2 < 0.001$); Stress x Group ($F(1, 65)= 0.25$, $p=0.619$, $\eta_p^2 = 0.004$; n= 39 [VB], n= 29 [C-section]) levels during the Non-Stress and Exam Stress period. Data are presented as mean ± S.E.M..



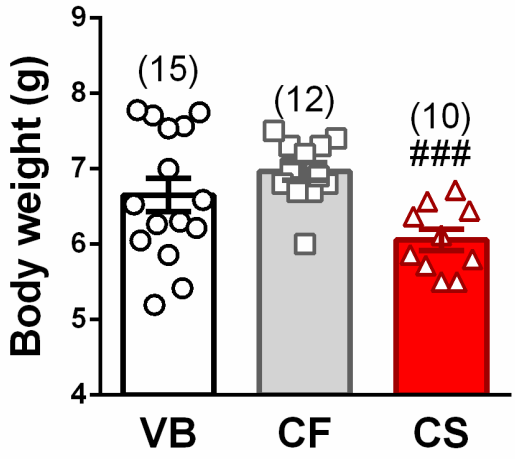

***Supplementary figure 2.8- Cognitive function did not differ in response to exam stress in C-section participants.***

**a)** Visuospatial memory as measured by the Paired Associates Learning (PAL) test, Total Errors Adjusted (Adj) (Stress ($F(1, 67)= 0.02$, $p=0.89$, $\eta_p^2< 0.001$); Group ($F(1, 67)= 0.462$, $p=0.499$, $\eta_p^2 < 0.007$); Stress x Group ($F(1, 67)= 0.076$, $p=0.783$, $\eta_p^2 = 0.001$; n= 37 [VB], n= 32 [C-section]). **b)** Response inhibition as measured by the Stop Signal Task (SST), stop signal reaction time (SSRT; calculated for last 20 sub-blocks) (Stress ($F(1, 67)= 1.44$, $p=0.24$, $\eta_p^2= 0.021$); Group ($F(1, 67)= 0.494$, $p=0.484$, $\eta_p^2 = 0.007$); Stress x Group ($F(1, 67)= 1.37$, $p=0.25$, $\eta_p^2 = 0.02$; n= 37 [VB], n= 32 [C-section]). **c)** Attentional Flexibility as measured by the Intra-Extra Dimensional Set Shift (IED), total errors made on stage 6 and 8 (Stress ($F(1, 67)= 0.28$, $p=0.598$, $\eta_p^2= 0.004$); Group ($F(1, 67)= 0.016$, $p=0.9$, $\eta_p^2 = 0.001$); Stress x Group ($F(1, 67)= 1.82$, $p=0.182$, $\eta_p^2 = 0.026$; n= 37 [VB], n= 32 [C-section]). **d)** Reversal Learning as measured by the IED, total errors on stage 2, 5, 7 and 9 (Stress ($F(1, 67)= 0.795$, $p=0.376$, $\eta_p^2= 0.012$); Group ($F(1, 67)= 0.009$, $p=0.93$, $\eta_p^2 = 0.001$); Stress x Group ($F(1, 67)= 1.81$, $p=0.183$, $\eta_p^2 = 0.026$; n= 37 [VB], n= 32 [C-section]). Data are presented as mean ± S.E.M..



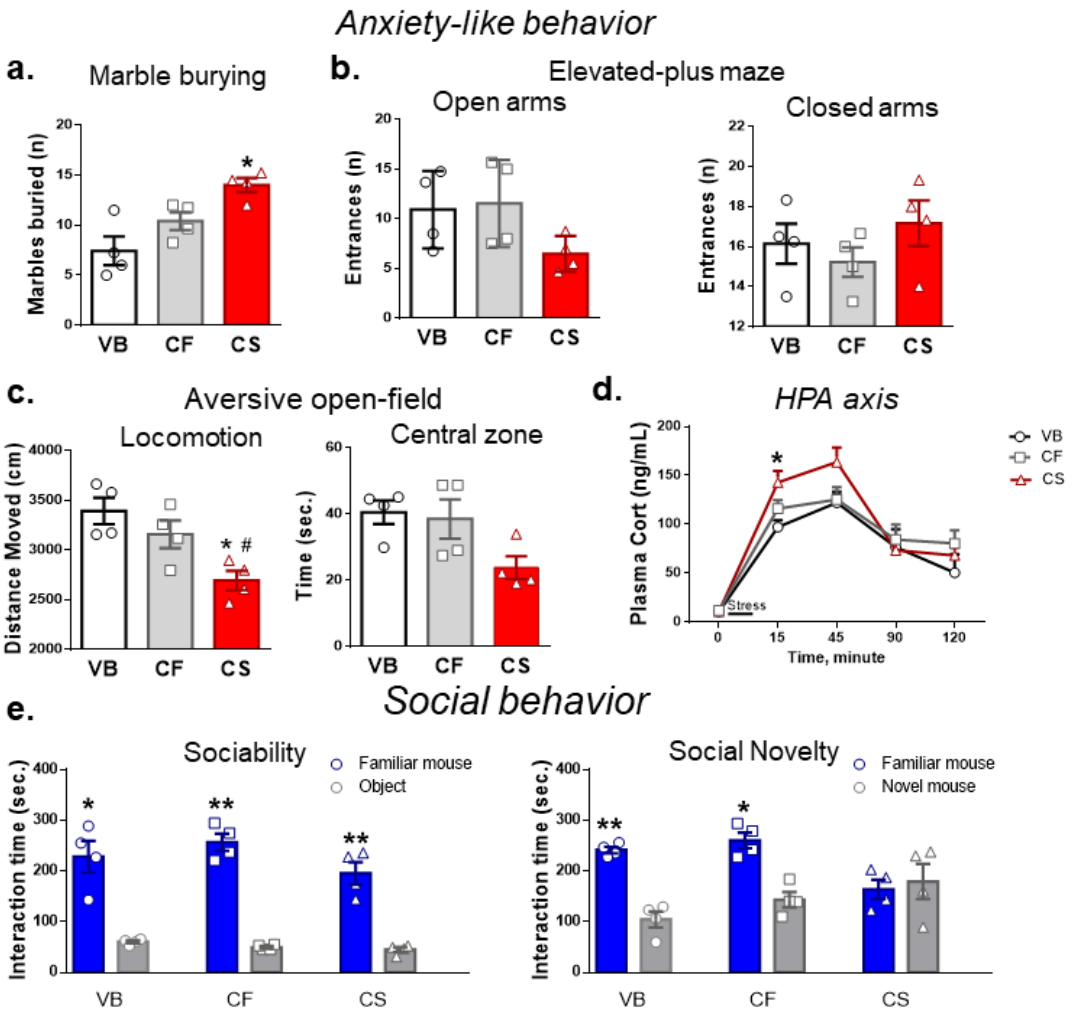

***Supplementary figure 2.9- The effect of mode of delivery on the microbial composition of adults.***

DNA was extracted from feces in a group of adults. **a)** Principal coordinate analysis (PCoA) plot, based on Bray-Curtis distance (p=0.8). **b)** Relative abundance of phylum in VB and CS born adults. The dominant phyla are labelled in the legend on the right. This plot was created using the ggplot2 package in R. Taxa below 0.5% sample abundance and the unclassified taxa were grouped into the "Other" category. ([An analysis of variance test was carried on Bray-Curtis beta diversity to calculate statistical significance using the adonis function in the vegan package in R.)]. VB, Vaginal birth; CS, C-section.



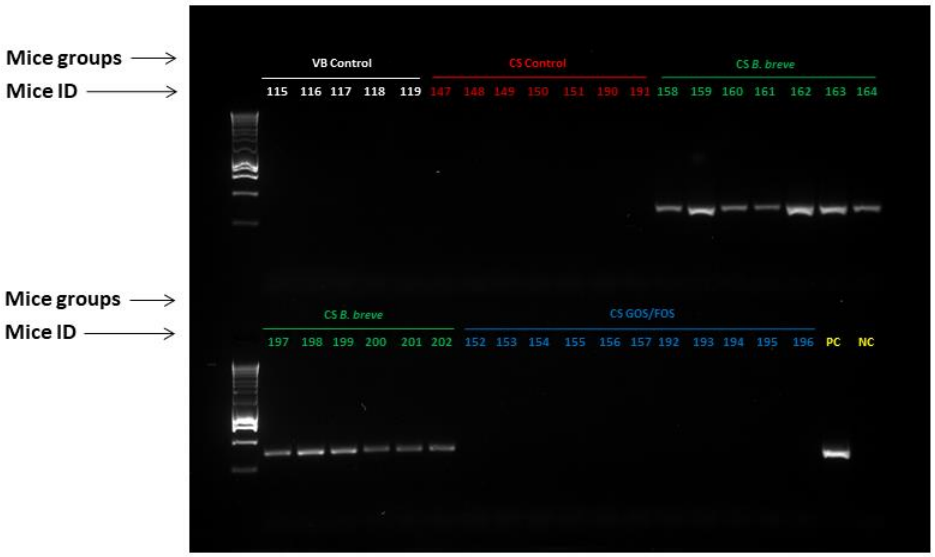

***Supplementary figure 2.10- Effects of the mode of delivery on body weight at P9.***

**a)** At P9, CS pups have a lower body weight in comparison to CF pups ($F(2,34)= 5.487$, $p= 0.009$ [One-way ANOVA followed by Tukey post hoc]). Scatter dot-plots represent mean ± S.E.M. Offspring in each cohort derived from three independent litters/group. Animal numbers per group are specified above each bar. *p< 0.05 CS *vs*. VB; ##p<0.01 CS *vs.* CF. VB, vaginal birth; CF, cross fostered; CS, C-section.




***Supplementary figure 2.11- Main neurobehavioral changes in C-section offspring across lifespan analysed by litter.***

**a)** Marble burying (F (2,9)= 9.778, p=0.006); **b)** number of entrances in the open arm of the elevated plus-maze (F (2,9)= 2.427, p=0.144 ) **;** number of entrances in the closed arms (F (2,9)= 3.758, p=0.407 ); **c)** time spent in the central zone (F(2,9) = 4.171, p= 0.052) of the aversive open field and total distance travelled (F(2,9)= 8.221, p= 0.009) **d**) Corticosterone (CORT) release in response to an acute stress. Time effect (F (4,36) = 61.905, p<0.001); group effect (F (2,9)= 2.547, p= 0.133); time x group (F(8,36)= 1.695, p=0.133 ([Repeated measures ANOVA]). **e**) Three-chamber test. C-section does not impact sociability. VB t (3) = 5.382, p=0.013, CF t (3) =14.061, p=0.001, CS t (3) =5.958, p<0.009. Reduced social novelty preference in CS. VB t (3) = 9.945, p=0.002, CF t (3) = 4.655, p=0.019, CS t (3) =-0.351, p= 0.749 ([Paired-Student T-test]). N=4 litters per group. *p< 0.05 and ***p<0.0001 CS *vs*. VB; #p<0.05 and ###p<0.0001 CS *vs.* CF. VB, vaginal birth; CF, cross fostered; CS, C-section. Data are presented as mean ± S.E.M..




***Supplementary figure 2.12- Qualitative determination of Bifidobacterium breve in faecal pellets.***
The occurrence of *Bifidobacterium breve* species was determined by qualitative PCR in VB, CS, CS B.breve and CS prebiotic groups. *Bifidobacterium breve* NCBIM2257 was used as positive control. Amplified products were subjected to gel electrophoresis in 1.5% agarose gels and visualized by ethidium bromide staining. VB, vaginal birth; CS, C-section.

## 2.7 Supplementary Tables

| | Dependent measure | | Cytokines | Mean ± S.E.M (n) | | | One-way ANOVA | | | Tukey post-hoc |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | VB | CF | CS | P-value | F-value | DF | |
| ConA | Conc. (pg/mL) | Thymus | IL-1b | 0.35±0.1(7) | 0.56±0.07 (12) | 0.56±0.05(11) | 0.111 | 2.389 | 2,29 | *ns* |
| | | | IL-2 | 204.5±16.5(7) | 204.3±22.1(11) | 224.4±27.1(12) | 0.789 | 0.239 | 2,29 | *ns* |
| | | | IL-4 | 292.8±99.8(7) | 407.2±68.2 (11) | 605.1±93.8(12) | 0.062 | 3.09 | 2,29 | *ns* |
| | | | IL-5 | 2.17±0.35(6) | 4.16±0.72 (12) | 4.72±1.19(11) | 0.243 | 1.494 | 2,28 | *ns* |
| | | | KCGRO | 9.59±1.2 (7) | 9.97±1.3 (11) | 11.10±0.6 (11) | 0.593 | 0.533 | 2,28 | *ns* |
| | | | IL-12p70 | 43.5±4.8(7) | 42.5±2.8 (12) | 45.7±3.2(12) | 0.861 | 0.15 | 2,30 | *ns* |
| | | Spleen | IL-1b | 9.0±0.9 (8) | 5.80±0.55 (13) | 8.4±1.6(12) | 0.095 | 2.557 | 2,30 | *ns* |
| | | | IL-2 | 7248.2±365.1(9) | 5894.6±453(13) | 7277.9±398(11) | **0.037** | 3.672 | 2,31 | ns |
| | | | IL-4 | 130.2±22.1(8) | 186.3±21(12) | 193.7±15.1(11) | 0.081 | 2.735 | 2,29 | *ns* |
| | | | IL-5 | 22.1±3.2(8) | 20.44±4.11(11) | 27.2±4.9(13) | 0.503 | 0.704 | 2,30 | *ns* |
| | | | KCGRO | 10.6±0.8(9) | 10.43±0.70(13) | 11.7±0.8(11) | 0.437 | 0.851 | 2,31 | *ns* |
| | | | IL-12p70 | 169.6±4.1(8) | 133.7±8.2(13) | 146.0±7.4(12) | **0.015** | 4.833 | 2,30 | VB *vs* CF* |

The table specifies the dependent measures and summarizes the main effects. The table compares the group effect of cytokines concentration (pg/mL) in thymocytes and splenocytes stimulated with Con-Afor VB, CF and CS. The table compares the group effect for IL-1β, IL-2, IL-4, IL-5, KCGRO, IL-12p70. Data was analyzed by one-way ANOVA followed by Tukey post hoc. The table also specifies the corresponding n number, degrees of freedom (DF) and F-values. Vaginal birth=VB; Cross-fostered=CF; C-section=CS Significant effects ($P < 0.05$) are given in bold font.

| | Dependent measure | | Cytokines | Mean ± S.E.M (n) | | | One-way ANOVA | | | Tukey post-hoc |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | VB | CF | CS | P-value | F-value | DF | |
| LPS | Conc. (pg/mL) | **Thymus** | IFNy | 0.3±0.1(7) | 0.6±0.2 (13) | 0.28±0.1 (11) | 0.071 | 2.912 | 2,30 | *ns* |
| | | | IL-1b | 0.6±0.1(8) | 0.7±0.1 (13) | 0.59±0.0 (11) | 0.555 | 0.601 | 2,31 | *ns* |
| | | | IL-2 | 0.6±0.1(8) | 0.79±0.1(13) | 0.74±0.2(11) | 0.526 | 0.658 | 2,31 | *ns* |
| | | | IL-4 | 19.4±11.3 (8) | 16.6±5.71(12) | 3.56±0.7 (11) | 0.189 | 1.767 | 2,30 | *ns* |
| | | | IL-5 | 0.13±0.1(8) | 0.2±0.04(12) | 0.09±0.0(11) | 0.379 | 1.005 | 2,30 | *ns* |
| | | | IL-6 | 81.5±15.8(8) | 90.9±12.05(12) | 75.43±7.7(11) | 0.617 | 0.492 | 2,30 | *ns* |
| | | | KCGRO | 38.6±4.7(8) | 45.2±6.02(13) | 40.87±4.3(12) | 0.685 | 0.383 | 2,32 | *ns* |
| | | | IL-10 | 4.7±0.8(7) | 6.9±1.32(13) | 4.78±0.4(11) | 0.227 | 1.562 | 2,30 | ns |
| | | | IL-12p70 | 13.6±1.3(8) | 17.5±1.83(13) | 8.54±1.2(11) | **0.001** | 8.831 | 2,31 | CF vs CS* |
| | | | TNFα | 40.3±5.27(8) | 43.50±4.56(13) | 33.51±3.2(11) | 0.244 | 1.482 | 2,31 | *ns* |

The table specifies the dependent measures and summarizes the main effects. The table compares the group effect of cytokines concentration (pg/mL) in thymocytes stimulated with LPS for VB, CF and CS. The table compares the group effect for IFNγ, IL1β, IL-2, IL-4, IL-5, IL-6, IL-10, KCGRO, IL-12p70, TNFα. Data was analyzed by one-way ANOVA followed by Tukey post hoc. The post hoc analyzes revealed significant difference in the levels of IL-12p70 between between CF and CS ($p<0.05$). Vaginal birth=VB; Cross-fostered=CF; C-section=CS. The table also specifies the corresponding n number, degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font.

| | | Dependent measure | | Cytokines | Mean ± S.E.M (n) | | | One-way ANOVA | | | Tukey post-hoc |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | VB | CF | CS | P-value | F-value | DF | |
| LPS 2µg/mL | | Conc. (pg/mL) | Spleen | IFNγ | 750.0 ±186.8 (10) | 480.3±114.2(12) | 864.5±327.0(10) | 0.43 | 0.868 | (2,29 ) | *ns* |
| | | | | IL1β | 35.3±6.3(9) | 29.2±4.1(12) | 47.9±24.2(10) | 0.628 | 0.473 | (2,28) | *ns* |
| | | | | IL-2 | 20.5±1.4(9) | 15.6±1.3(12) | 20.92±1.8(11) | **0.032** | 3.874 | (2,29) | *ns* |
| | | | | IL-4 | 1.34±0.1(9) | 1.1±0.1(11) | 1.47±0.14(10) | 0.078 | 2.805 | (2,27) | *ns* |
| | | | | IL-5 | 1.03±0.2(9) | 0.8±0.1(12) | 1.07±0.21(10) | 0.358 | 1.066 | (2,28) | *ns* |
| | | | | IL-6 | 2276.6±305.0(10) | 2183.3±308.1(12) | 3092.7±855.5(10) | 0.429 | 0.872 | (2,29) | *ns* |
| | | | | KCGRO | 114±11.4(10) | 113.1 ±9.0(12) | 125.9±19.4(10) | 0.776 | 0.256 | (2,29) | *ns* |
| | | | | IL-10 | 393±54.3(10) | 341.7±43.6(12) | 397.14±76.5(11) | 0.755 | 0.284 | (2,30) | ns |
| | | | | IL-12p70 | 77.4±8.9(10) | 55.9±6.5(11) | 85.2±17.8(10) | 0.202 | 1.695 | (2,28) | ns |
| | | | | TNFα | 830.4±49.3(9) | 566.9±57.3(11) | 866.1±245.9(10) | 0.298 | 1.267 | (2,27) | *ns* |

***Supplementary Table 2. 1- Summary of the statistical tests and outcomes for the cytokines analyzes in splenocytes stimulated with Lipopolysaccharide from CS, CF and VB offspring.***

The table specifies the dependent measures and summarizes the main effects. The table compares the group effect of cytokines concentration (pg/mL) in splenocytes stimulated with Lipopolysaccharide (LPS) for VB, CF and CS. The table compares the group effect for IFNγ, IL1β, IL-2, IL-4, IL-5, IL-6, IL-10, KCGRO, IL-12p70, TNFα. Data was analyzed by One-way ANOVA followed by Tukey post hoc. Vaginal birth=VB; Cross-fostered=CF; C-section=CS. The table also specifies the corresponding n number, degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font.

| | Taxa | Taxonomic level | Median [1stquartile;3rdquartile] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| **Early-life** | *Acidobacteria* | p | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.011** | **0.021** | N/A | 0.098 |
| | *Proteobacteria* | p | 0.04138 [0.00596;0.45960] | 0.51542 [0.25449;0.77717] | 0.05550 [0.04317;0.38229] | **0.024** | **0.048** | **0.048** | 0.491 |
| | *Deferribacteres* | p | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.032** | 0.066 | 0.358 | 0.066 |
| | *Firmicutes* | p | 0.87032 [0.48448;0.96583] | 0.42689 [0.12893;0.60411] | 0.70625 [0.58908;0.74191] | **0.035** | 0.125 | 0.071 | 0.427 |
| | *Cyanobacteria* | p | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00005] | 0.00002 [0.00001;0.00408] | **0.042** | 0.194 | 0.09 | 0.053 |
| | Uncultured *Cyanobacteria* | f | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 1.00E-05 [0e+00;1e-05] | **0.002** | **0.01** | 0.358 | **0.018** |
| | *Veillonellaceae* | f | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00003] | 0.01758 [0.00001;0.02425] | **0.003** | **0.009** | 0.158 | **0.009** |
| | *Pasteurellaceae* | f | 0.00320 [0.00037;0.00479] | 0.27615 [0.03471;0.51854] | 0.00141 [0.00088;0.15332] | **0.005** | 0.089 | **0.008** | 0.315 |
| | *Orbaceae* | f | 0.00010 [0.00000;0.00017] | 0.00002 [<0.00001;0.00007] | 0.00000 [0.00000;0.00000] | **0.009** | **0.009** | 0.22 | **0.034** |
| | *Acidobacteria* Unknown Family | f | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.011** | **0.021** | N/A | 0.098 |
| | *Deferribacteraceae* | f | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.032** | 0.066 | 0.358 | 0.066 |
| | *Lactobacillaceae* | f | 0.60149 [0.42740;0.95999] | 0.32948 [0.08108;0.52279] | 0.02594 [0.01455;0.21948] | **0.032** | 0.09 | 0.09 | 0.069 |
| | *Alcaligenaceae* | f | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | **0.037** | N/A. | **0.049** | 0.198 |
| | *Phyllobacteriaceae* | f | 1.00E-05 [0e+00;3e-05] | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | **0.043** | 0.932 | 0.061 | 0.151 |
| | Uncultured *Cyanobacteria* | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 1.00E-05 [0e+00;1e-05] | **0.002** | **0.01** | 0.358 | **0.018** |
| | *Veillonella* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00002] | 0.01758 [0.00001;0.02425] | **0.003** | **0.009** | 0.175 | **0.009** |
| | *Actinobacillus* | g | 0.00320 [0.00037;0.00479] | 0.27615 [0.03471;0.51854] | 0.00141 [0.00088;0.15332] | **0.005** | 0.089 | **0.008** | 0.315 |
| | *Odoribacter* | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 2.00E-05 [0e+00;2e-05] | **0.008** | **0.013** | 1 | **0.025** |
| | *Gilliamella* | g | 0.00010 [0.00000;0.00017] | 0.00002 [<0.00001;0.00007] | 0.00000 [0.00000;0.00000] | **0.009** | **0.009** | 0.22 | **0.034** |

| | Taxa | Taxonomic level | Median [1stquartile;3rdquartile] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| | *Blastocatella* | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.011** | **0.021** | N/A | 0.098 |
| | *Eubacterium coprostanoligenes* group | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;2e-05] | **0.022** | 0.078 | 0.179 | **0.037** |
| | *Mucispirillum* | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.032** | 0.066 | 0.358 | 0.066 |
| | *Lactobacillus* | g | 0.60149 [0.42740;0.95999] | 0.32948 [0.08108;0.52279] | 0.02594 [0.01455;0.21948] | **0.032** | 0.09 | 0.09 | 0.069 |
| | Uncultured *Prevotellaceae* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | **0.037** | N/A | 0.049 | 0.198 |
| | *Sutterella* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | **0.037** | N/A | 0.049 | 0.198 |
| | *Herminiimonas* | g | 0.00E+00 [0e+00;1e-05] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;0e+00] | **0.041** | 0.311 | 0.077 | 0.077 |
| | *Alloprevotella* | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;4e-05] | **0.043** | **0.041** | 0.54 | 0.28 |
| | *Ruminiclostridium* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00001] | 0.00001 [<0.00001;0.00091] | **0.046** | 0.125 | 0.212 | 0.053 |
| | *Ruminiclostridium* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;<0.00001] | **0.049** | **0.032** | 0.037 | 1 |
| **Adolesc.** | *Tenericutes* | p | 0.00019 [0.00004;0.00050] | 0.02848 [0.01911;0.03942] | 0.00461 [0.00028;0.01049] | **0.004** | **0.023** | **0.007** | 0.213 |
| | *Saccharibacteria* | p | 0.00023 [0.00013;0.00116] | 0.00262 [0.00123;0.00348] | 0.00544 [0.00211;0.01004] | **0.036** | **0.203** | 0.146 | 0.063 |
| | *Anaeroplasmataceae* | f | 0.00015 [0.00004;0.00050] | 0.02848 [0.01909;0.03940] | 0.00432 [0.00022;0.01043] | **0.004** | **0.023** | **0.007** | 0.213 |
| | *Saccharibacteria* Unknown Family | f | 0.00023 [0.00013;0.00116] | 0.00262 [0.00123;0.00348] | 0.00544 [0.00211;0.01004] | **0.036** | 0.203 | 0.146 | 0.063 |
| | *Rikenellaceae* | f | 0.11658 [0.09044;0.12660] | 0.11056 [0.10432;0.14343] | 0.15878 [0.14587;0.17826] | **0.04** | 0.124 | 0.77 | **0.039** |
| | *Clostridiales* vadinBB60 group | f | 0.01059 [0.00396;0.01436] | 0.02721 [0.01154;0.03404] | 0.01497 [0.01284;0.01793] | **0.05** | 0.487 | 0.093 | 0.093 |
| | *Anaeroplasma* | g | 0.00015 [0.00004;0.00050] | 0.02848 [0.01909;0.03940] | 0.00432 [0.00022;0.01043] | **0.004** | **0.023** | **0.007** | 0.213 |
| | *Eubacterium* brachy group | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 1.00E-05 [1e-05;1e-05] | **0.007** | 0.076 | 0.34 | **0.01** |
| | *Anaerosporobacter* | g | 3.00E-05 [1e-05;5e-05] | 1.00E-05 [0e+00;2e-05] | 0e+00 [0e+00;0e+00] | **0.011** | 0.094 | 0.199 | **0.011** |

| | Taxa | Taxonomic level | Median [1stquartile;3rdquartile] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| | *Lachnospiraceae* UCG 008 | g | 0.00084 [0.00054;0.00211] | 0.00551 [0.00414;0.00672] | 0.00164 [0.00082;0.00292] | **0.013** | 0.056 | **0.014** | 0.424 |
| | *Clostridiales* vadinBB60 group | g | 0.00018 [0.00004;0.00024] | 0.00309 [0.00099;0.00329] | 0.00050 [0.00036;0.00072] | **0.015** | **0.042** | **0.042** | 0.076 |
| | *Lachnospiraceae* UCG 005 | g | 0.00038 [0.00004;0.00158] | 0.00004 [0.00001;0.00014] | 0.00000 [0.00000;0.00001] | **0.015** | 0.107 | 0.107 | **0.024** |
| | *Lachnospiraceae* uncultured | g | 0.04657 [0.04082;0.05149] | 0.05906 [0.05118;0.06618] | 0.03353 [0.02971;0.03908] | **0.02** | 0.061 | 0.061 | 0.11 |
| | *Erysipelatoclostridium* | g | 2.00E-05 [1e-05;3e-05] | 0.00E+00 [0e+00;1e-05] | 0e+00 [0e+00;0e+00] | **0.022** | 0.563 | **0.044** | **0.044** |
| | *Ruminococcaceae* UCG 009 | g | 0.00039 [0.00019;0.00087] | 0.00013 [0.00000;0.00020] | 0.00014 [0.00009;0.00027] | **0.023** | 0.486 | **0.047** | **0.047** |
| | *Acetatifactor* | g | 3.00E-05 [2e-05;5e-05] | 1.00E-05 [1e-05;4e-05] | 0.00E+00 [0e+00;1e-05] | **0.024** | 0.172 | 0.172 | **0.028** |
| | *Candidatus Saccharimonas* | g | 0.00023 [0.00013;0.00116] | 0.00262 [0.00123;0.00348] | 0.00544 [0.00211;0.01004] | **0.036** | 0.203 | 0.146 | 0.063 |
| | *Lachnospiraceae* FCS020 group | g | 0.00013 [0.00011;0.00019] | 0.00012 [0.00006;0.00018] | 0.00005 [0.00004;0.00006] | **0.038** | 0.124 | 0.626 | **0.039** |
| | *Eubacterium coprostanoligenes* group | g | 0.00169 [0.00052;0.00448] | 0.00008 [0.00002;0.00071] | 0.00095 [0.00036;0.00122] | **0.04** | 0.158 | **0.044** | 0.328 |
| | *Parabacteroides* | g | 0.00021 [0.00001;0.00037] | 0.00004 [0.00002;0.00012] | 0.00000 [0.00000;0.00001] | **0.042** | 0.067 | 0.236 | 0.067 |
| **Adult** | *Verrucomicrobia* | p | 0.00174 [0.00048;0.01566] | 0.03489 [0.01124;0.06378] | 0.01377 [0.00070;0.02204] | **0.043** | 0.249 | **0.035** | 0.328 |
| | *Acidaminococcaceae* | f | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;<0.00001] | **0.032** | 0.071 | . | 0.071 |
| | *Verrucomicrobiaceae* | f | 0.00174 [0.00048;0.01566] | 0.03489 [0.01124;0.06378] | 0.01377 [0.00070;0.02204] | **0.043** | 0.249 | **0.035** | 0.328 |
| | *Christensenellaceae* | f | 2.00E-05 [1e-05;4e-05] | 7.00E-05 [4e-05;1e-04] | 5.00E-05 [1e-05;9e-05] | **0.049** | 0.386 | **0.038** | 0.269 |
| | *Mollicutes* uncultured | f | 0.00005 [0.00003;0.00011] | 0.00013 [0.00010;0.00026] | 0.00012 [0.00008;0.00027] | **0.049** | 0.908 | 0.096 | 0.062 |
| | *Bacteroidales* S24.7 group | f | 2.00E-05 [1e-05;4e-05] | 4.00E-05 [3e-05;6e-05] | 4.00E-05 [3e-05;6e-05] | **0.011** | 0.862 | **0.022** | **0.022** |
| | *Desulfovibrio* | g | 0.00397 [0.00277;0.00605] | 0.00244 [0.00104;0.00466] | 0.00198 [0.00141;0.00219] | **0.017** | 0.817 | 0.15 | **0.006** |
| | *Candidatus Arthromitus* | g | 5.00E-05 [2e-05;5e-05] | 1.00E-05 [0e+00;2e-05] | 0.00E+00 [0e+00;2e-05] | **0.028** | 0.781 | **0.044** | 0.061 |

| | Taxa | Taxonomic level | Median [1stquartile;3rdquartile] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| | *Phascolarctobacterium* | g | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;0.00000] | 0.00000 [0.00000;<0.00001] | **0.032** | 0.071 | . | 0.071 |
| | *Ruminococcaceae* NK4A214 group | g | 0.00105 [0.00081;0.00141] | 0.00070 [0.00057;0.00092] | 0.00128 [0.00078;0.00142] | **0.038** | **0.046** | 0.067 | 0.918 |
| | *Akkermansia* | g | 0.00174 [0.00048;0.01566] | 0.03489 [0.01124;0.06378] | 0.01377 [0.00070;0.02204] | **0.043** | 0.249 | **0.035** | 0.328 |
| | *Senegalimassilia* | g | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | 1.00E-05 [0e+00;2e-05] | **0.044** | 0.226 | 0.226 | **0.045** |
| | *Eubacterium* ruminantium group | g | 0.00020 [0.00001;0.00206] | 0.00000 [0.00000;0.00001] | 0.00000 [0.00000;0.00003] | **0.045** | 0.842 | 0.08 | 0.08 |
| | *Lachnospiraceae* uncultured bacterium | g | 0.00E+00 [0e+00;2e-05] | 2.00E-05 [0e+00;5e-05] | 0e+00 [0e+00;0e+00] | **0.045** | 0.06 | 0.124 | 0.553 |
| | *Porphyromonadaceae* uncultured | g | 0e+00 [0e+00;0e+00] | 0e+00 [0e+00;0e+00] | 0.00E+00 [0e+00;1e-05] | **0.047** | 0.099 | 0.355 | 0.175 |
| | *Erysipelotrichaceae* uncultured bacterium | g | 0e+00 [0e+00;0e+00] | 1.00E-05 [0e+00;3e-05] | 0.00E+00 [0e+00;2e-05] | **0.049** | 0.274 | **0.045** | 0.264 |
| | *Mollicutes* uncultured | g | 0.00005 [0.00003;0.00011] | 0.00013 [0.00010;0.00026] | 0.00012 [0.00008;0.00027] | **0.049** | 0.908 | 0.096 | 0.062 |

***Supplementary Table 2. 2- Summary of the statistical differences and outcomes for taxonomy for early-life, adolescence and adulthood for VB, CF and CS.***

Significant differentially abundance taxa between CS, CF, VB groups in ealy-life, adolescence and adulthood at phylum(p), family (f) and genus (g) level, indicated by the p-value. Vaginal birth =VB; Cross-fostered=CF; C-section=CS. The p-values are correct for multiple testing at a defined false discovery. Significant effects ($p<0.05$) are given in bold.

| Age | Diversity metric | Taxonomic level | Median [1stquartile;3rdquartile] | | | P-value | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| Early-life | chao1 | 210.82500 [168.05530; 434.83285] | 167.55556 [142.35294; 252.66667] | 210.82500 [168.05530;434.83285] | 246 [184.75000;322.00000] | 0.279 | 1 | 0.279 | 0.279 |
| | simpson | 0.59513 [0.36188; 0.70911] | 0.58874 [0.10365; 0.77676] | 0.59513 [0.36188;0.70911] | 0.87489 [0.57677;0.90721] | 0.06 | 0.075 | 0.542 | 0.075 |
| | shannon | 2.16485 [1.30430; 2.80319] | 1.89436 [0.56167; 3.23007] | 2.16485 [1.30430;2.80319] | 4.08773 [1.82870;4.28035] | 0.107 | 0.1 | 0.486 | 0.1 |
| | PD_whole_tree | 5.00302 [4.50525; 5.60048] | 5.21185 [5.10231; 5.45896] | 5.00302 [4.50525;5.60048] | 5.84350 [4.24261;6.79752] | 0.711 | 0.634 | 0.634 | 0.634 |
| | observed_species | 172.50000 [128.25000; 323.25000] | 145 [100.00000; 201.00000] | 172.5 [128.25000;323.25000] | 205.00000 [128.50000;269.00000] | 0.368 | 0.819 | 0.366 | 0.366 |
| Adolesc. | chao1 | 724.38158 [680.43694; 814.99446] | 735.55561 [714.53363; 792.31075] | 724.38158 [680.43694;814.99446] | 777.80000 [693.43341;798.74887] | 0.968 | 0.908 | 0.908 | 0.908 |
| | simpson | 0.96354 [0.96109; 0.96562] | 0.9708 [0.95878; 0.97444] | 0.96354 [0.96109;0.96562] | 0.96513 [0.96175;0.96651] | 0.55 | 0.643 | 0.57 | 0.57 |
| | shannon | 6.09444 [6.00854; 6.15921] | 6.24047 [6.06030; 6.33626] | 6.09444 [6.00854;6.15921] | 6.00689 [5.87900;6.15395] | 0.4 | 0.495 | 0.495 | 0.495 |
| | PD_whole_tree | 24.57861 [22.85232; 27.56155] | 24.69626 [23.76503; 26.78379] | 24.57861 [22.85232;27.56155] | 26.89073 [21.92052;28.67326] | 0.983 | 0.908 | 0.908 | 0.908 |
| | observed_species | 632.00000 [607.00000; 770.50000] | 668 [637.75000; 711.25000] | 632 [607.00000;770.50000] | 726.50000 [637.25000;751.25000] | 0.915 | 0.908 | 0.908 | 0.908 |
| Adult | chao1 | 840.06606 [719.12816; 893.71028] | 805.04152 [751.45427; 831.49694] | 840.06606 [719.12816;893.71028] | 786.12240 [713.24712;858.50561] | 0.832 | 0.681 | 0.681 | 0.681 |
| | simpson | 0.97644 [0.97564; 0.98103] | 0.97844 [0.97327; 0.98057] | 0.97644 [0.97564;0.98103] | 0.97724 [0.97177;0.97994] | 0.951 | 0.918 | 0.918 | 0.918 |
| | shannon | 6.58155 [6.46689; 6.73930] | 6.65903 [6.45822; | 6.58155 [6.46689;6.73930] | 6.59537 [6.45072;6.72102] | 0.984 | 0.954 | 0.954 | 0.954 |

| Age | Diversity metric | Taxonomic level | Median [1stquartile;3rdquartile] | | | P-value | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | overall | CF x CS | CF x VB | CS x VB |
| | | | 6.75114] | | | | | | |
| | PD_whole_tree | 26.95793 [25.05227; 28.26768] | 24.53608 [23.86014; 25.43093] | 26.95793 [25.05227;28.26768] | 25.90536 [23.68774;28.73040] | 0.113 | 0.862 | 0.071 | 0.355 |
| | observed_species | 814.00000 [689.50000; 865.75000] | 779.5 [722.50000; 809.50000] | 814 [689.50000;865.75000] | 763.00000 [693.00000;827.25000] | 0.846 | 0.7 | 0.7 | 0.7 |

***Supplementary Table 2. 3- Summary of the statistical analyses for diversity in VB, CF,CS groups for ealy-life, adolescence and adulthood at phylum(p), family (f) and genus (g) level, indicated by the p-value.***

Vaginal birth =VB; Cross-fostered=CF; C-section=CS. The p-values are correct for multiple testing at a defined false discovery. Significant effects ($p<0.05$) are given in bold.

| SCFA | | Dependent measure | Mean ±S.E.M (n) | | | One-way ANOVA | | | Tukey post-hoc |
|---|---|---|---|---|---|---|---|---|---|
| | | | VB | CF | CS | P-value | F-value | DF | |
| Adolescent | **Acetate** | Conc. (µmol/g) | 33.67±1.62(8) | 30.29 ±1.37(8) | 36.96±1.87(7) | **0.03** | 4.178 | 2, 20 | *ns* |
| | **Proprionate** | | 5.56±0.88(8) | 5.87±.67(8) | 6.17±0.93(7) | 0.877 | 0.132 | 2, 20 | *ns* |
| | **Iso-butyrate** | | 3.04±0.57(8) | 2.65±0.55(8) | 3.05±0.68(7) | 0.861 | 0.15 | 2, 20 | *ns* |
| | **Butyrate** | | 16.58±0.94(8) | 17.47±1.16(8) | 19.84±1.32(7) | 0.148 | 2.107 | 2, 20 | *ns* |
| | **Total SCFA** | | 58.84±3.05(8) | 56.28±2.69(8) | 66.01±3.90(7) | 0.117 | 2.391 | 2, 20 | *ns* |
| Adult | **Acetate** | | 25.6±1.58(14) | 21.97±1.19 (12) | 24.70±1.57(13) | 0.219 | 1.584 | 2, 37 | *ns* |
| | **Proprionate** | | 2.87±0.2(14) | 2.58±0.16(12) | 2.81±0.18(13) | 0.503 | 0.7 | 2, 37 | *ns* |
| | **Iso-butyrate** | | 0.17±0.01(14) | 0.16±0.02(12) | 0.16±0.01(13) | 0.86 | 0.151 | 2, 37 | *ns* |
| | **Butyrate** | | 10.54±1.21(13) | 8.21±0.48(12) | 10.8±1.0(13) | **0.036** | 3.674 | 2, 38 | CF *vs* CS* |
| | **Total SCFA** | | 40.27±2.56(14) | 32.93±1.53(12) | 38.47±2.53(13) | 0.083 | 2.664 | 2, 36 | *ns* |

***Supplementary Table 2. 4- Summary of the statistical tests and outcomes for the Short chain fatty acids (SCFA) analyzes from CS, CF and VB offspring. The table specifies the dependent measures and summarizes the main effects.***

The table compares the group effect of short chain fatty acids (concentration (conc.) (µmol/g) cecum content in the adolescent and adult VB, CF and CS. The table compares the group effect for acetate, propionate, iso-butyrate, butyrate and total SCFA. Data was analyzed by one-way ANOVA followed by Tukey´s post hoc. The post hoc analyzes revealed significant differences in the acetate between CF and CS mice in the adolescence and in butyrate between CF and CS in the adulthood. Vaginal birth=VB; Cross-fostered=CF; C-section=CS. The table specifies the Mean ± S.E.M. The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font.

| | | Vaginally-born | C-section | P-value |
|---|---|---|---|---|
| **Age** | | 20.22 ± 0.24 | 20.34 ± 0.22 | 0.72 |
| **BMI** | | 23.66 ± 0.46 | 24.58 ± 0.63 | 0.21 |
| **Years of Education** | | 16.03 ± 0.22 | 15.83 ± 0.19 | 0.5 |
| **Units of Alcohol per week** | | 7.42 ± 1.04 | 9.96 ± 1.53 | 0.17 |
| **Smoker (%)** | | 4 (10) | 2 (5.6) | 0.473 |
| **Social Class (%)** | **1 (Professional)** | 18 (45) | 22 (61.1) | 0.232 |
| | **2 (Managerial/Technical)** | 6 (15) | 1 (2.8) | |
| | **3 (Non-Manual)** | 3 (7.5) | 6 (16.7) | |
| | **4 (Skilled Manual)** | 3 (7.5) | 3 (8.3) | |
| | **5 (Semi-Skilled)** | 4 (10) | 1 (2.8) | |
| | **6 (Unskilled)** | 5 (12.5) | 3 (8.3) | |
| | **7 (Other gainfully occupied & unknown)** | 1 (2.5) | 0 (0) | |
| **CTQ (Abuse)** | **Emotional** | 5.76 (0.38) | 6.23 (0.43) | 0.45 |
| | **Physical** | 5.86 (0.14) | 5.57 (0.28) | 0.59 |
| | **Sexual** | 4.7 (0.26) | 5.17 (0.35) | 0.33 |
| **CTQ (Neglect)** | **Emotional** | 6.95 (0.55) | 7.83 (0.61) | 0.32 |
| | **Physical** | 5.11 (0.3) | 5.91 (0.39) | 0.14 |
| **CTQ** | **Minimization/Denial** | 10.38 (0.61) | 10.54 (0.59) | 0.85 |

***Supplementary Table 2. 5- Participant Demographics.***

The table specifies the demographic information for the Vaginally-born and C-section participants. The table compares body mass index (BMI); years of education; average of units of alcohol intake per week; the percentage of smoker participants; social class; childhood trauma questionnaire (CTQ) in Vaginally-born and C-section participants. The table specifies the Mean ± S.E.M. The table also specifies the corresponding P-values.

| | Dependent measure | Mixed-effects model | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | VB | | | CF | | | CS | | |
| | | P-value | F-value | DF | P-value | F-value | DF | P-value | F-value | DF |
| **Marble burying** | Number of marbles buried (n) | .209 | 1.720 | (1,15) | 0.681 | 0.176 | (1,14) | 0.323 | 1.062 | (1,12 ) |
| **Elevated-plus maze** | Entrances in the open arms (n) | **0.0001** | 38.983 | (1,15) | **0.001** | 19.475 | (1,14) | 0.185 | 1.981 | (1,12 ) |
| | Entrances in the closed arms (n) | 0.354 | 0.917 | (1,15) | 0.446 | 0.614 | (1,14) | 0.903 | 0.015 | (1,12 ) |
| **Aversive open-field** | Time in central zone (sec.) | 0.106 | 2.959 | (1,15) | 0.458 | 0.582 | (1,14) | 0.13 | 2.645 | (1,12 ) |
| | Total distance (cm) | 0.198 | 1.814 | (1,15) | 0.368 | 0.865 | (1,14) | 0.343 | 0.976 | (1,12 ) |
| **Sociability** | Interaction time (sec.) (Mouse) | 0.291 | 1.199 | (1,15) | 0.815 | 0.057 | (1,14) | 0.664 | 0.198 | (1,12 ) |
| | Interaction time (sec.) (Object) | 0.283 | 0.602 | (1,15) | 0.549 | 0.378 | (1,14) | 0.348 | 0.955 | (1,12 ) |
| **Social Novelty** | Interaction time (sec.) (Novel mouse) | 0.938 | 0.006 | (1,15) | 0.554 | 0.368 | (1,14) | 0.604 | 0.283 | (1,12 ) |
| | Interaction time (sec.) (Familiar mouse) | 0.467 | 0.505 | (1,15) | 0.053 | 4.475 | (1,14) | **0.048** | 4.848 | (1,12 ) |
| **Stress response** | T0 | 0.306 | 1.123 | (1,15) | 0.104 | 3.053 | (1,13) | 0.246 | 1.487 | (1,12 ) |
| | T15 | 0.933 | 0.007 | (1,13) | 0.922 | 0.01 | (1,13) | 0.295 | 1.208 | (1,12 ) |
| | T45 | 0.362 | 0.9 | (1,12) | 0.063 | 4.12 | (1,13) | 0.296 | 1.194 | (1,12 ) |
| | T90 | 0.571 | 0.337 | (1,14) | 0.024 | 6.651 | (1,12) | 0.592 | 0.305 | (1,11 ) |
| | T120 | 0.952 | 0.004 | (1,13) | 0.57 | 0.343 | (1,11) | 0.925 | 0.009 | (1,11 ) |

***Supplementary Table 2. 6- Summary of the statistical tests and outcomes for Mixed-model design.***

The table specifies the dependent measures and summarizes the main effects of Mixed-model design. The table compares an estimative for the litter effect within each group for the main neurobehavioral outcomes. The table also specifies the corresponding degrees of freedom (DF) and F-values. Significant effects ($P < 0.05$) are given in bold font.

| | Median [1st Quartile;3rd Quartile] | | |
|---|---|---|---|
| **Diversity metric** | **VB** | **CS** | **P-value** |
| chao1 | 316 [278;361] | 347 [313;396] | 0.029 |
| simpson | 0.96 [0.95;0.97] | 0.96 [0.96;0.97] | 0.679 |
| shannon | 5.45 [5.25;5.85] | 5.67 [5.40;5.83] | 0.236 |
| PD_whole_tree | 17.3 [15.1;20.5] | 18.7 [17.1;22.2] | 0.075 |
| observed_species | 276 [236;320] | 305 [275;333] | 0.044 |

***Supplementary Table 2. 7- Alpha diversity statistics for VB and CS groups for a group of adult males.***

A Mann-Whitney U test was performed on these. The p-values are unadjusted, as they relate to different diversity metrics and are thus not directly comparable. VB, Vaginal birth; CS, C-section.

| | | Median [1st Quartile;3rd Quartile] | | |
|---|---|---|---|---|
| **Taxa** | **Taxonomic Level** | **VB** | **CS** | **P-value** |
| Actinobacteria | p | 4.29 [2.95;5.87] | 4.06 [2.52;6.64] | 0.847 |
| Bacteroidetes | p | 13.7 [7.31;19.7] | 15.7 [9.64;22.0] | 0.448 |
| Firmicutes | p | 77.4 [71.5;81.8] | 77.3 [72.1;82.9] | 0.934 |
| Proteobacteria | p | 0.30 [0.20;0.61] | 0.33 [0.15;0.66] | 0.815 |
| Verrucomicrobia | p | 0.62 [0.02;2.16] | 0.02 [0.00;0.33] | **0.027** |
| Actinomycetaceae | f | 0.01 [0.00;0.01] | 0.01 [0.01;0.02] | 0.098 |
| Bifidobacteriaceae | f | 2.73 [1.98;3.70] | 2.71 [1.30;3.96] | 0.945 |
| Coriobacteriaceae | f | 1.55 [0.87;2.15] | 1.13 [0.83;2.28] | 0.491 |
| Bacteroidaceae | f | 5.45 [3.91;11.0] | 9.19 [5.74;14.0] | 0.092 |
| Porphyromonadaceae | f | 1.05 [0.52;2.07] | 0.92 [0.53;1.46] | 0.424 |
| Prevotellaceae | f | 0.46 [0.01;4.30] | 0.12 [0.01;1.82] | 0.624 |
| Rikenellaceae | f | 0.96 [0.52;1.55] | 1.04 [0.37;1.86] | 0.836 |

| | | Median [1st Quartile;3rd Quartile] | | |
|---|---|---|---|---|
| **Taxa** | **Taxonomic Level** | **VB** | **CS** | **P-value** |
| Enterococcaceae | f | 0.01 [0.00;0.03] | 0.01 [0.00;0.02] | 0.195 |
| Lactobacillaceae | f | 0.01 [0.00;0.09] | 0.01 [0.01;0.03] | 0.689 |
| Streptococcaceae | f | 0.34 [0.13;0.66] | 0.37 [0.16;0.73] | 0.526 |
| Clostridiaceae_1 | f | 0.57 [0.21;1.12] | 0.44 [0.11;1.09] | 0.629 |
| Eubacteriaceae | f | 0.01 [0.01;0.02] | 0.02 [0.01;0.03] | 0.572 |
| Lachnospiraceae | f | 32.1 [27.5;42.0] | 33.3 [29.6;39.0] | 0.424 |
| Peptostreptococcaceae | f | 1.19 [0.62;2.39] | 1.54 [0.60;2.06] | 0.879 |
| Ruminococcaceae | f | 28.6 [24.7;32.2] | 29.5 [23.7;34.8] | 0.679 |
| Erysipelotrichaceae | f | 1.07 [0.54;2.52] | 1.67 [0.84;2.23] | 0.356 |
| Acidaminococcaceae | f | 0.07 [0.01;1.15] | 0.47 [0.01;1.52] | 0.244 |
| Veillonellaceae | f | 3.74 [1.38;5.49] | 1.54 [0.18;5.44] | 0.19 |
| Sutterellaceae | f | 0.10 [0.02;0.22] | 0.05 [0.01;0.18] | 0.436 |
| Desulfovibrionaceae | f | 0.07 [0.03;0.12] | 0.04 [0.02;0.15] | 0.639 |
| Enterobacteriaceae | f | 0.01 [0.00;0.06] | 0.02 [0.01;0.09] | 0.25 |
| Pasteurellaceae | f | 0.01 [0.00;0.03] | 0.02 [0.00;0.07] | 0.33 |
| Verrucomicrobiaceae | f | 0.62 [0.02;2.16] | 0.02 [0.00;0.33] | **0.027** |
| Actinomyces | g | 0.01 [0.00;0.02] | 0.01 [0.01;0.02] | 0.136 |
| Akkermansia | g | 0.65 [0.02;2.18] | 0.02 [0.00;0.34] | **0.025** |
| Alistipes | g | 0.96 [0.54;1.56] | 1.06 [0.40;1.89] | 0.858 |
| Anaerostipes | g | 1.26 [0.58;1.59] | 1.14 [0.59;2.03] | 0.689 |
| Asaccharobacter | g | 0.10 [0.04;0.17] | 0.05 [0.02;0.13] | 0.209 |
| Bacteroides | g | 5.90 [4.00;11.0] | 9.67 [6.02;15.6] | 0.098 |

| | | Median [1st Quartile;3rd Quartile] | | |
|---|---|---|---|---|
| **Taxa** | **Taxonomic Level** | **VB** | **CS** | **P-value** |
| Barnesiella | g | 0.23 [0.12;0.69] | 0.23 [0.15;0.48] | 0.684 |
| Bifidobacterium | g | 2.75 [1.99;3.76] | 2.85 [1.31;3.99] | 0.912 |
| Bilophila | g | 0.05 [0.01;0.09] | 0.03 [0.01;0.08] | 0.535 |
| Blautia | g | 5.58 [4.52;9.30] | 6.10 [4.48;8.48] | 0.793 |
| Butyricicoccus | g | 0.43 [0.31;0.59] | 0.41 [0.28;0.66] | 0.783 |
| Butyricimonas | g | 0.02 [0.00;0.04] | 0.02 [0.00;0.05] | 0.792 |
| Clostridium_IV | g | 0.50 [0.23;1.42] | 0.40 [0.26;0.89] | 0.6 |
| Clostridium_sensu_stricto | g | 0.58 [0.21;1.13] | 0.45 [0.11;1.09] | 0.62 |
| Clostridium_XI | g | 1.19 [0.63;2.43] | 1.55 [0.61;2.10] | 0.858 |
| Clostridium_XlVa | g | 1.06 [0.77;1.94] | 1.26 [0.92;1.67] | 0.408 |
| Clostridium_XlVb | g | 0.05 [0.02;0.17] | 0.04 [0.01;0.14] | 0.517 |
| Clostridium_XVIII | g | 0.53 [0.18;0.92] | 0.74 [0.27;1.24] | 0.16 |
| Collinsella | g | 0.90 [0.63;1.83] | 0.84 [0.50;1.62] | 0.416 |
| Coprococcus | g | 3.32 [1.20;6.18] | 2.13 [1.07;3.76] | 0.195 |
| Dialister | g | 1.98 [0.74;4.11] | 0.64 [0.01;3.40] | 0.067 |
| Dorea | g | 1.41 [0.81;1.96] | 1.64 [0.94;2.56] | 0.264 |
| Eerthella | g | 0.00 [0.00;0.02] | 0.00 [0.00;0.02] | 0.814 |
| Enterococcus | g | 0.01 [0.01;0.03] | 0.01 [0.00;0.02] | 0.209 |
| Escherichia.Shilla | g | 0.01 [0.00;0.06] | 0.01 [0.00;0.05] | 0.71 |
| Eubacterium | g | 0.01 [0.01;0.02] | 0.01 [0.01;0.03] | 0.699 |
| Faecalibacterium | g | 13.1 [9.81;16.0] | 11.9 [7.84;15.7] | 0.535 |
| Flavonifractor | g | 0.03 [0.02;0.04] | 0.02 [0.02;0.10] | 0.61 |

| | | Median [1st Quartile;3rd Quartile] | | |
|---|---|---|---|---|
| **Taxa** | **Taxonomic Level** | **VB** | **CS** | **P-value** |
| Gemmir | g | 1.11 [0.57;1.78] | 1.15 [0.64;2.51] | 0.572 |
| Gordonibacter | g | 0.01 [0.00;0.03] | 0.01 [0.00;0.02] | 0.571 |
| Haemophilus | g | 0.01 [0.00;0.04] | 0.02 [0.00;0.06] | 0.374 |
| Holdemania | g | 0.01 [0.01;0.01] | 0.01 [0.01;0.02] | 0.581 |
| Lachnospira | g | 0.23 [0.02;0.72] | 0.24 [0.03;1.12] | 0.836 |
| Lactobacillus | g | 0.01 [0.00;0.09] | 0.01 [0.01;0.03] | 0.689 |
| Odoribacter | g | 0.07 [0.02;0.17] | 0.06 [0.02;0.16] | 0.783 |
| Oscillibacter | g | 0.20 [0.09;0.29] | 0.17 [0.08;0.39] | 0.858 |
| Parabacteroides | g | 0.59 [0.28;0.84] | 0.41 [0.25;0.83] | 0.491 |
| Parasutterella | g | 0.01 [0.00;0.07] | 0.01 [0.00;0.02] | 0.562 |
| Phascolarctobacterium | g | 0.01 [0.00;0.55] | 0.22 [0.00;1.36] | 0.214 |
| Prevotella | g | 0.07 [0.00;4.29] | 0.01 [0.00;1.71] | 0.276 |
| Roseburia | g | 7.48 [6.28;13.2] | 10.4 [5.73;12.3] | 0.639 |
| Ruminococcus | g | 4.59 [3.23;6.60] | 5.35 [1.66;7.42] | 0.526 |
| Ruminococcus2 | g | 1.16 [0.93;1.81] | 1.23 [0.72;1.87] | 0.783 |
| Streptococcus | g | 0.34 [0.12;0.68] | 0.37 [0.14;0.65] | 0.699 |
| Sutterella | g | 0.01 [0.00;0.15] | 0.02 [0.00;0.11] | 0.78 |
| Turicibacter | g | 0.04 [0.01;0.14] | 0.08 [0.01;0.20] | 0.416 |
| Veillonella | g | 0.04 [0.01;0.12] | 0.06 [0.01;0.19] | 0.62 |

***Supplementary Table 2. 8- Differentially abundance taxa between VB and CS groups in adults at phylum(p), family (f) and genus (g) level, as tested with a Mann-Whitney U-test.***

The p-values are correct for multiple testing at a defined false discovery. Significant effects ($p<0.05$) are given in bold. Vaginal birth =VB; C-section=CS.

| Gene name | Assay ID | Ref. Seq. | Exon Location | Primers |
|---|---|---|---|---|
| *Actb* | REF4352341E (VIC labeled) | NM_007393.1 | | |
| *Tjp1* | Mm.PT.58.12952721 | NM_001163574 | 25-26 | 1: 5'-GCCACTACAGTATGACCATCC-3'<br>2: 5'-AATGAATAATATCAGCACCATGCC-3' |
| *Ocln* | Mm.PT.58.30118962 | NM_008756 | 6-7 | 1: 5'-GTTGATCTGAAGTGATAGGTGGA-3'<br>2: 5'-CACTATGAAACAGACTACACGACA-3' |
| *Tlr4* | Mm00445274_m1 | NM_021297.2 | 2-3 | Not Available |
| *Nr3c1* | Mm.PT.56a.42952901 | NM_008173 | 1-2 | 1: 5' -GAT CCT CAA GAC CTT CCA AGA-3'<br>2: 5' -AGT CAT TTC TTC CAG CAC ACA- 3' |
| *Nr3c2* | Mm.PT.56a.30752774 | NM_001083906(1) | | 1: 5' -GAT TAC TGC TCT GGC TCC TAG-3'<br>2: 5' -GAC TCA TCG TAC TCC TGC TTG -3' |
| *Crhr1* | Mm.PT.58.13604366 | NM_007762(1) | | 1: 5'-TGC CTT TCC CCA TCA TTG TG-3'<br>2: 5' -GCC CTG GTA GAT GTA GTC AGT A-3' |
| *Oxtr* | Mm.PT.58.10758.10711295 | NM_001081147 | 1-2 | 1: 5'-ATGGCAATGATGAAGGCAGA-3'<br>2: 5'-CGCACAGTGAAGATGACCTT-3' |

***Supplementary Table 2. 9- PrimeTime® qPCR assays used in this study.***

ß-Actin, household gene (Actb) ; Tight junction protein-1 (Tjp1); Occludin (Ocln) ; Toll-like receptor 4 (Tlr4); Glucocorticoid receptor (Nr3c1); Mineralocorticoid receptor (Nr3c2); Corticotropin-releasing factor receptor 1 (Crhr1), Oxytocin receptor (*Oxtr*).

# Chapter 3. Reversal of Fortune: Early-life Oxytocin Blunts Selective Behavioural & Physiological Effects Induced by Caesarean-section Birth Delivery in Mice

**Livia H. Morais**[1,2], Anna V. Golubeva[1], Karen A. Scott[1], Sophie Casey[1], Ana Paula Ramos Costa[1], Gerard Moloney[1,2], Timothy G. Dinan[1,2], John F. Cryan[1,2*]

[1] APC Microbiome Institute, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

To be submitted to: Neuropsychopharmacology

*Corresponding author:

John F. Cryan

j.cryan@ucc.ie

## 3.1 Abstract

The oxytocin (OXT) system has been strongly implicated in the regulation of social behaviour and anxiety, potentially contributing to the aetiology of a wide range of psychopathologies. Alterations in the microbiota-gut brain axis have also been implicated in the development of both social and anxiety-related behaviours. Birth by Caesarean-section (C-section) results in alterations in microbiota diversity and complexity in early-life and has recently been associated with long-term social and anxiety-like behaviour deficits. In this study, we assessed whether OXT intervention in the early postnatal period could reverse C-section-mediated effects on stress, anxiety-like behaviour, social behaviour, and physiology in early-life and adulthood. Following C-section or *per vaginum* birth, pups were reared by foster dams, and OXT (0.2 or 2 μg/20μl; s.c.) or saline was administered daily from postnatal days one to five.

In early-life, high-dose OXT administration reversed C-section-mediated maternal attachment impairments. Moreover, treatment with low-dose OXT restored social memory deficits, some aspects of anxiety-like behaviour and disrupted gastrointestinal motility in adulthood. Furthermore, as a consequence of OXT intervention in early life, OXT plasma levels in adult male mice were increased, baseline corticosterone levels were decreased, and the dysregulation of immune response in C-section animals was attenuated. These findings indicate that there is an early developmental window sensitive to manipulations of the OXT system that can prevent lifelong behavioural and physiological impairments associated with mode of delivery.

## 3.2 Introduction

Delivery by Caesarean-section (C-section), when medically indicated, is a crucial life-saving procedure. However, in recent decades, the use of elective C-section has dramatically increased worldwide, with rates exceeding World Health Organization guidelines (of 10-15%), especially in middle and high-income countries (Betran et al., 2016). This trend raises significant concerns given the growing evidence for an association between C-section delivery and increased risk for immune and metabolic disorders (Algert et al., 2009; Darmasseelane et al., 2014; Decker et al., 2010; Horta et al., 2013; Mårild et al., 2012). Moreover, birth by C-section results in a different pattern of microbiota seeding early in life (Dominguez-Bello et al., 2010; Hill et al., 2017; Martinez et al., 2017; Penders et al., 2006), interfering with the wiring of the entire microbiota-gut-brain axis (Moya-Pérez et al., 2017). Recently, we have demonstrated that mice delivered by C-section develop enduring abnormal behavioural and physiological phenotypes in addition to a reduction in gut microbiota diversity and altered oxytocin receptor mRNA expression in the amygdala (Morais et al., 2017).

OXT is a key modulator of mammalian maternal-offspring attachment and social behaviour (Carter, 2003; Donaldson and Young, 2008; Insel, 2003; Johnson and Young, 2017). Moreover, the OXT system is activated by stressful and anxiogenic stimuli and acts as key-modulator of the hypothalamic-pituitary-adrenal (HPA) axis (Neumann and Slattery, 2016). Consistent with the important role of OXT in social behaviour promotion and stress regulation, OXT dysregulation has been associated with anxiety and autism spectrum disorders (ASDs) (Kosaka et al., 2016; Liu et al., 2005; Mamrut et al., 2013). Therefore, there is a growing interest in the OXT system as a potential target in the treatment of neuropsychiatric disorders associated with stress and social dysfunction (Andari et al., 2010, 2017; Bakermans-Kranenburg and van I Jzendoorn, 2013; Parker et al., 2017). Indeed early-life administration of oxytocin can reverse long-lasting social behavioural deficits and restored oxytocin immunoreactivity in the PVN in the contactin-associated protein-like 2 (*CNTNAP2*) model of ASD (Peñagarikano et al., 2015). Moreover, OXT treatment after birth prevents social and cognitive deficits and normalises OXT system function in adult mice deficient for *Magel2* gene, which is involved in Prader-Willi Syndrome and ASD

and (Meziane et al., 2015). OXT developmental effects are not limited to OXT system and behaviour, it influences the development of HPA-axis (Kramer et al., 2003), and other neurotransmitter systems such as GABaergic (Tyzio et al., 2014), noradrenergic and serotoninergic systems (Eaton et al., 2012). Thus, demonstrating organisational effect during development (Miller and Caldwell, 2015).

Recently, there have been numerous studies investigating the link between gut microbiota and social behaviour (Arentsen et al., 2015; Desbonnet et al., 2014; Golubeva et al., 2017; Hsiao et al., 2013; Kim et al., 2017). Moreover, there is growing evidence showing that the microbiota can interact with the OXT system by acting through immune-system to produce beneficial effects in the host (Varian et al., 2017). For example, treatment with *Lactobacillus reuteri* was shown to increase OXT plasma levels and OXT-positive cells in the paraventricular nucleus (PVN) of hypothalamus, and accelerate wound healing in mice (Poutahidis et al., 2013; Varian et al., 2017). In another study, the same bacteria were able to reverse social deficits and increase brain levels of oxytocin of mice born to obese dams on high fat diet (Buffington et al., 2016). Similarly, we have recently demonstrated that birth by C-section induces alterations in OXT receptor mRNA expression in the amygdala which are reversed by restoring bifidobacteria in the mouse gut (Morais et al., 2017). However, it remains unclear whether interventions targeting the OXT system can ameliorate the enduring effects of C-section on physiology and behaviour. Therefore, in this study, we investigated the long-term effects of early postnatal OXT treatment on mice delivered by C-section.

## 3.3 Materials and Methods

### 3.3.1 Animals

The experiments were carried out in male Swiss mice of different ages. 8-week-old female and male breeders were obtained from Harlan laboratories, UK. Breeding began after 1-2 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%) with food and water given *ad libitum* unless specified otherwise. Male offspring were weaned at postnatal (P) day 21 and group-housed in 3-4 mice per cage. Each experimental group included the offspring from 3-10 independent litters. In addition, 10-week-old Swiss male mice, purchased from Harlan

laboratories, UK, were used as conspecifics in the three-chamber test. All experimental procedures were conducted in accordance with the Directive 2010/63/EU and were approved by the Animal Experimentation Ethics Committee of University College Cork # 2012/036.

### 3.3.2 Mode of Delivery: Experimental Groups

Mice were time-mated, and the presence of a vaginal plug in females was marked as a gestational day 0.5 (G0.5). Males were removed from the cages, and pregnant females were not disturbed except for cage cleaning. Pregnant dams were randomly assigned to one of two experimental groups (using a generator of random numbers): (1) Vaginal delivery: the offspring were delivered naturally and raised by their biological mother. (2) C-section: the offspring were delivered by C-section surgery and raised by a foster dam that gave birth on the same day. Treatments were assigned to the entire litter; each of the offspring in a litter received the same treatment. (See **Oxytocin administration** for further details).

### 3.3.3 C-section Surgery

At full term (G20.5), female mice were euthanized by cervical dislocation. All further procedures were performed in nearly aseptic conditions. The abdominal skin was prepped with 70% ethanol; the uterus was excised with sterile surgical tools and placed on sterile gauze, preheated to +37C with a heating pad. The pups were removed from the uterus by applying a gentle pressure with a sterile swab, and further massaged for 1-2 minutes to clear the amniotic membrane and stimulate pulmonary breathing. Once spontaneous breathing was recorded, the pups were immediately transferred to a foster mother. To facilitate nursing, pups were briefly smeared with the bedding material from the foster mother's cage prior to transfer.

### 3.3.4 Oxytocin Administration

From P1 to P5, male and female pups were temporarily removed from their nest (5 min) weighed and given a daily subcutaneous (s.c.) injection as previously described (Schaller et al., 2010, Meziane et al., 2015). Pups received a s.c. injection of either 0.2 µg or 2 µg of

OXT (Tocris, Bioscience, UK) dissolved in 20 µL of isotonic saline, or 20 µL of isotonic saline alone (control) (Meziane et al., 2015; Schaller et al., 2010).

### 3.3.5 Experimental Design

Effects of C-section on behaviour were evaluated in both male and female offspring in early-life (P9/P10) and only in males in adulthood (weeks 10-16). Mice were habituated to the room 30 minutes prior to each test. The exact order of behavioural tests, as well as resting intervals between them were optimized to reduce cumulative stress effects and the potential confounding carryover effects from previous tests (see **Figure 3. 1a**).

### 3.3.6 Behavioural Assessments

#### *3.3.6.1 Attachment Behaviour (Homing Test)*

The homing test evaluates the tendency of pups to recognize their mother's and siblings' nest and is an indirect measure of attachment behaviour (Macrì et al., 2010). At P10, a clean mouse cage was divided into the three equal-size quadrants by wire-mesh dividers. One of the side quadrants was uniformly covered with wood shavings from the home cage, thus containing familiar odour stimuli. The opposite side quadrant was covered with wood shavings from the cage of another litter (born at approximately the same time); the middle quadrant was covered with clean bedding material. Pups were placed individually in the middle part for 1 minute; the dividers were then removed, and the pups could freely move around for 2 minutes. Total time spent in each area was recorded.

#### *3.3.6.2 Three-chamber Test*

Sociability and preference for social novelty were assessed in a three-chamber apparatus, as described previously (Desbonnet et al., 2014). Animals were placed in a rectangular apparatus divided into three chambers (left, right and a smaller central chamber) by transparent partitions with small circular openings allowing easy access to all compartments. The test was composed of three sequential 10 min trials: (1) habituation (a test animal was allowed to explore the three empty chambers); (2)

sociability (an unfamiliar animal was positioned in a wire mesh cage in either the left- or right- side chamber); (3) social novelty preference (a novel animal was positioned in either right- or left- side chamber, opposite the one occupied by the now familiar animal). Each trial started from the central chamber. All animals were age- and sex-matched; each chamber was cleaned and lined with fresh bedding between trials. Behaviours were recorded by a video camera mounted above the apparatus, and the time of active interaction was measured.

#### *3.3.6.3 Defensive Marble Burying Test*

The defensive marble burying (DMB) test measures repetitive and anxiety-like behaviours; the number of marbles buried during the test correlates with anxiety (Sweeney et al., 2014). Clean cages were filled with a 4-cm layer of chipped cedar wood bedding. Twenty glass marbles (15mm diameter) were gently laid on top of the bedding, equidistant from each other in a 4×5 arrangement. The mouse was then placed in the cage and allowed to explore it for 30 min. At the end of the test, the number of marbles buried (> 2/3 marble covered by bedding material) was recorded.

#### *3.3.6.4 Elevated Plus Maze Test*

The elevated plus maze (EPM) is one of the most widely used behavioural tests to assess anxiety-related behaviours (Holmes, 2001; Rodgers, 1997b). The Plexiglas maze consists of a plus-shaped apparatus with two open and two enclosed arms (50 cm × 5 cm × 15 cm walls) elevated from the floor by 1m. Mice were placed in the centre of the EPM apparatus facing an open arm and allowed to explore it for 5 minutes. The number of entries into open and closed arms were scored. When all four paws of a test mouse were standing on the arm, this position was considered as an "entry".

#### *3.3.6.5 Aversive Open Field (OF) Test*

The open field (OF) test is used to assess locomotor activity, as well as the response to a novel, stressful environment (O'Leary et al., 2014). Light was set at 1000 lux. A test mouse was placed in the centre of an open field arena (Perspex box with white

base: 30 x 30 x 20 cm) and allowed to explore the arena for a 10-minute period. The distance moved and the time spent in the central zone (16 x 15 cm) of the open field were recorded using Ethovision videotracking system (Noldus Information Technology).

### *3.3.6.6 In vivo Intestinal Motility*

In vivo intestinal motility was measured as previously described (Golubeva et al., 2017). Mice were single-housed and habituated to new cages for three hours. Following the acclimatisation, mice received a 200µL oral gavage of carmine (C1022; Sigma Aldrich) suspended in 0.5% carboximethylcelulose (CMC) sodium salt (Sigma; St Louis, MO, USA). Cages were checked every 10 minutes, and the time between the gavage and the appearance of the first coloured faecal bolus was recorded.

### *3.3.6.7 Oxytocin Measurement*

Oxytocin measurement was performed as previously described (Neumann et al., 2013). Briefly, trunk blood was collected and centrifuged (5 min, 5000 g, 4 °C), and plasma samples were kept at −20°C until oxytocin concentration was measured using a highly sensitive and specific radioimmunoassay performed by RIAgnosis, Munich.

### *3.3.6.8 Gene Expression Analysis with qRT-PCR*

Total RNA was extracted from the paraventricular nucleus of the hypothalamus (PVN) using the mirVana™ miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific). RNA concentration was quantified using the ND-1000 spectrophotometer (NanoDrop®). Equal amounts of RNA were reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies, Carlsbad, CA). cDNA was stored at −20 °C prior to the assay. Gene expression was analysed using TaqMan Gene Expression Assays on the AB7300 system (Applied Biosystems, Thermo Fisher Scientific). Changes in gene expression levels were calculated using the ΔΔCt method (Livak and Schmittgen, 2001); the average of three technical replicates for each biological sample was first related to β-actin expression, then the fold changes vs. the control (VB) group were calculated.

### *3.3.6.9 Analysis of Cytokine Release in Stimulated Murine Splenocyte*

Splenocytes were isolated as follows: immediately upon collection, cellular contents of spleen and thymus were gently dissociated in RPMI medium (R8758, supplemented with 10% FBS and 5% penicillin/streptomycin, Sigma), treated with Red Blood Cells lysis buffer (Sigma) to clear the culture from erythrocytes, passed through a 70 μm filter, and resuspended in fresh RPMI medium (as above) for seeding. Splenocytes were seeded in 6 well plates at $16x10^6$ cells/well in 4 mL. Following a 2.5 h rest period, cells were stimulated with lipopolysaccharide (LPS-2 μg/mL) for 24 h. Following stimulation, the supernatants were harvested to assess IL-10 and TNF-α concentration (pg/mL) using the Proinflammatory Panel 1 (mouse) V-PLEX MULTI-SPOT® Meso Scale Discovery kits (MSD, Rockville, MD, USA) as per manufacturer's instructions. The median lower limits of detection for each cytokine are IL-10 0.95 pg/mL, TNF-α 0.13 pg/mL.

### *3.3.6.10 Statistical Analyses*

Statistical analyses were carried out using SPSS version 19 (Armonk, NY, USA). Parametric data were analysed by Two-way ANOVA for mode of delivery and treatment factors followed by LSD post-hoc tests or Paired Student t-test and represented as mean ± S.E.M. Non-parametric data were analysed by Kruskal-Wallis followed by U- Mann Whitney tests and data are represented as median ± interquartile range. $p<0.05$ was considered statistically significant. The main statistical differences are represented in the graphs

## 3.4 Results

### 3.4.1 Postnatal Oxytocin Reversed C-section Mediated Maternal Attachment Deficits

Birth by C-section was recently shown to have a negative impact on the offspring-maternal attachment (Morais et al., 2017). To test whether the postnatal treatment with oxytocin (**Figure 3. 1a**) could ameliorate these effects, pups homing behaviour was tested at P10 **(Figure 3. 1b)**. As expected, VB pups from all treatment groups could discriminate between the familiar and non-familiar stimuli and move towards the mother's nest (**Figure 3. 1b**), whereas CS offspring failed to show a preference for the familiar stimuli (**Figure 3. 1b**). Strikingly, the postnatal treatment with the higher dose of OXT re-established the preference for the mother's bedding. To exclude the possibility that differences in offspring phenotype investigated in this study are due to cross-fostering effects on nurturing *per se*, we assessed maternal care behaviour at P6 **(Supplementary figure 3. 1)**. Of interest, cross-fostering CS offspring did not impair maternal care behaviour **(Supplementary figure 3. 1).** Interestingly, when pups were treated with oxytocin there was a significant increase on the time the mother spent engaged in high care behaviours (licking, grooming, and arched back nursing the pups) in both VB and CS groups **(Supplementary figure 3. 1).**

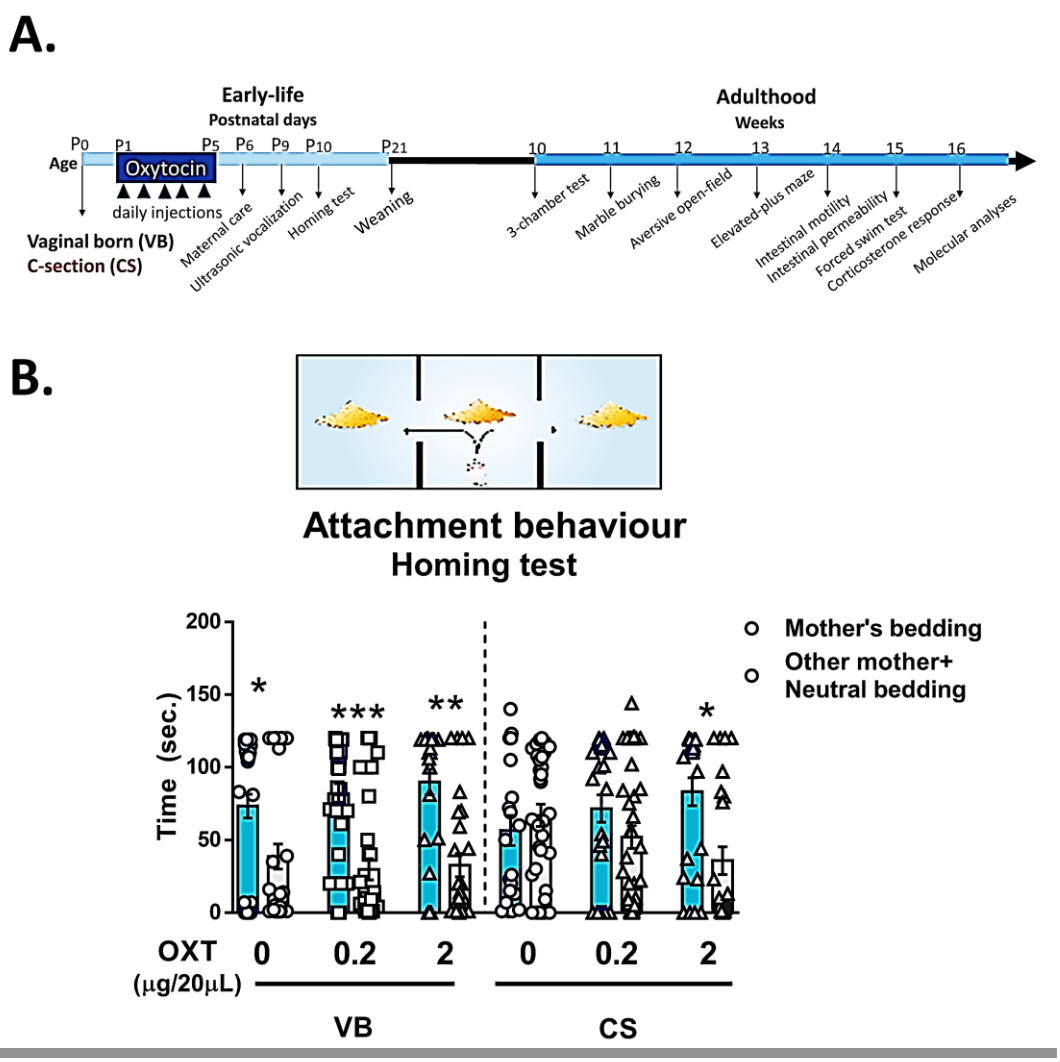



***Figure 3. 1- Early postnatal administration of OXT rescued C-section mediated effects on attachment behaviour. (a) Schematic representation of OXT treatment and behavioural testing sequence.***

Following birth by C-Section or per vaginum, pups received daily injections of oxytocin (0.2 or 2 µg/20µl saline; s.c.) from postnatal days 1-5 and were subjected to a sequence of behavioural tests. **(b)** High-dose OXT administration reversed C-section mediated effects on attachment behaviour. VB control t (35) = 2.531, p<0.0001, n=36; VB 0.2 OXT t (35) =4.281, p<0.0001, n=36; VB 2 OXT t (23) =3.444, p<0.0022, n=24; CS control t (22) = 0.5392, p=0.5952, n=23; CS 0.2 OXT t (35) = 1.571, p=0.1252, n=36; CS 2 OXT t (24) = 2.462, p=0.0214, n=25. Male and Female offspring in each cohort derived from three independent litters/ group. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). *p< 0.05, *p< 0.01 and ***p<0.0001. Paired Student's t-test comparing time spent in the mother's bedding with time spent in other bedding (mother's bedding + neutral bedding) as a measure of attachment behaviour. OXT, oxytocin; VB, vaginal birth; CS, C-section.

### 3.4.2 Postnatal Treatment With Low-dose OXT Restored the Preference for Social Novelty in C-section Born Adult Mice

To investigate if OXT could attenuate the enduring social behavior deficits induced by C-section, we performed the 3-chamber test in adulthood **(Figure 3. 2)**. Previous results did not detect differences in sociability due to C-section delivery mode(Morais et al., 2017). Here, we confirmed these findings and demonstrated that OXT treatment does not interfere with this pattern (**Figure 3. 2a**). However, when CS mice were given the choice to interact with a novel or with a familiar mouse, they exhibited decreased preference for social novelty **(Figure 3. 2b)** which was completely restored by treatment with low-dose OXT early in life (**Figure 3. 2b**).

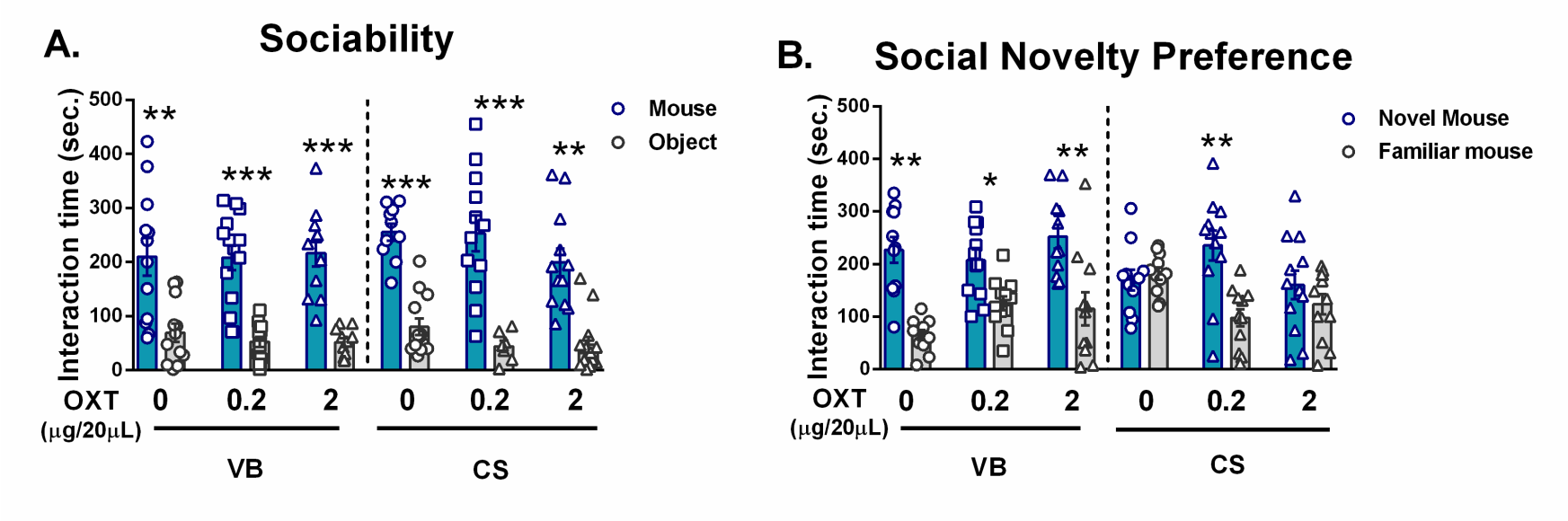



***Figure 3. 2- Early postnatal OXT restores social novelty preference in adult CS mice.***

(**a)** There were no significant differences on sociability across all groups in the three-chamber test. VB control t (11) = -3893, p=0.003, n=12; VB 0.2 OXT t (12) =-6144, p<0.0001, n=13; VB 2 OXT t (11) =5.066, p<0.0001, n=12; CS control t (11) = 6.298, p=0.0001, n=12; CS 0.2 OXT t (11) = 5.395, p<0.0.001, n=12; CS 2 OXT t (11) = 5.118, p<0.0001, n=12. Paired Student's t test comparing interaction time with mouse to an object. (**b)** Low-dose oxytocin rescued the preference for social novelty in CS. VB control t (11) = 4.681, p=0.001, n=12; VB 0.2 OXT t (11) =3.064, p=0.011, n=12; VB 2 OXT t (10) =3.404, p=0.007, n=11; CS control t (11) = 0.594, p=0.564, n=12; CS 0.2 OXT t (11) = 3.671, p=0.004, n=12; CS 2 OXT t (10) = 0.970, p=0.355, n=12. Paired Student's t test comparing interaction time with a familiar mouse to a novel mouse. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Male offspring in each cohort derived from three independent litters/ group. *p< 0.05, *p< 0.01 and ***p<0.0001. OXT, oxytocin; VB, vaginal birth; CS, C-section.

### 3.4.3 Early-life Oxytocin Improved But Does Not Completely Reverse the Anxiety-associated Phenotype Induced by C-section

To extend our investigation on the effects of the early-life pharmacological manipulation of OXT on the C-section-associated behavioural phenotype, adult VB and CS offspring were tested in a variety of anxiety-like-behavioural paradigms. In early-life, anxiety-like behaviour was assessed in the isolation-induced USV test (**Supplementary figure 3. 2**). Although there was no significant effect of the mode of delivery in this test, the treatment with low-dose OXT significantly increased the number of calls in both VB and CS groups **(Supplementary figure 3. 2)**.

In adulthood, as expected, CS showed increased anxiety-like behaviour as they buried significantly more marbles in comparison to VB in the marble burying test **(Supplementary figure 3. 2a)**. Interestingly, postnatal injection with the low-dose OXT attenuated these effects **(Supplementary figure 3. 2a)**. Additionally, we measured anxiety-like behaviour in the EPM **(Supplementary figure 3. 2b).** Although we confirmed that C-section reduces the number of entrances into the open arms of the EPM in comparison to the control group, there were no significant effects of OXT treatment on this test **(Supplementary figure 3. 2b)**. Similarly, when mice were tested in the aversive open-field, C-section reduced the total distance moved with no effects of OXT on ameliorating these effects **(Supplementary figure 3. 3a)**. Moreover, treatment with OXT significantly reduced the distance travelled in the open-field arena in the VB group **(Supplementary figure 3. 3b)**.

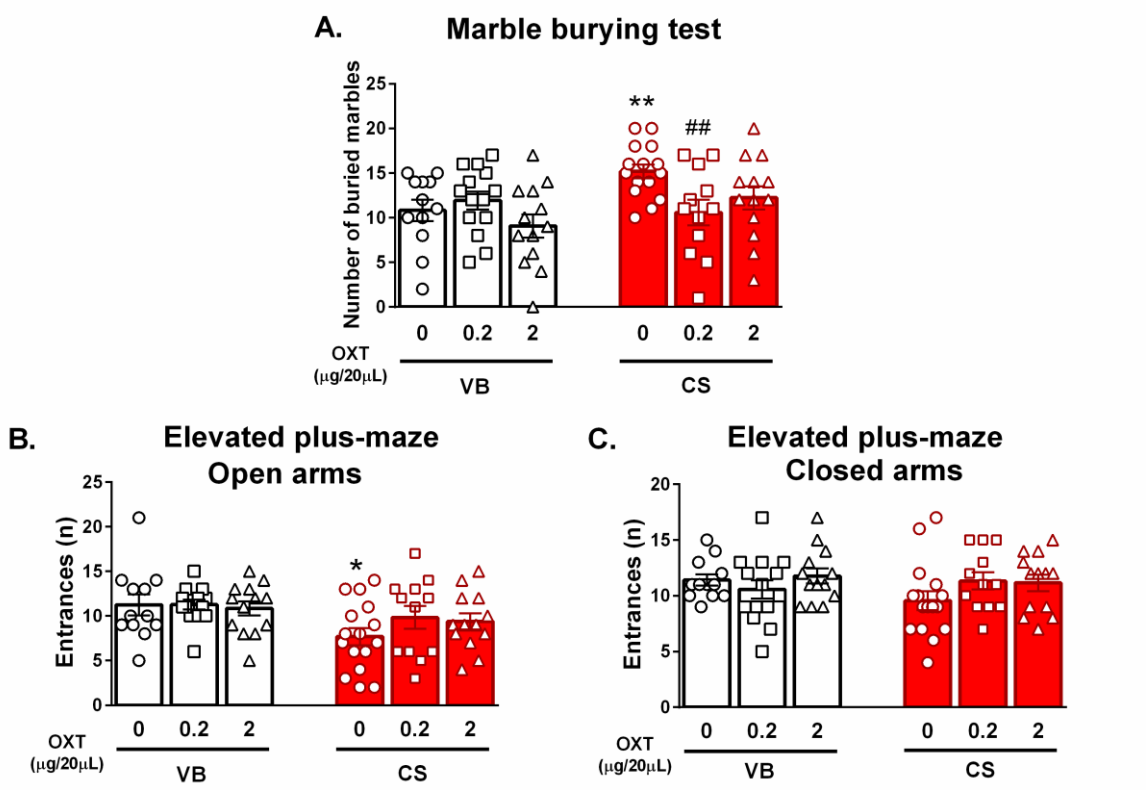



***Figure 3. 3- Effects of postnatal OXT administration on C-section-associated anxiety phenotype.***

(**a)** Early postnatal treatment with low-dose OXT attenuated the number of marbles buried in the CS group. Mode of delivery effect (F (1, 73) = 4.716, p=0.033); treatment effect (F (2, 73) = 2.258, p= 0.112); mode of delivery x treatment (F (2,73) = 3.351, p=0.041) ([VB control n= 12, VB 0.2 OT n=14, VB 2 OT control n=13, CS control n=15, CS 0.2 OXT n=12, CS 2 OXT n=13]). (**b)** OXT administration did not improve C-section-induced anxiety-like behaviour in the EPM. Mode of delivery effect (F (1, 74) = 7.614, p=0.007); treatment effect (F (2, 74) = 0.663, p= 0.518); mode of delivery x treatment (F (2,74) = 0.820, p=0.444). **(c)** There were no significant differences in the number of entrances of the closed arms of the EPM. Mode of delivery effect (F (1, 74) = 0.843, p=0.361); treatment effect (F (2, 74) = 0.829, p= 0.441); mode of delivery x treatment (F (2,74) = 1.497, p=0.231). ([VB control n= 12, VB 0.2 OT n=14, VB 2 OT control n=13, CS control n=16, CS 0.2 OXT n=12, CS 2 OXT n=13]). Two-way ANOVA followed by LSD post-hoc. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Male offspring in each cohort derived from three independent litters/ group. *p< 0.05, *p< 0.01 and ***p<0.0001. OXT, oxytocin; VB, vaginal birth; CS, C-section.

### 3.4.4 Early-life Postnatal OXT Improved Intestinal Motility Deficits in Adult C-section Offspring

Birth by C-section has been associated with alterations in gastrointestinal function in mice (Morais et al., 2017). To further characterize whether the pharmacological treatment with OXT could, as well, have beneficial effects on the gastrointestinal function in adult C-section offspring, we used the carmine red assay as an *in vivo* marker of motility. As expected, we confirmed that CS mice have faster gastrointestinal transit when compared with VB mice. Surprisingly, the treatment with low-dose OXT early in life reversed these effects **(Figure 3. 4).** To test whether this effects of OXT on gastrointestinal motility would correlate with changes in gastrointestinal permeability, we measured fluorescence in plasma of VB and CS offspring following gavage with fluorescein isothiocyanate (FITC)-labelled dextran. However, no significant alterations were observed across the groups **(Supplementary figure 3. 4)**.

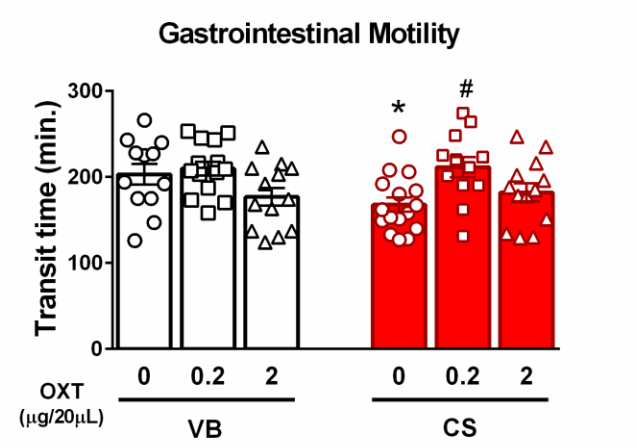



***Figure 3. 4- Oxytocin improved gastrointestinal motility in C-section.***

CS exhibited faster intestinal transit in comparison to VB, which was fully reversed by the low-dose OXT treatment in early-life. Mode of delivery effect (F (1, 74) = 1.278, p=0.262); treatment effect (F (2, 74) = 5.238, p= 0.007); mode of delivery x treatment (F (2, 74) = 2.523, p=0.087). ([VB control n= 12, VB 0.2 OT n=14, VB 2 OT control n=13, CS control n=16, CS 0.2 OXT n=12, CS 2 OXT n=13]). Two-way ANOVA, followed by LSD post-hoc. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Male

offspring in each cohort derived from three independent litters/ group. *p<0.05 for mode of delivery effect. #p< 0.05 for treatment effect within the group. OXT, oxytocin; VB, vaginal birth; CS, C-section.

### 3.4.5 Early-life OXT Modulates Immune Response and Neuroendocrine Function in Adult Mice

To combine the pharmacological evidence of a role of OXT in ameliorating specific C-section behavioural phenotype with the established link between the gut-brain axis, OXT and immune system (Varian et al., 2017), we assessed whether early-life treatment with OXT could modulate CS immune response in adulthood. Interestingly, when splenocytes from CS mice were challenged with LPS they produced significantly more TNFα than the splenocytes from VB mice (

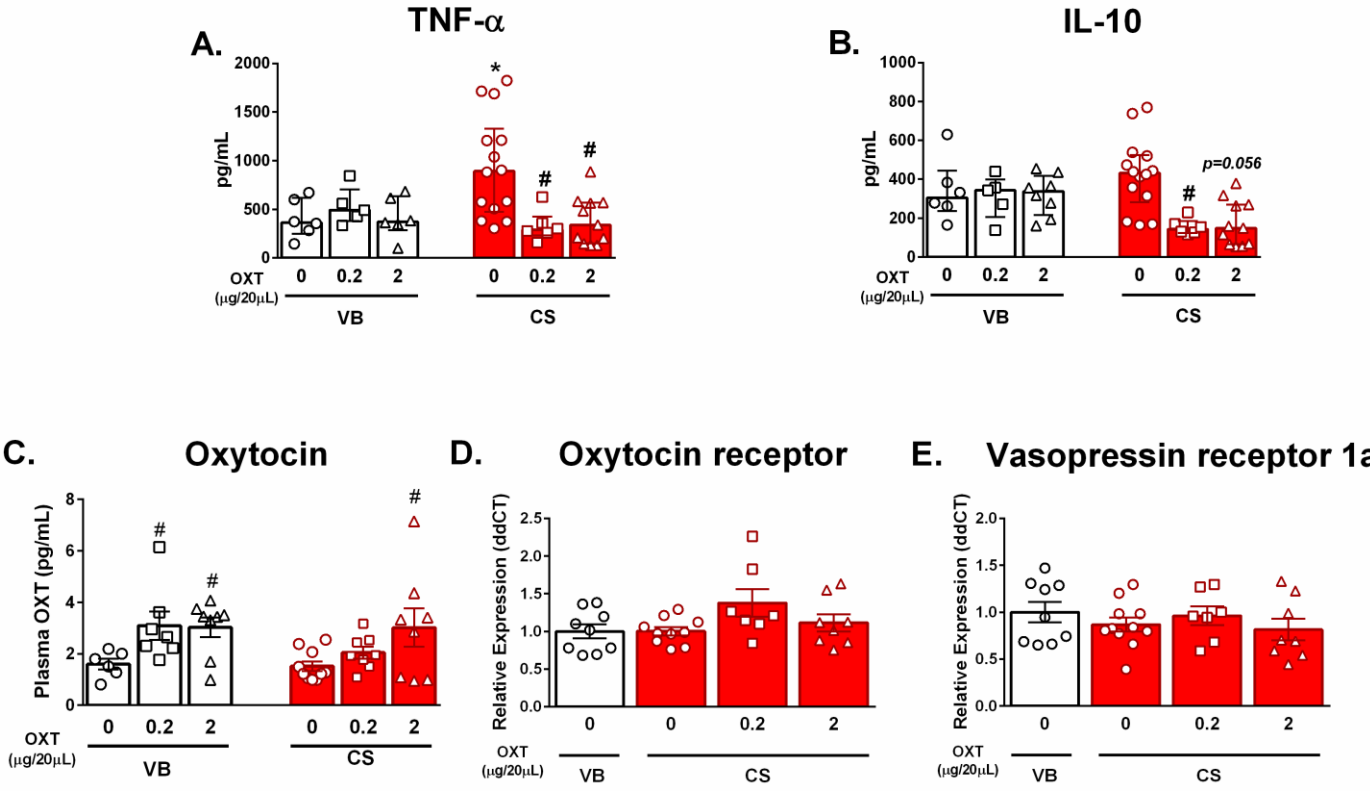


**Figure 3.** 5**a**). This aberrant immune response was attenuated by administration of low-dose and high-dose OXT early in life (**Figure 3. 5b**). Moreover, treatment with low-dose and high-dose OXT reduced IL-10 production in CS splenocytes.

OXT modulates immune system function through multiple approaches that include neuro-endocrine immune network (Wang et al., 2015). Here we showed that early-postnatal OXT can decrease baseline corticosterone levels in both VB and CS groups. However, OXT had no effect on HPA-axis response to an acute stressor **(Supplementary figure 3. 5).**

Decreased HPA-axis activity has been associated to alterations in OXT plasma levels (Grewen et al., 2010). Next, we assessed the long-term effects of the mode of delivery to the OXT system by measuring the concentration of OXT in the plasma of VB and CS adult mice. Although, there was no significant effect of the mode of delivery, a sustained increase in the peptide concentration was observed in VB and CS mice followed high-dose OXT administration in early in life (**Figure 3. 5c**). However, only VB group increased plasma OXT in response to low-dose OXT (**Figure 3. 5c**). In addition, we determine OXT and AVP1a receptor mRNA expression in the PVN of CS mice. However, no differences were detected between the groups **(Figure 3. 5d-e)**

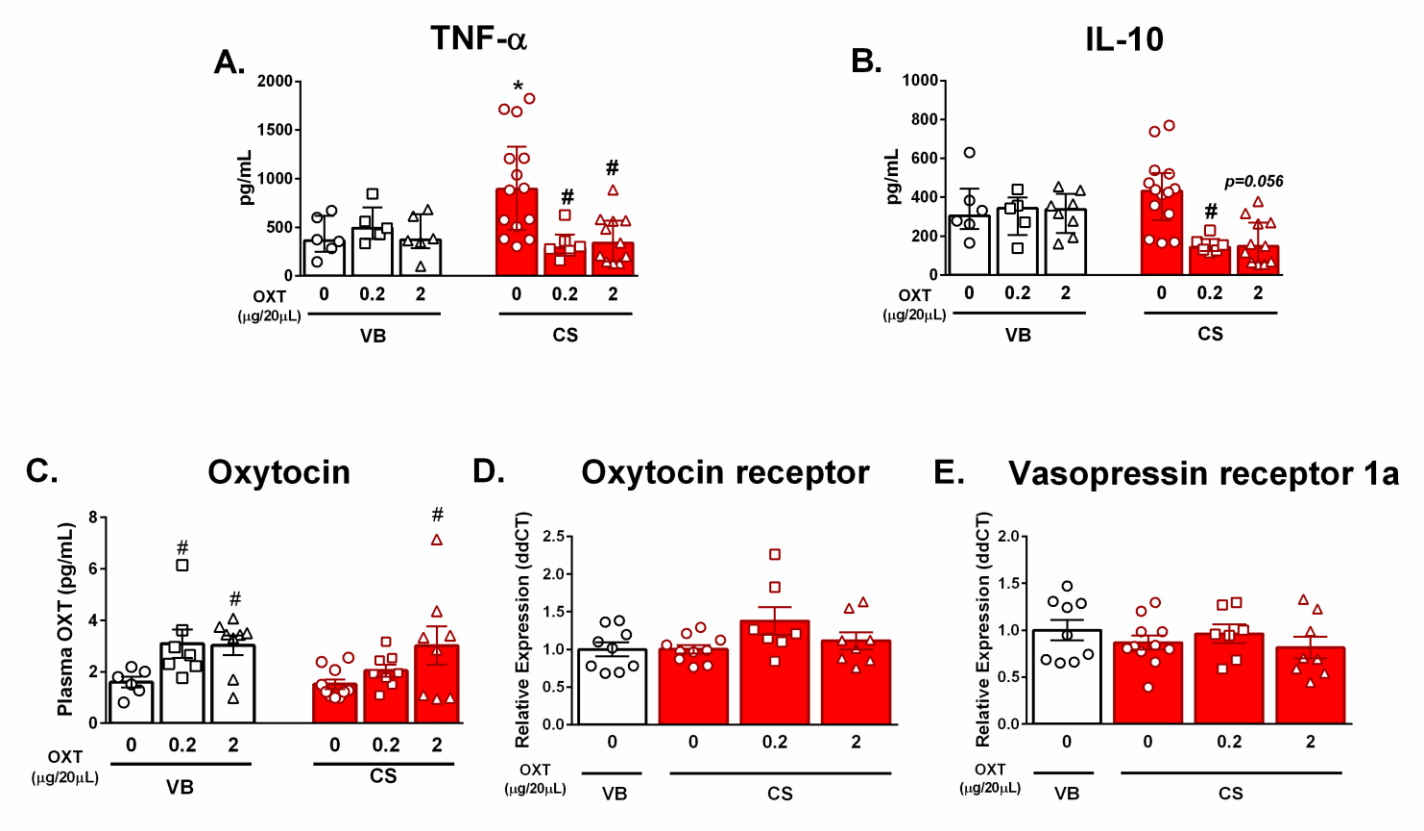



***Figure 3. 5- Early-life oxytocin administration induced long-lasting effects on neuro-endocrine-immune function.***

**(a)** Early-life OXT attenuates TNF-α secretion in CS splenocytes stimulated with LPS. Mode of delivery effect ($x^2$= 0.019; df=1; p=0.892) and treatment effect ($x^2$= 0.7.314; df=1; p=0.026) ([VB control n= 6, VB 0.2 OT n=5, VB 2 OT control n=6, CS control n=12, CS 0.2 OXT n=6, CS 2 OXT n=11]). **(b)** Early-life OXT decreases IL-10 in CS group. Mode of delivery effect ($x^2$= 1.820; df=1; p=0.117) and treatment effect ($x^2$= 22.805; df=1; p=0.003) ([VB control n= 6, VB 0.2 OT, n=5, VB 2 OT control n=8, CS control n=14, CS 0.2 OXT n=6, CS 2 OXT n=10]). Kruskal-Wallis test followed by Mann-Whitney

U test. Scatter dot-plots represent Median ± Interquartile range. **(c**) OXT administration in early-life increases OXT plasma levels in adulthood. Mode of delivery effect (F (1, 41) = 1.116, p=0.297); treatment effect (F (1, 41) = 5.893, p= 0.006); mode of delivery x treatment (F (2,41) = 0.886, p=0.420). ([VB control n= 6, VB 0.2 OT n=7, VB 2 OT control n=8, CS control n=10, CS 0.2 OXT n=8, CS 2 OXT n=8]). Two-way ANOVA, followed by LSD post-hoc**. d)** There were no significant effects on OXT receptor mRNA expression in the PVN. VB x CS **(**t=0.622; df=18; p=0.542). CS x CS + treatment (F (2,23) =0.850, p=0.440). ([VB control n= 9, CS control n=11, CS 0.2 OXT n=7, CS 2 OXT n=8]). **(e)** There were no significant effects on AVP1a receptor mRNA expression in the PVN. **(**t=1.018; df=18; p=0.322), CS x CS + treatment (F (2,23) = 0.531, p=0.595). ([VB control n= 9, CS control n=11, CS 0.2 OXT n=7, CS 2 OXT n=8]).**d-e**) Student's t -test and One-way ANOVA. **c-e)** Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.) Male offspring in each cohort derived from three independent litters/ group. *p<0.05 for mode of delivery effect. $^{\#}$p< 0.05 for treatment effect within the group. OXT, oxytocin; VB, vaginal birth; CS, C-section.

## 3.5 Discussion

It is well established that birth by C-section results in a different pattern of microbiota seeding early in life(Dominguez-Bello et al., 2010; Hill et al., 2017; Martinez et al., 2017; Penders et al., 2006). These alterations are critically implicated in aberrant development of the microbiota-gut-brain axis, resulting in long-term neurobehavioural consequences (Morais et al., 2017). Recent discoveries point to a pivotal a role for OXT in regulating the microbiota-gut-brain axis, as well as general well-being through its influence on neuro-immune-endocrine pathways (Erdman, 2016). Here we demonstrate that pharmacological manipulation of the OXT system during the early postnatal period improves social, anxiety, endocrine and immune responses later in life in the C-section model of early-life disruption of gut microbiota.

One of the main findings of this study is that early-life treatment with OXT prevented adult CS offspring from immune hyper-reactivity and decreased the production of both pro- (TNFα) and anti-inflammatory IL-10 cytokine production in splenocytes stimulated with LPS. Alterations in the initial microbial acquisition due to C-section has been previously associated with abnormal immune responses in humans (Cho and Norman, 2013) and in mouse models (Hansen et al., 2014). On the other hand, OXT has emerged as key factor regulating immune homeostasis through several different neuro-immune-endocrine networks(Varian et al., 2017; Wang et al., 2015) . Here we observed a reduction in basal HPA-axis activity in adult mice that received OXT early in life as indicated by **Supplementary figure 3. 5a**. However, there were no differences in the corticosterone response to acute stress in these animals (**Supplementary figure 3. 5b**). In addition, OXT is known to be involved in immune system maturation and by reinforcing immunotolerance by activation of a subset of CD4+Foxp3+CD25+ regulatory T cells (Tregs) (Poutahidis et al., 2013). Interestingly, some early-life bacteria colonizers that are decreased in the mouse model of C-section (i.e. Bifidobacteria, Bacteroides, Lactobacillus) (Morais et al., 2017) are known to promote Foxp3(+) Treg development (Hansen et al., 2014; Johansson et al.,

2012; Round and Mazmanian, 2009). Thus, the relative contribution of such OXT-microbiota-immune interactions to the behavioural effects observed should be explored in future studies. Another noteworthy aspect is that OXT can modulate immune system maturation by activation of OXTR and AVPR present in the PVN (Wang et al., 2015) and in immune organs such as the thymus and spleen (Elands et al., 1990). However, mRNA expression of OXTR and AVPR in the PVN were not affected by C-section model suggesting this may not be the mechanism of immune dysfunction at play.

OXT, and the closely related AVP system, are fundamental for establishing maternal-offspring attachment and social behaviour in mammals (Carter, 2003; Donaldson and Young, 2008; Insel, 2003; Johnson and Young, 2017). The most consistent finding in relation to behavioural changes is that postnatal treatment with OXT reversed C-section-mediated social recognition deficits. At P10, CS offspring failed to show preference for a familiar nest which was completely restored by the high-dose of OXT. Importantly, the C-section-associated attachment deficits are not due to differences in maternal care received by the foster dam. Interestingly, OXT treatment applied to the offspring increases maternal care in all groups, supporting the role of OXT in strengthening maternal-offspring bonds (Insel and Young, 2001). Furthermore, early orientation to specific odour cues is critical to mammalian offspring survival and to social behaviour developmental trajectories (Hammock, 2015). As expected, adult CS offspring showed deficits in social recognition and social novelty preference which was fully restored by low-dose OXT treatment early-in-life. Our laboratory has previously demonstrated that C-section alters OXTR mRNA expression in the amygdala (Morais et al., 2017), an important brain region involved in recognition of socially relevant cues (Ferguson et al., 2001). Here we extended the investigation to the effects of the delivery mode on peripheral levels of OXT (in plasma) and on OXTR and AVPR mRNA expression in the PVN. Although mode of delivery did not affect plasma OXT or PVN expression of OXTR and AVPR in the PVN, postnatal pharmacological manipulation of the OXT system increased the peptide plasma levels in adulthood in both VB and CS groups. These results are in line with recent studies

demonstrating long-term pro-social behavioural effects and neuropeptide system alterations in rodents following OXT system manipulation during the developmental phase (Bales and Perkeybile, 2012; Meziane et al., 2015; Mogi et al., 2014; Peñagarikano et al., 2015; Schaller et al., 2010).

As previously shown, CS offspring exhibited enduring behavioural effects on anxiety-like behaviours in the MB, EPM and aversive OF tests. In addition to its role on social behaviour, OXT has been associated with anxiolytic and anti-stress effects in the brain (Neumann and Slattery, 2016). Here we demonstrated that postnatal treatment with a low-dose of OXT reduced anxiety in the marble burying test but not in the EPM or in the OF. Although these three behavioural models evaluate the tendency of mice to engage in exploratory behaviour in an aversive space (Crawley, 1985), the MB is also widely thought as a measure of repetitive behaviour (Silverman et al., 2010a). This suggests the possibility that postnatal OXT may be differentially acting in neuronal mechanisms and circuits that are involved in obsessive-compulsive-behaviours which may be also be relevant to ASDs. In this study, some tests of the anxiety- and stress-related behaviours did not reveal differences between CS and VB groups (**Supplementary figure 3. 2, Supplementary figure 3. 3b** and **Supplementary figure 3. 5b**). It may be that these measures are more sensitive to handling effects, masking differences between the groups. Handling pups during the 1$^{st}$ week after birth is reported to affect the quality of maternal care received and can have long-lasting behavioural effects to the offspring (Bales and Perkeybile, 2012; Villescas et al., 1977).

In this study, we observed an interesting physiological effect of postnatal OXT on restoring gastrointestinal transit in CS adult mice without compromising gut permeability. To date, only a few studies have investigated the role of OXT in the gut and OXT/OXTR signalling in the enteric nervous system. These studies have demonstrated that OXT slows gastrointestinal motility, protects the intestinal barrier from inflammation and decreases macromolecular permeability (Welch et al., 2014). Although speculative, the restoration of the gastrointestinal transit could reflect an indirect developmental effect of OXT in the

microbiota-gut-brain axis network. Additional investigation into the role of postnatal OXT on the development of the gastrointestinal system in C-section mice would be of value.

The results obtained from this study demonstrate that there is an early developmental window sensitive to manipulations of the OXT system, which may be capable of preventing some of the lifelong behavioural and physiological impairments induced by the mode of delivery. Interestingly, the higher dose of OXT was more effective in preventing the CS-mediated effects in early postnatal days whereas the low dose of OXT was associated with long-term changes. A similar dose response curve has been reported in several studies involving OXT and other peptides (Bales and Perkeybile, 2012; Popik et al., 1992). A better understanding of the dynamic role of the OXT system during development would be required for the interpretation of these results. It is worth noting that exogenous exposure to OXT early in life can interact with receptor systems beyond OXT and AVP (Bales and Perkeybile, 2012). In the present experiments, OXT was given subcutaneously and resulted in changes in CNS function. Although the passage of peptides through the blood-brain barrier is still not well understood, the dose and method of administration employed for this study, was previously reported to have effects within the brain (Meziane et al., 2015).

There is a growing interest in manipulation of the OXT system for potential therapeutic use. Our study opens an avenue for the investigation on how manipulation OXT system influences the wiring of the gut-brain axis when the normal seeding of the microbiome has been disrupted. Although the translation of findings in animal models are limited, OXT is present in human breast milk (Takeda et al., 1986) and breast-feeding initiation may also be adversely affected by C-section (Hobbs et al., 2016). As a rise in unplanned C-section deliveries are a global trend, the data from this study suggest that targeting OXT in parallel with re-establishment of the gut microbiome may serve as a novel and worthy target for improving health outcomes associated with the procedure.

## 3.6 Supplementary Material

### 3.6.1 Supplementary Materials and Methods

#### 3.6.1.1 *Maternal Care*

Maternal care was assessed at P6 in order to exclude possible cross-fostering confounding effects. Home cages were transported to the experimental room 30 minutes prior to the experiment. The behaviour of each dam was monitored for 30 minutes in the morning in their home cage. The time spent on nursing, pup licking/grooming, carrying and time on-nest were collectively considered as high care behaviours. The data is presented as time engaged in high care behaviours.

#### 3.6.1.2 *Isolation-induced Ultrasonic Vocalization*

Ultrasonic vocalizations (USV) are produced by mice pups during the first two weeks of life when separated from the mother and littermates (Insel et al., 1986). At P9 pups were isolated one by one and placed in a clean plastic container inside a sound attenuating chamber. Emission of ultrasonic vocalizations was monitored by an ultrasound sensitive microphone (US Mini-3 bat detector, Summit, Birmingham, USA) tuned in the range of 60-80 kHz and suspended above the isolated pup. The microphone was connected via an Ultravox Noldus automated system to a personal computer and the recordings were carried out with a sampling range of 70 kHz for 3 minutes. The recordings were analysed using computer analysis software (UltraVox 2.0; Noldus Information Technology). The number of USVs during the test period was recorded.

#### 3.6.1.3 *Forced-swim Test (FST)*

Depression behaviour was used as a mild acute stressor as previously described (Robertson et al., 2017). In this test, mice were placed into a cylinder of tepid water (23-25 $^{o}$C) to a depth of 17 cm for 6 min. Behaviour was recorded by a camera positioned above the swim tank. After removal from the cylinder, animals were placed into a separate cage for recovery. The immobility time was blinded scored during the last 4 min. by two investigators.

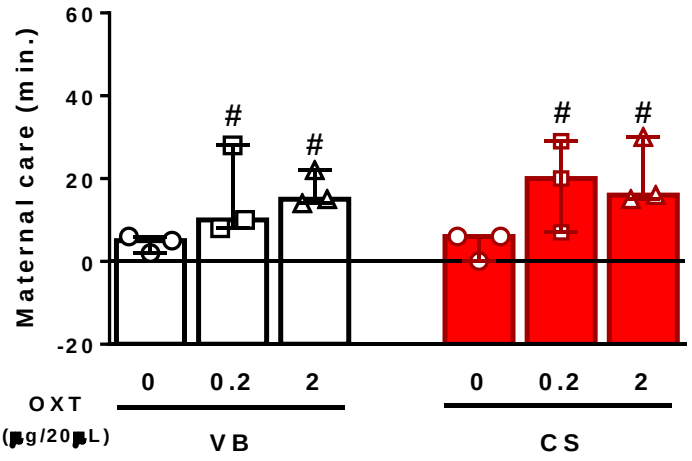
#### 3.6.1.4 *HPA Axis Response*

Blood and subsequent plasma samples were taken to assess the HPA axis response to a mild acute stress. A blood sample was taken at a baseline time point at week 13 and 30 min after the FST onset at week 15. Blood samples (50-70µl) were taken from the tail vein and placed in heparin-coated capillary tubes. Bleeding was performed in a separate room. Total corticosterone was measured according to the manufacturer's protocol, Corticosterone ELISA kit (Enzo Life Sciences, Farmingdale). Plasma dilution was 1:40.

#### 3.6.1.5 *In Vivo Intestinal Permeability Assay (FITC-dextran)*

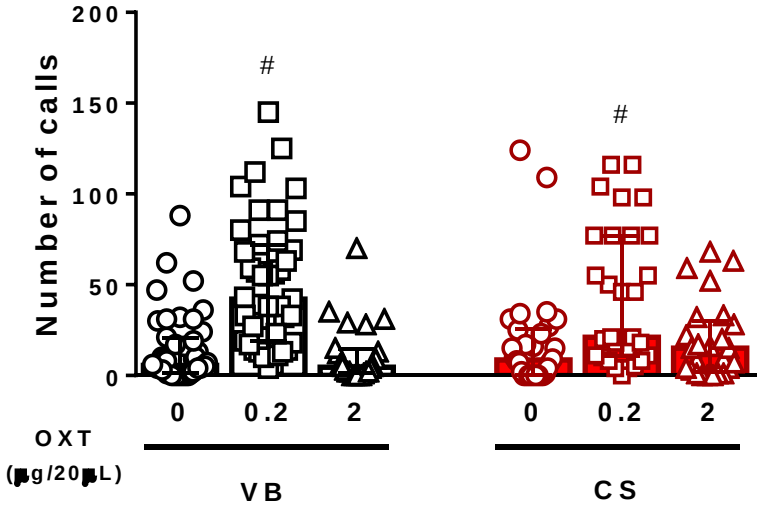
Intestinal permeability was indirectly measured by plasma concentration of FITC-dextran as described before (Golubeva et al., 2017). Following overnight fasting, mice were orally gavaged with 600 mg/kg FITC-dextran (FD4, Sigma) in PBS. Blood was taken from the tail vein 2h after the gavage. FITC was measured in plasma at 490 nm excitation/520 nm emission wavelengths. For a standard curve, serial dilutions of FITC were prepared in PBS.

## 3.7 Supplementary Results

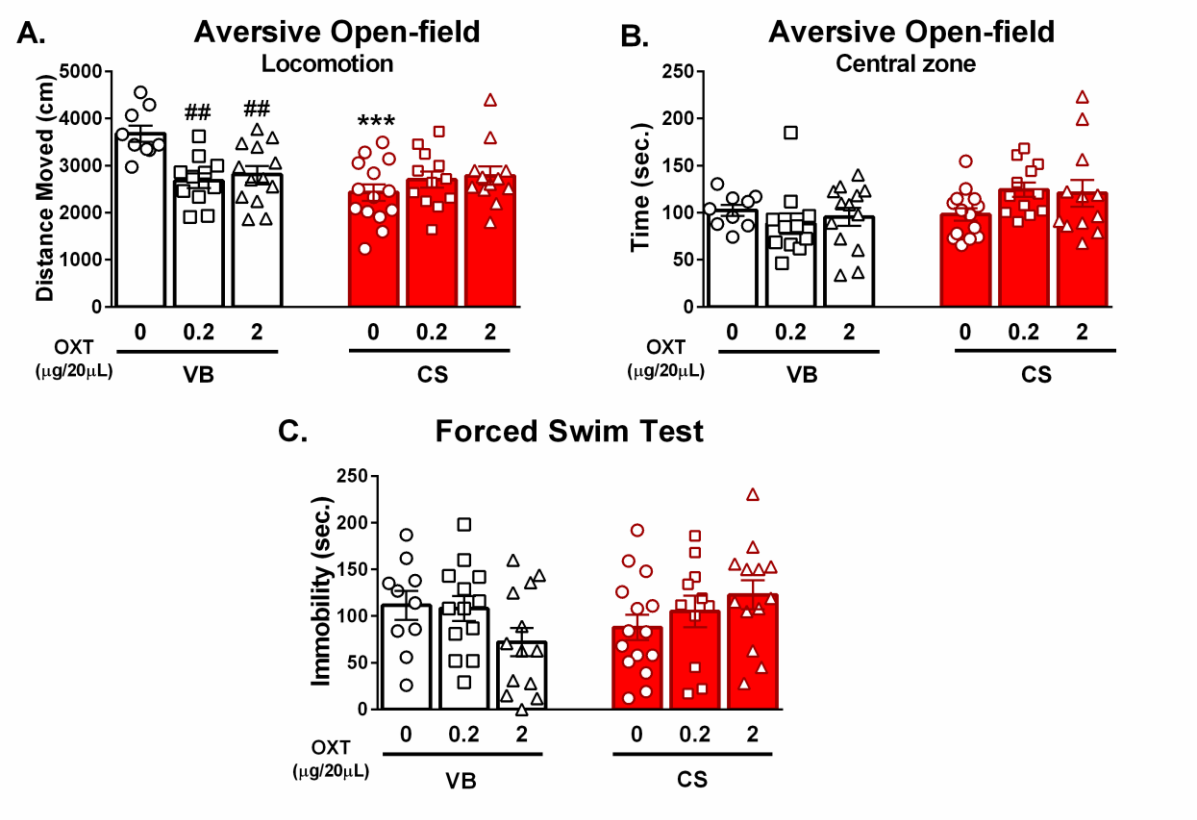



***Supplementary figure 3. 1- Mode of delivery did not affect time spent on maternal care behaviours.***

To exclude the possibility that differences in offspring phenotype investigated in this study are due to variation in maternal care per se, we assessed maternal care behaviour on P6. There was no significant effect of the mode of delivery ($x^2$= 0.282; df=1; p=0.595). However, when pups were treated with OXT there was a significant increase on the time the mother spent engaged in high care behaviours across all the groups ($x^2$= 11.615; df=2; p=0.003). n=3 dams per group. Kruskal-Wallis test followed by Mann-Whitney U test. Scatter dot-plots represent Median ± Interquartile range. $^{\#}p< 0.05$ for treatment effect within the group. OXT, oxytocin; VB, vaginal birth; CS, C-section.

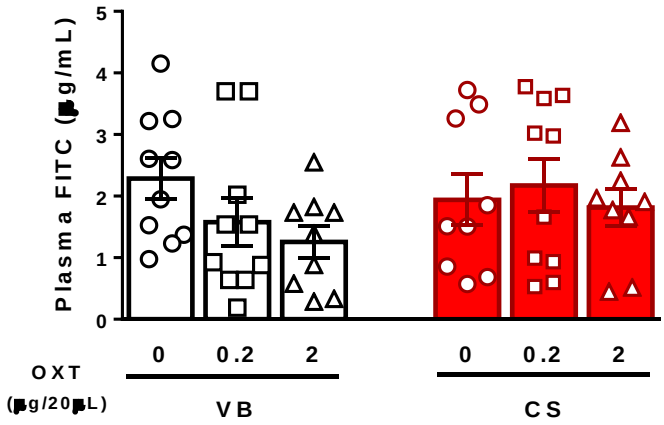



***Supplementary figure 3. 2- Low-dose OXT increases the number of calls followed isolation-induced USV.*** Isolation-induced USV was measured at P9. There was no significant effect of the mode of delivery ($x^2$= 0.034; df=1; p=0.853). However, when pups were treated with low-dose OXT there was a significant increase on the number of calls emitted in both birth conditions ($x^2$= 51.080; df=2; p=0.0001). ([VB control n= 44, VB 0.2 OT n=43, VB 2 OT control n=24, CS control n=30, CS 0.2 OXT n=29, CS 2 OXT n=25]). Male and Female offspring. Kruskal-Wallis test followed by Mann-Whitney U test. Scatter dot-plots represent Median ± Interquartile range. $^{\#}p< 0.05$ for treatment effect within the group. OXT, oxytocin; VB, vaginal birth; CS, C-section.



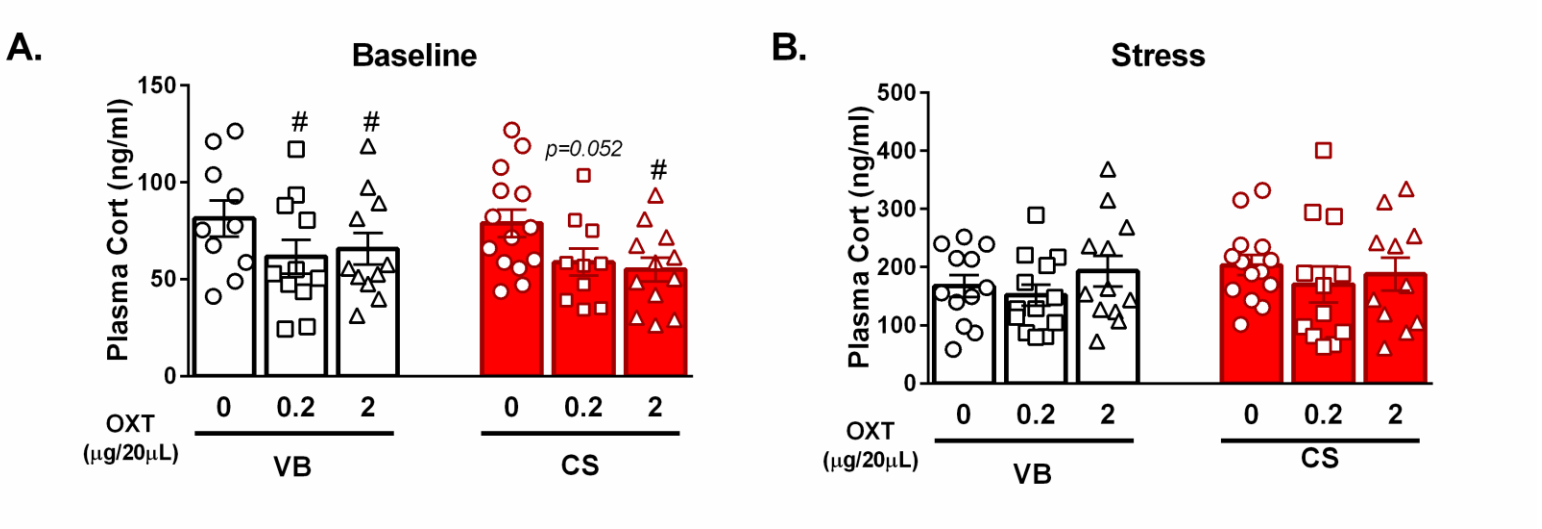

***Supplementary figure 3. 3- Effects of postnatal OXT administration on the aversive open-field and on the forced-swim test.***

**a)** Early postnatal treatment did not reverse C-section-mediated effects on the total distance travelled in an open-field arena. Mode of delivery effect (F (1, 64) = 7.296, p=0.009); treatment effect (F (2, 64) = 2.291, p= 0.109); mode of delivery x treatment (F (2,64) = 8.538, p=0.001) ([VB control n= 9 VB 0.2 OT n=11, VB 2 OT control n=12, CS control n=14, CS 0.2 OXT n=12, CS 2 OXT n=12]). **b)** There were no significant effects on time spent in central zone of an aversive-open field. Mode of delivery effect (F (1, 64) = 5.994, p=0.017); treatment effect (F (2, 64) = 0.771, p= 0.261); mode of delivery x treatment (F (2,64) = 0.2.255, p=0.113) ([VB control n= 9 VB 0.2 OT n=11, VB 2 OT control n=12, CS control n=14, CS 0.2 OXT n=12, CS 2 OXT n=12]). **c)** There were no significant differences in the FST. Mode of delivery effect (F (1, 74) = 0.881, p=0.351); treatment effect (F (2, 74) = 0.295, p= 0.745); mode of delivery x treatment (F (2,74) = 2.300, p=0.107). ([VB control n= 12, VB 0.2 OT n=14, VB 2 OT control n=13, CS control n=16, CS 0.2 OXT n=12, CS 2 OXT n=13]). Male offspring. Two-way ANOVA, followed by LSD post-hoc. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Male offspring in each cohort derived from three independent litters/ group. ***p<0.0001 for mode of delivery effect. $^{\#\#}$p< 0.01 for treatment effect within the group. OXT, oxytocin; VB, vaginal birth; CS, C-section.




***Supplementary figure 3. 4- Effects of early-life treatment with OXT to gastrointestinal permeability.***

There was no significant effect of mode of delivery or OXT treatment in gastrointestinal permeability as measured by plasma Fluorescein isothiocyanate (FITC)-dextran concentration (µg/mL). Mode of delivery effect (F (1, 74) = 3.449, p=0.068); treatment effect (F (2, 74) = 0.178, p= 1.774); mode of delivery x treatment (F (1, 74) = 0.3291, p=0.721). ([VB control n= 12, VB 0.2 OT n=10, VB 2 OT n=10, CS control n=14, CS 0.2 OXT n=11, CS 2 OXT n=10]). Male offspring. Two-way ANOVA, followed by LSD post-hoc. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Offspring in each cohort derived from three independent litters/ group. Two-way ANOVA, followed by LSD post-hoc. *p< 0.05, *p< 0.01 and ***p<0.0001. OXT, oxytocin; VB, vaginal birth; CS, C-section.




***Supplementary figure 3. 5- Early postnatal OXT restores social novelty preference in adult C-section born mice.***

***a)*** There were no significant differences on sociability across all groups in the three-chamber test. VB control t (11) = -3893, p=0.003, n=12; VB 0.2 OXT t (12) =-6144, p<0.0001, n=13; VB 2 OXT t (11) =5.066, p<0.0001, n=12; CS control t (11) = 6.298, p=0.0001, n=12; CS 0.2 OXT t (11) = 5.395, p<0.0.001, n=12; CS 2 OXT t (11) = 5.118, p<0.0001, n=12. Paired Student's t test comparing interaction time with mouse to an object. **b)** Low-dose oxytocin rescues the preference for social novelty in CS. VB control t (11) = 4.681, p=0.001, n=12; VB 0.2 OXT t (11) =3.064, p=0.011, n=12; VB 2 OXT t (10) =3.404, p=0.007, n=11; CS control t (11) = 0.594, p=0.564, n=12; CS 0.2 OXT t (11) = 3.671, p=0.004, n=12; CS 2 OXT t (10) = 0.970, p=0.355, n=12. Male offspring. Paired Student's t test comparing interaction time with a familiar mouse to a novel mouse. Scatter dot-plots represent Mean ± Standard Error of the Mean (S.E.M.). Offspring in each cohort derived from three independent litters/ group. #p< 0.05 for treatment effect within the group. VB, vaginal birth; CS, C-section.

# Chapter 4. Strain Differences in the Susceptibility to Gut-Brain Axis and Neurobehavioural Alterations Induced by Maternal Immune Activation in Mice

**Livia H. Morais**[1,2], Daniela Felice[1], Anna V. Golubeva[1], Gerard Moloney[1,2], Timothy G. Dinan[1,3], John F. Cryan[1,2*]

[1] APC Microbiome Institute, University College Cork, Cork, Ireland

[2] Department of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3] Department of Psychiatry and Neurobehavioural Science, University College Cork, Cork, Ireland

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*Corresponding author:

John F. Cryan

j.cryan@ucc.ie

## 4.1 Abstract

There is a growing realisation that the severity of the core symptoms of autism spectrum disorders and schizophrenia are associated with gastrointestinal dysfunctions. Nonetheless, the mechanisms behind such comorbidity remain unknown. Several genetic and environmental factors have been linked to a higher susceptibility to neurodevelopmental abnormalities. The maternal immune activation (MIA) rodent model was shown to be a valuable tool for elucidating the basis of this interaction. We induced maternal immune activation (MIA) with polyinosinic-polycytidylic acid (poly I:C) at gestational day 12.5 and assessed behavioural, physiological and molecular aspects relevant to the gut-brain axis in the offspring of an outbred (NIH Swiss) and an inbred (C57BL6/J) mouse strain. Our results showed that the current MIA protocol induces social deficits in both strains. However, alterations in anxiety and depression-like behaviours were more pronounced in NIH Swiss mice. These strain-specific behavioural effects in the NIH Swiss mice were associated with marked changes in important components of gut-brain axis communication: the endocrine response to stress and gut permeability. In addition, changes in vasopressin receptor 1a mRNA expression in the hypothalamus were observed in NIH Swiss mice only. Taken together, these data suggest that genetic background is a critical factor in susceptibility to the gut-brain axis effects induced by MIA.

**Key-words**

Gut-brain axis; poly (I:C); maternal immune activation; autism spectrum disorders; schizophrenia; gut permeability; vasopressin; gene-environment; behaviour.

## 4.2 Introduction

A growing body of evidence suggests that exposure to maternal immune activation (MIA) during pregnancy can increase the risk of neuropsychiatric disorders such as autism spectrum disorder (ASD) and schizophrenia (SZ) (Atladóttir et al., 2010; Brown, 2006; Estes and McAllister, 2016). Epidemiological studies suggest that there is a specific time window in the second trimester of gestation when infection and associated inflammation leads to long-lasting consequences on brain development and behaviour (Patterson, 2002). In addition, neurodevelopmental disorders such as ASD and SZ have a significant genetic basis (van Os et al., 2010; Ronald et al., 2011; Ziats et al., 2015). Epidemiological data from twin studies suggests strong heritability associated with ASD and SZ. Moreover, genetic alterations such as rare *de novo* mutations, microdeletions, cytogenetic abnormalities, and single nucleotide polymorphisms are shown to trigger disease-related symptoms (Turner et al., 2000). Hence, understanding the interactions between genetic and environmental factors is important in unravelling the neurobiology of neurodevelopmental disorders (Chaste and Leboyer, 2012; Stamou et al., 2013).

In view of the emerging role of prenatal infection as an environmental factor implicated in the aetiology of ASD and SZ, translational animal models based on MIA have been shown to be valuable tools for elucidating disease mechanisms and for developing new therapeutic approaches for the treatment of these conditions (Knuesel et al., 2014; Reisinger et al., 2015). In this regard, administration of influenza virus, lipopolysaccharide (LPS, a cell wall component of gram negative bacteria) and polyinosinic-polycytidylic acid (poly (I:C); a synthetic double-stranded RNA) to pregnant dams have been used (Basta-Kaim et al., 2012; Hsiao et al., 2013; Lombardo et al., 2017; Meyer, 2014; Oskvig et al., 2012; Shi et al., 2003; Straley et al., 2017).

In particular, the rodent poly (I:C) model of MIA has contributed significantly to the understanding of specific changes that occur in ASD and SZ. For example, poly (I:C) given during the second gestational trimester results in decreased pre-pulse inhibition and increased startle sensitivity (Meyer et al., 2005; Wolff and Bilkey, 2010), reduced sociability (Xuan and Hampson, 2014), cognitive impairments (Ozawa et al., 2006),

communication deficits, repetitive behaviours (Malkova et al., 2012), altered gut permeability and perturbations of the gut microbiota (Hsiao et al., 2013).

Of the comorbidities associated with ASD and SZ, gastrointestinal (GI) dysfunctions are among the most prevalent (Buie et al., 2010; Molloy and Manning-Courtney, 2003). The GI symptoms identified include increased GI permeability (Severance et al., 2012, 2015), altered intestinal motility (Nikolov et al., 2009) and changes in intestinal microbiota composition (Dinan et al., 2014). Importantly, GI disturbances have also been associated with disease severity, including the development of emotional dysregulation, which is a major determinant of quality of life (Hill et al., 2017; Mazurek et al., 2013). Given that alterations in gut-brain signalling during development have a significant impact on programming of the brain and behavioural function (Dinan and Cryan, 2017a; Neufeld and Foster, 2009), there is an urgent need to better understand the GI associated neurobehavioral symptoms ASD and SZ (Hsiao, 2014; Nithianantharajah et al., 2017).

Previous studies using the poly (I:C) model have mainly focused on a single mouse strain (Babri et al., 2014; Schwartzer et al., 2013). Although inbred strains are commonly used in biomedical research, they tend to be considerably more expensive than outbred lines (Peters and Festing, 1985). In contrast, outbred stocks are often used in toxicology and pharmacology research (Chia et al., 2005). As defined by (Festing, 1993) they are a closed population (for at least four generations) of genetically variable animals that are bred to maintain maximum heterozygosity. Of the many outbred strains available, National Institutes of Health (NIH) Swiss mice are a widely used albino strain (derived from a nucleus colony obtained from the National Institutes of Health, Bethesda, Maryland , USA in 1935, Envigo) that has been particularly important in establishing the effects of gut-brain interactions and neurodevelopmental disorders in germ free models (Bercik et al., 2011b; Hoban et al., 2016b; Luczynski et al., 2016b)

In this study we investigated whether genetic background could influence the effect of maternal immune activation on important pathways of the gut-brain axis and behaviour in inbred and outbred mice. We hypothesised that MIA would differentially affect behavioural, neuroendocrine function and gut physiology in the NIH Swiss mice in comparison to C57BL6/J. To this end, NIH Swiss and C57BL6/J dams were

exposed to poly (I:C) on GD 12.5 and behavioural, physiological and molecular parameters were assessed in the offspring in early-life, adolescence and adulthood.

## 4.3 Experimental Procedures

### 4.3.1 Animals

C57BL/6J and NIH Swiss adult mice were obtained from Harlan. The animals were kept under a 12 h light/dark cycle with lights on at 07:30 am and off at 7:30 pm. Mice were kept under controlled temperature and humidity (21±1 $^{o}$C, 55.5%) and food and water *ad libitum*. Animals were housed in breeding pairs and the presence of a vaginal plug was set as gestational day (G)0.5. Pregnant females were left undisturbed except for cage cleaning until G12.5, when they were weighed and randomly assigned to one of two treatments: poly (I:C) or saline. Each group contained three to five pregnant females. All pups remained with the mother until weaning at postnatal (P) day 21 when male mice were housed in same-sex littermate groups containing three to four mice. No significant differences in the average litter size at birth, spontaneous abortion rate, and postnatal vitality between groups were found. Strain differences in body weight were observed.

### 4.3.2 Prenatal Immune Activation

Pregnant dams received either a single intraperitoneal (i.p.) injection of 20 mg/kg poly (I:C) (potassium salt; Sigma-Aldrich, Sigma, St. Louis, MO) or saline on G12.5 (Hsiao et al., 2013).

### 4.3.3 Experimental Design

All behavioural tests were performed during the light phase of the circadian cycle (9:00 to 17:00). All experimental mice underwent the same sequence of behavioural procedures. Maternal care behaviour was observed on P6, in order to ensure that differences in the offspring were not due to differences in maternal care during postnatal development. Behavioural analyses of MIA offspring were carried out in

male mice throughout the life-span, starting on P9 and continuing into adulthood (week 16) (**Figure 4. 1**).

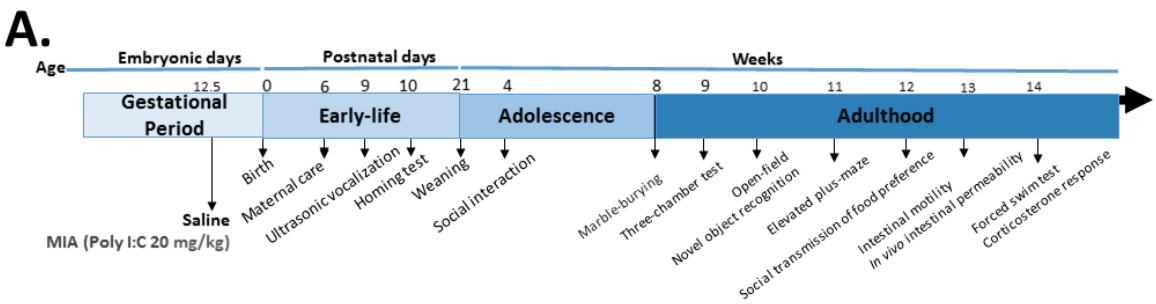



***Figure 4. 1- Experimental time line.***

A graphical representation of the treatment with poly (I:C) or saline on gestational day 12.5 and the sequence of behavioural testing performed in the offspring.

## 4.3.4 Behavioural Assessments

### *4.3.4.1 Maternal Care*

Maternal care was assessed at P6. Home cages were transported to the experimental room 30 minutes prior to the experiment. The behaviour of each dam was monitored for 30 minutes in the morning in their home cage. The time spent on nursing, pup licking/grooming, carrying and time on-nest were collectively considered as high care behaviours. The data is presented as time engaged in high care behaviours.

### *4.3.4.2 Isolation-induced Ultrasonic Vocalization*

Ultrasonic vocalizations (USV) are produced by mice pups during the first two weeks of life when separated from the mother and littermates (Insel et al., 1986). At P9 pups were isolated one by one and placed in a clean plastic container inside a sound attenuating chamber. Emission of ultrasonic vocalizations was monitored by an ultrasound sensitive microphone (US Mini-3 bat detector, Summit, Birmingham, USA) tuned in the range of 60-80 kHz and suspended above the isolated pup. The microphone was connected via an Ultravox Noldus automated system to a personal computer and the recordings were carried out with a sampling range of 70 kHz for 3 minutes. The recordings were analysed using computer analysis software (UltraVox

2.0; Noldus Information Technology). The number of USVs during the test period were recorded.

#### *4.3.4.3 Homing Test*

The homing test evaluates the ability of pups to recognise their own nest and is an indirect measure of maternal-offspring attachment behaviour (Macrì et al., 2010). At P10 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 with clean bedding material. Individual pups were placed in the middle section for 1 minute, the dividers were then removed and the pups were allowed to freely move around for 2 minutes. Total time spent in each area was noted.

#### *4.3.4.4 Reciprocal Social Interaction*

Reciprocal social interaction was performed based on (Peñagarikano et al., 2015) At week 4, animals were acclimatized to the experiment room for 30 minutes prior to the test. The reciprocal social interaction consisted of two sessions of 10 minutes: the first 10 minutes were considered a habituation session and the subsequent 10 minutes were considered the actual test session. Mice were placed in a new cage and allowed to interact with an unfamiliar mouse matched in strain, age and sex. The time spent in active social interaction (nose-to-nose sniffing, nose-to-anus sniffing and following/crawling on each other) was measured during both sessions.

#### *4.3.4.5 Defensive Marble Burying*

The defensive marble burying test measures repetitive and anxious behaviour, with a higher number of marbles buried indicating higher levels of anxiety or stereotyped behaviour (Thomas et al., 2009). Clean cages were filled with a 4-cm layer of chipped cedar wood bedding. At week 8 animals were habituated to the room for 30 minutes prior to the test. Twenty glass marbles (15mm diameter) were gently laid on top of the

bedding, equidistant from each other in a 4×5 arrangement. During the testing phase, each mouse was placed in the cage and allowed to explore for 30 minutes. At the end of the test, animals were placed back into the testing cage and the number of buried marbles were counted. A marble was considered as fully buried when > 80% of the marble was covered by bedding material and considered to be partially buried when 50-80% of marble was covered by bedding. Total buried marbles was calculated as the sum of fully buried and partially buried marbles.

#### *4.3.4.6 Three-chamber Test*

The three-chamber test is a widely used experimental paradigm to investigate social approach, measuring the tendency of the subject mouse to approach another mouse and engage in social investigation (Crawley, 2007). At 9 weeks of age, animals were placed in a rectangular apparatus divided into three chambers (left and right and a smaller centre chamber) by transparent partitions with small circular openings allowing easy access to all compartments. The test was composed of three sequential 10 minute trials: (1) habituation (test animal allowed to freely explore the three empty compartments); (2) sociability (an unfamiliar animal was placed in an inner mesh wire cage in either the left or right compartment); (3) social novelty preference (a novel animal was placed into the previously empty inner cage in the chamber, opposite the now familiar animal). All animals were strain, age- and sex-matched, with each chamber cleaned and lined with fresh bedding between trials. For each of the three stages, behaviours were recorded by a video camera mounted above the apparatus and time of active interaction was measured.

#### *4.3.4.7 Novel Object Recognition*

At 10 weeks mice were tested in the novel object recognition test, a widely used test to assess recognition memory in rodents. On day 1, animals were habituated to a square open field arena (Perspex sides and base: 34.5 x 42.7 cm) in a dimly lit room by individually placing animals into the apparatus for 10 minutes. On day 2, two identical objects were positioned on adjacent corners approximately 5 cm from each wall of the open field and each animal was introduced for a 10-minute exploration period. Animals were then placed directly back into their home cages. After a 24hr inter-trial

interval, one familiar object was replaced with a novel object and each animal was introduced for a further 10 minutes. On each day, animals were acclimatised to the testing room for approximately 30 minutes prior to the test. Object exploration was defined as when the animal's nose came within 1 cm from the object. In between trials, objects and testing arenas were cleaned with alcohol wipes and rinsed with water. A discrimination index was calculated as the difference between time spent investigating the novel and familiar objects, divided by the total time exploring both objects [Discrimination index= (Novel Object Exploration time – Familiar Object Exploration time)/(Novel Object Exploration time + Familiar Object Exploration time)].

#### *4.3.4.8 Open-field*

The open field test is used to assess locomotor activity and the anxiety response to a novel environment. At 10 weeks, mice were placed in the centre of a grey open field arena (40 x 32 x 23 cm, L x W x H) and allowed to explore the arena for a 10 minute period. The total distance travelled in the open field was measured using the Ethovision videotracking system (Noldus Information Technology). A centre zone was demarcated as (20 x 15 cm, L x W). Time spent in each zone and the frequency of entries into each zone were also measured. Mice were placed back into the home cage immediately after testing. The box was cleaned with 10% alcohol and allowed to dry prior to the next test.

#### *4.3.4.9 Elevated Plus Maze*

The elevated plus maze (EPM) is a widely used behaviour assay to assess anxiety-like behaviour (Jacobson et al., 2007). The Plexiglas maze consisted of a plus-shaped apparatus with two open and two enclosed arms (50 cm × 5 cm × 15 cm walls) elevated 1m above the floor. At week 11, the animal was placed in the centre of the EPM apparatus facing an open arm and was allowed to explore for a total of 6 minutes. The apparatus was cleaned with ethanol after each subject to prevent olfactory cues from the previous mouse. The time spent into open and closed arms was considered.

#### *4.3.4.10 Social Transmission of Food Preference*

This test was performed as previously described by (Desbonnet et al., 2015). At 12 weeks of age, mice were deprived of food for 18 hours before the test, whereas water was available *ad libitum*. Food choices consisted of either 1% ground cinnamon or 2% powdered red cocoa (Drinking Chocolate, Cadbury Ltd.) made with ground mouse chow. Naïve mice (strain/sex/aged matched) were used as demonstrators. The demonstrator mice and the experimental mice were co-housed in a same new cage 18 hours before the beginning of the test. Their fur was marked using blue marker to enable identification during subsequent social interactions. Demonstrator mice received only one of the types of food (cinnamon or cocoa) during a 1 h sampling session and food containers were weighed before and after it. A minimum of 0.2 g of consumed food was required for inclusion in the test. Demonstrator mice were placed back into their respective home cages for a 20 minute interaction with cage-mates. Subsequently cage-mates were individually tested for preference of cued food and novel food for 30 min. Containers were weighed immediately before and after each choice session. Observer mice were then placed back into their respective home cages and the choice session was repeated 24 hours later.

#### *4.3.4.11 Forced-swim Test*

The forced swim test (FST) is a widely used experimental paradigm to assess depression-like behaviour in rodents (Cryan et al., 2001). At week 14, animals were placed into a cylinder of tepid water (23-25 oC) to a depth of 17 cm. Behaviour was recorded by a camera positioned above the swim tank. The immobility time was scored during the last 4 min of the 6 min test. After removal from the cylinder, animals were placed into a separate cage for recovery.

### 4.3.5 *In Vivo* Intestinal Motility

At week 13, mice were single-housed and habituated to new cages for three hours. Following the acclimatisation, mice received 200µL oral gavage of Carmine (C1022; Sigma Aldrich) suspended in 0.5% carboximethylcelulose (CMC) sodium salt (Sigma; St Louis, MO, USA). Time until the first coloured bolus was recorded.

### 4.3.6 *In Vivo* Intestinal Permeability Assay (FITC-dextran)

For this procedure mice were food-deprived overnight. Early the next morning, FITC-dextran (MW = 4 kDa; FD4, Sigma), was administered orally by gavage, 600 mg/kg, 80 mg/ml in Phosphate buffered saline (PBS) (pH 7.4). A blood sample (100 µl in heparin-coated glass capillary) was taken from the tail vein 2h after the oral gavage. Samples were kept on ice, centrifuged at ~ 3500 g for 15 min, plasma was aspirated, transferred to amber tubes, kept on ice and analysed on the day of sampling. Plasma was measured undiluted, 25 µl in a 384 well plate. FITC was measured with a Victor spectrometer. The excitation maximum was 490 nm, the emission maximum was 520 nm (measured at 535 nm). For a standard curve, serial dilutions of FITC were prepared in PBS.

### 4.3.7 HPA Axis Response

Blood and subsequent plasma samples were taken to assess the HPA axis response to a mild acute stress. A blood sample was taken 5 min before and at different time-points following the FST test at week 14. Samples were taken at: 1) baseline (5 min before the onset) 2) 15 min after the onset of FST, 3) 30 min after the onset of FST 4) 60 min after the onset of FST and 5) 120 min after the onset of FST. Blood samples (50-70µl) were taken from the tail vein and placed in heparin-coated capillary tubes. Bleeding was performed in a separate room. During the recovery period, animals were single housed in separate cages; after the last bleed animals were returned to the home cage. Total corticosterone was measured according to the manufacturer's protocol, Corticosterone ELISA kit (Enzo Life Sciences, Farmingdale). Plasma dilution was 1:40.

### 4.3.8 Oxytocin Measurement

Oxytocin measurement was performed as previously described by (Neumann et al., 2013). At 15 weeks of age, trunk blood was collected and centrifuged (5 min, 5000 rpm, 4 °C), and plasma samples were kept at −20°C until oxytocin concentration was measured using a highly sensitive and specific radioimmunoassay performed by *RIAgnosis*, Munich.

### 4.3.9 RNA Isolation and Synthesis of cDNA

Total RNA was isolated from the ventral hypothalamus using the mirVana™ miRNA Isolation Kit as per manufacturer's instructions (Thermo Fisher Scientific). 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 °C until time of assay.

### 4.3.10 qRT-PCR

Gene expression was analysed using Gene Expression Assays on an AB7300 system (Applied Biosystems, Thermo Fisher 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) and normalised by the average of the four experimental groups.

### 4.3.11 Statistical Analyses

Statistical analyses were carried out using SPSS version 19 (Armonk, NY, USA). Data was analysed by Two-way ANOVA for strain and treatment factors followed by LSD post hoc tests. Additionally, one-sample *t*-test (with test value=0) was performed for NOR data. All data are presented as mean ± S.E.M. Corticosterone data was analysed by Mixed-design ANOVA. $p<0.05$ was considered statistically significant. In all datasets, the individual values outside the group mean ± 2 x standard deviation range were considered as outliers.

## 4.4 Results

### 4.4.1 Maternal Immune Activation Did Not Influence Maternal Care

Firstly we determined the influence of strain on the impact of MIA on maternal care maternal care behaviour on P6 (**Table 4.1**). Although we did not find significant effect

of treatment ($F_{(1,10)}$=0.351, p=0.567) or interaction between treatment and strain ($F_{(1,10)}$=754, p=0.408). There was a significant main effect of mouse strain, with C57BL6/J dams spending more time in high care behaviours than NIH Swiss dams ($F_{(1,10)}$=6.396, p=0.030) (**Table 4.1**).

| | **C57BL6/J** | | **NIH Swiss** | |
|---|---|---|---|---|
| | Saline | MIA | Saline | MIA |
| | *n*=3 litters | *n*=5 litters | *n*=3 litters | *n*=3 litters |
| **Time spent in maternal care (min.)** | 3.56±1.46 | 4.58±3.70 | 16.44±1.83$^{\#}$ | 10.91±5.38 |

***Table 4. 1- Maternal immune activation (MIA) did not affect time spent on maternal care behaviours.*** Total time spent on high care behaviours (nursing, pup licking/ grooming, carrying and time on-nest) during the 30 minute test was noted. *Data is represented as mean ± S.E.M. $^{\#}$indicates p<0.05 compared to the C57BL6/J control group.*

### 4.4.2 Maternal Immune Activation Did Not Affect Isolation-induced Ultrasonic Vocalization

We next investigated communication deficits in the offspring by measuring pup ultrasonic vocalization at P9 (**Table 4.2**). While maternal exposure poly (I:C) had no effect on the number of 70kHz calls elicited by the offspring from the two strains (treatment effect [$F_{(1,49)}$= 1.53, p=.222]; treatment x strain [$F_{(14,49)}$= 0.00, p=0.994]), NIH Swiss strain showed a higher number of calls overall ($F_{(1,49)}$=17.282 p=0.001) (**Table 4.2**).

**Table 4.2: Maternal immune activation (MIA) did not affect isolation-induced ultrasonic vocalization.** Total number of 70kHz calls was recorded for 3 minutes.

| | **C57BL6/J** | | **NIH Swiss** | |
|---|---|---|---|---|
| | Saline | MIA | Saline | MIA |
| | *n*=10 pups | *n*=8 pups | *n*=24 pups | *n*=11 pups |
| **Number of calls** | 9.7±3.6 | 8.4±5.38 | 37.5±8.2$^{\#}$ | 27.91±14.05 |

***Table 4. 2- Maternal immune activation (MIA) did not affect isolation-induced ultrasonic vocalization.***

Total number of 70kHz calls was recorded for 3 minutes. Data is represented as mean ± S.E.M. $^{\#}$indicates p<0.05 compared to the C57BL6/J control group.

### 4.4.3 Maternal Immune Activation Did Not Affect Homing Behaviour in a Strain Dependent Manner

Further, we investigated the effects of MIA on maternal attachment by using the homing behaviour test (**Figure 4. 2**). When comparing the time spent with the familiar or non-familiar bedding, a Two-way ANOVA showed a significant effect of strain ($F_{(1,52)}$=11.824, p=0.001) .However, no significant effect of treatment with MIA ($F_{(1,52)}$=0.21, p=0.887) or of the interaction between strain and treatment ($F_{(1,52)}$=0.169, p=0.382) were found (**Figure 4. 2a-b**).

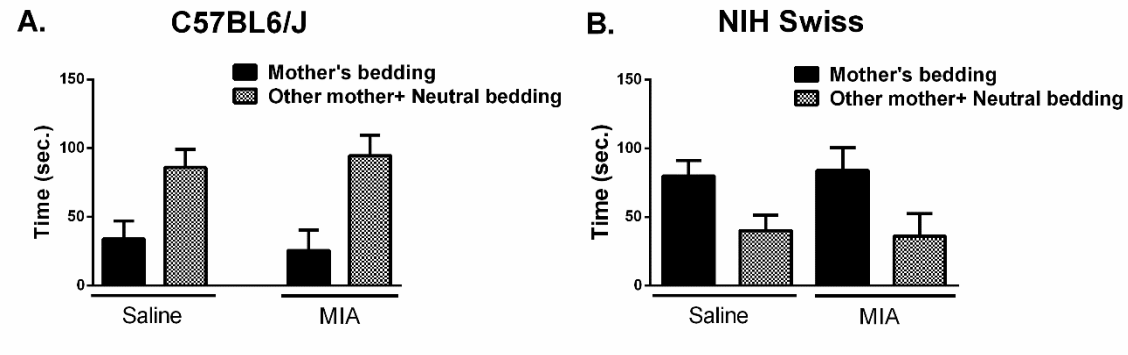



***Figure 4. 2- Effects of maternal immune activation (MIA) on homing behaviour.***

(**A)** The preference for the familiar bedding was not affected in the C57BL6/J offspring. (**A)** The preference for the familiar bedding was not affected in the NIH Swiss offspring. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 11, MIA C57BL6/J n= 8, Sal NIH Swiss n= 25, MIA NIH Swiss n= 12]).

### 4.4.4 Maternal Immune Activation Altered Reciprocal Social Interaction in Adolescence

Social interaction during adolescence is essential for normal development of socio-affective response in adulthood (Trezza et al., 2011). Thus, we investigated the effects of MIA on reciprocal social interaction in adolescent offspring (**Figure 4. 3**). Interestingly, Two-way ANOVA of the time spent in social interaction during the habituation session showed that MIA treatment to the dams induced significant deficits in social interaction behaviour in the offspring of both strains (F(1,36)=14.625, p=0.0001) (**Figure 4. 3a-b**). However, no significant effect of strain (F(1,36)=0.744,

p=0.394) or interaction between the factors (F(1,36)=0.004, p=0.952) was found. Interestingly, at the test session, there was a significant interaction between strain and treatment (F(1,36)=4.824, p=0.035) with MIA affecting social interaction time only in the C57BL6/J offspring. Regardless, no significant effect of strain (F(1,36)=3.531, p=0.068) or treatment (F(1,36)=3.025, p=0.091) was found (**Figure 4. 3c-d**).

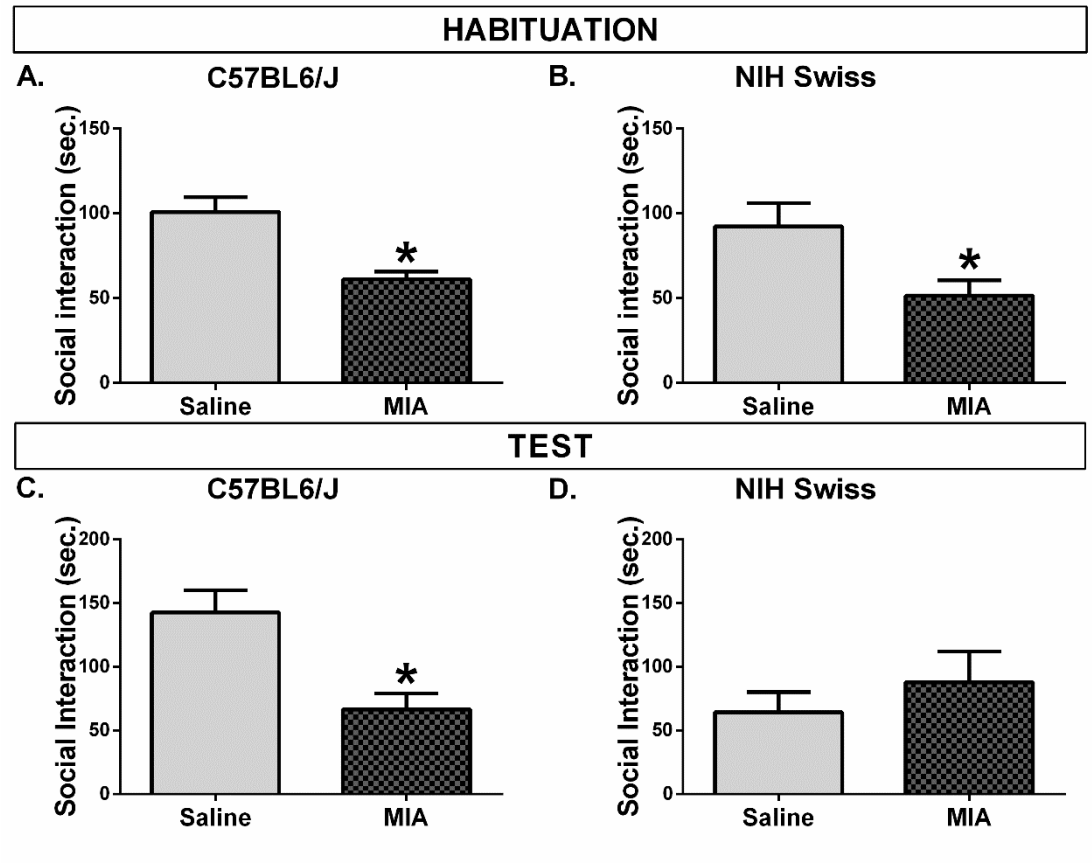



***Figure 4. 3- Maternal immune activation alters reciprocal social interaction in adolescent offspring.*** **(A,B)** Maternal immune activation offspring displayed decreased social interaction in the habituation phase in both **(A)** C57BL6/J **(B)** NIH Swiss strains. **(C)** Social interaction time remained significantly reduced in the C57BL6/J mice during the test session. **(D)** There were no significant differences between NIH Swiss groups during the test session. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 9, MIA C57BL6/J n= 9, Sal NIH Swiss n=12, MIA NIH Swiss n= 10]).* indicates $p<0.05$ compared with the respective control group.

### 4.4.5 Maternal Immune Activation Had a Modest Effect on C57BL6/J Offspring Behaviour in the Marble Burying Test

At week 8, repetitive behaviour was evaluated using the marble burying test (**Table 4.3**). Although no differences were found between MIA and control groups for the fully buried marbles (treatment ([$F_{(1,38)}$=0.528, p=0.472]; strain x treatment [$F_{(1,38)}$=3.286, p=0.584]), there was a significant strain effect with NIH Swiss mice burying significantly more marbles than C57BL6/J strain ($F_{(1,38)}$=51.315, p<0.0001). For partially buried marbles, Two-way ANOVA revealed a significant effect of the strain x treatment interaction ($F_{(1,38)}$=7.041, p=0.012) but no main effect of strain ($F_{(138)}$=0.026, p=0.872) or treatment ($F_{(1,38)}$=1.713, p=0.199). Interestingly, post-hoc comparisons showed that C57BL6/J mice buried significant more marbles after MIA, whereas MIA had no effect in NIH Swiss mice. Finally, for the total number of buried marbles, Two-way ANOVA showed a significant effect of strain ($F_{(1,38)}$=46.000, p<0.0001) such that NIH Swiss mice buried more marbles than C57BL6/J mice, but no significant effect of treatment ($F_{(1,38)}$=3.259, p=0.079) or the strain x treatment interaction ($F_{(1,38)}$=0.371, p=0.546).

| **Number of buried marbles** | **C57BL6/J** | | **NIH Swiss** | |
|---|---|---|---|---|
| | Saline | MIA | Saline | MIA |
| | *n*=10 | *n*=9 | *n*=12 | *n*=11 |
| Fully buried (>80%) | 1.6±0.47 | 1.77±1.11 | 8.5±1.16# | 9.6±1.26# |
| Partially buried (50-80%) | 1.9±0.50 | 4.6±0.79* | 3.7±0.61 | 2.6±0.67 |
| Total buried (Fully+Partially buried marbles) | 3.3±0.55 | 6.5±1.6 | 12.20±1.19# | 13.18±1.1# |

***Table 4. 3- Maternal immune activation (MIA) increased the number of partially buried marbles in C57BL6/J.***

Data is represented as mean ± S.E.M. #indicates p<0.05 compared to the C57BL6/J control and MIA groups.

### 4.4.6 Maternal Immune Activation Mediated Effects on the 3-chamber Test

At week 9, sociability and social novelty preference was assessed using the 3-chamber test. For sociability, Two-way ANOVA revealed significant effects of treatment ($F_{(1,37)}$=22.490, p<0.0001). Individual group comparisons showed a significant decrease in time spent with the conspecific in C57BL6/J (**Figure 4. 4a**) NIH Swiss (**Figure 4. 4b**). Additionally, there was a significant effect of strain with NIH Swiss mice spending less on social interaction than C57BL6/J ($F_{(1,37)}$=11.584, p<0.002), although, no significant effect of the interaction between MIA and strain was found ($F_{(1,37)}$=0.361, p<0.552) (**Figure 4. 4b**). Two-way ANOVA revealed no significant differences in the time spent interacting with the object. When social memory was tested, Two-way ANOVA revealed significant effects of treatment ($F_{(1,37)}$=29.214, p<0.0001) and strain x treatment interaction ($F_{(1,37)}$=7.132, p=0.011) with decreased time spent with the novel mouse in the MIA C57BL6/J (**Figure 4. 4c**) and in the MIA NIH Swiss (**Figure 4. 4d**) groups. Moreover, there was significant effect of strain ($F_{(1,37)}$=5.683, p=0.022) with NIH Swiss spending less time interacting with the novel mouse than C57BL6/J.

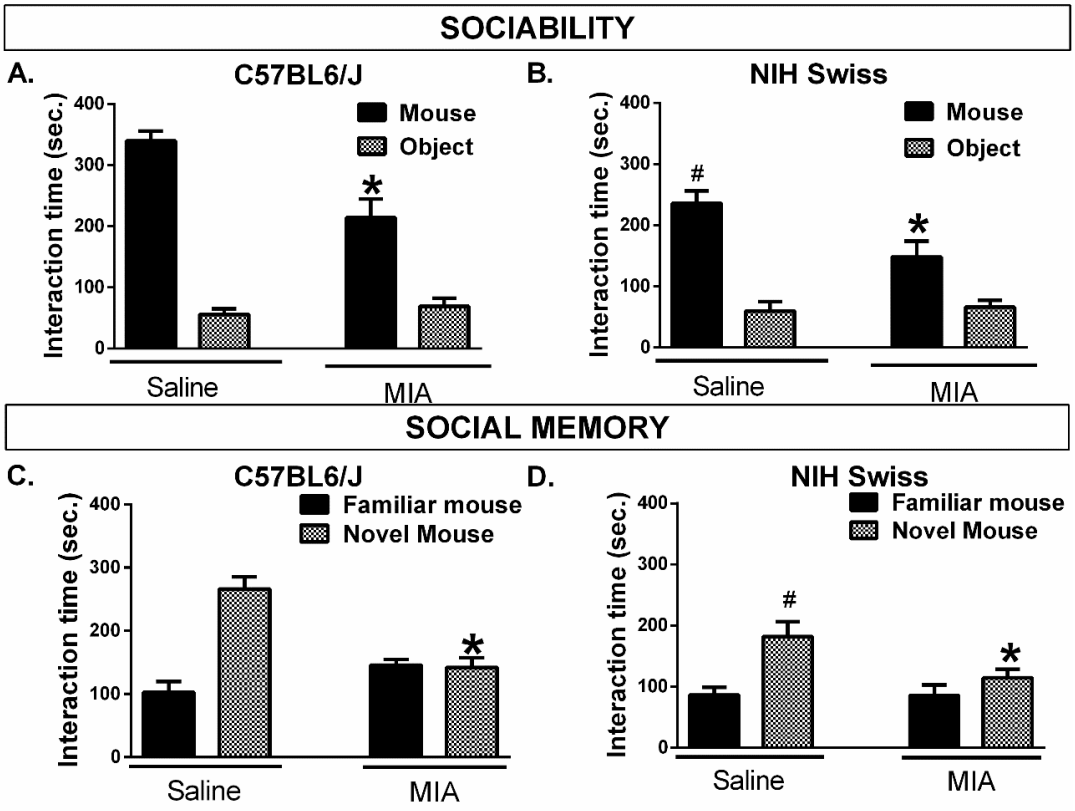



***Figure 4. 4- Maternal immune activation induced deficits in social behaviour in adult offspring.*** **(A,B)** MIA decreases sociability in both **(A)** C57BL6/J and **(B)** NIH Swiss offspring. **(C,D)** Further, MIA induced social preference deficits in both **(C)** C57BL6/J and **(D)** NIH Swiss groups. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n=11, MIA NIH Swiss n= 11]). * indicates $p<0.05$ compared with the respective control group. $^{\#}$indicates $p<0.05$ compared to the C57BL6/J control.

### 4.4.7 Maternal Immune Activation Affects Social Transmission of Food Preference

In addition, social behavioural deficits were assessed in the social transmission of food preference test at week 12. Food preference in observer mice was tested 1 hour (**Fig. Figure 4. 5a-b**) and 24h (**Figure 4. 5c-d**) after exposure to a demonstrator mouse. At the 1h test, Two-way ANOVA showed a significant effect of the interaction between strain and treatment ($F_{(1,37)}=6.863$, $p=0.013$) but no significant effect of strain ($F_{(1,37)}=0.433$, $p=0.515$) or treatment ($F_{(1,37)}=1.033$, $p=0.316$). Post hoc comparisons revealed a significant decrease in the preference for the cued food in MIA C57BL6/J mice (**Figure 4. 5a**). A similar pattern was observed at the 24h test. Two-way ANOVA

showed a significant interaction between treatment and strain ($F_{(1,38)}$=6.376, p=0.016) whereas there were no main effects of treatment ($F_{(1,38)}$=2.341, p=0.134) or strain ($F_{(1,38)}$=1.203, p=0.280). Post-hoc analyses revealed that the deficits for the cued food preference remained in the MIA C57BL6/J compared with its control group (**Figure 4. 5d**). Additional strain comparisons showed that NIH Swiss have a decreased preference for the cued food in comparison to C57BL6/J at the both time points investigated (**Figure 4. 5b** and **Figure 4. 5d**).

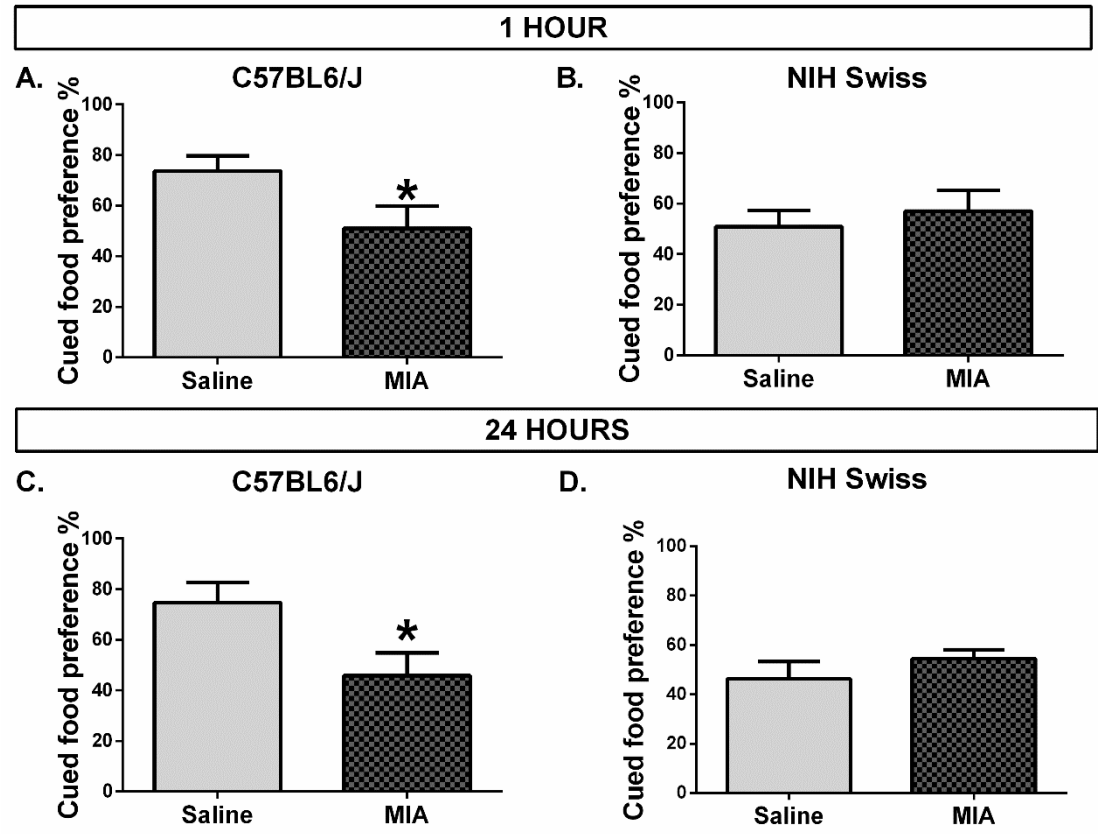



***Figure 4. 5- Effects of Maternal immune activation in social transmission of food preference.***

**(A)** Reduced preference for cued food was observed in MIA C57BL6/J offspring at 1 h test. **(B)** No differences were found in the preference for cued food in the NIH Swiss mice at 1 h test. **(A,B)** [Sal C57BL6/J n= 9, MIA C57BL6/J n= 9, Sal NIH Swiss n=12, MIA NIH Swiss n= 10].**(C)** Reduced preference for cued food was observed in MIA C57BL6/J offspring at 24 h test. **(D)** No differences were found in the preference for cued food in the NIH Swiss mice at 24 h test. Data is represented as mean ± S.E.M. **(C,D)** [Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n=12, MIA NIH Swiss n= 10].* indicates $p<0.05$ compared with the respective control group. $^{\#}$indicates $p<0.05$ compared to the C57BL6/J control group.

### 4.4.8 Maternal Immune Activation Induces Depression-like Behaviour in a Strain-dependent Manner

The immobility time in the FST was used as measure of depression-like behaviour. Two-way ANOVA showed a significant strain effect ($F_{(1,37)}$=68.571, p<0.0001) with the NIH Swiss strain spending significantly less time immobile than the C57BL6/J strain (**Figure 4. 6a-b**). Two-way ANOVA showed no significant effect of the treatment ($F_{(1,37)}$=3.580, p=0.066) or in the strain x treatment interaction ($F_{(1,37)}$=3.384, p=0.074). Further individual group comparisons revealed that MIA significantly affected immobility time in the NIH Swiss mice in comparison with its control group (**Figure 4. 6b**). A Two-way Mixed-design ANOVA over the 6-min time revealed a significant effect of time ($F_{(5,38)}$=38.503, p<0.0001) and a significant effect strain ($F_{(1, 38)}$=102.044, p<0.0001) with the NIH Swiss strain showing less immobility overall. Additionally, there was a significant effect of the strain in respect with the time ($F_{(1,38)}$=5.718, p=0.022). However, no interaction between time and treatment was found ($F_{(1,38)}$=0.059, p=0.202) or between time, treatment and strain ($F_{(1,38)}$=2.526, p=0.120) (**Figure 4. 6c-d**).

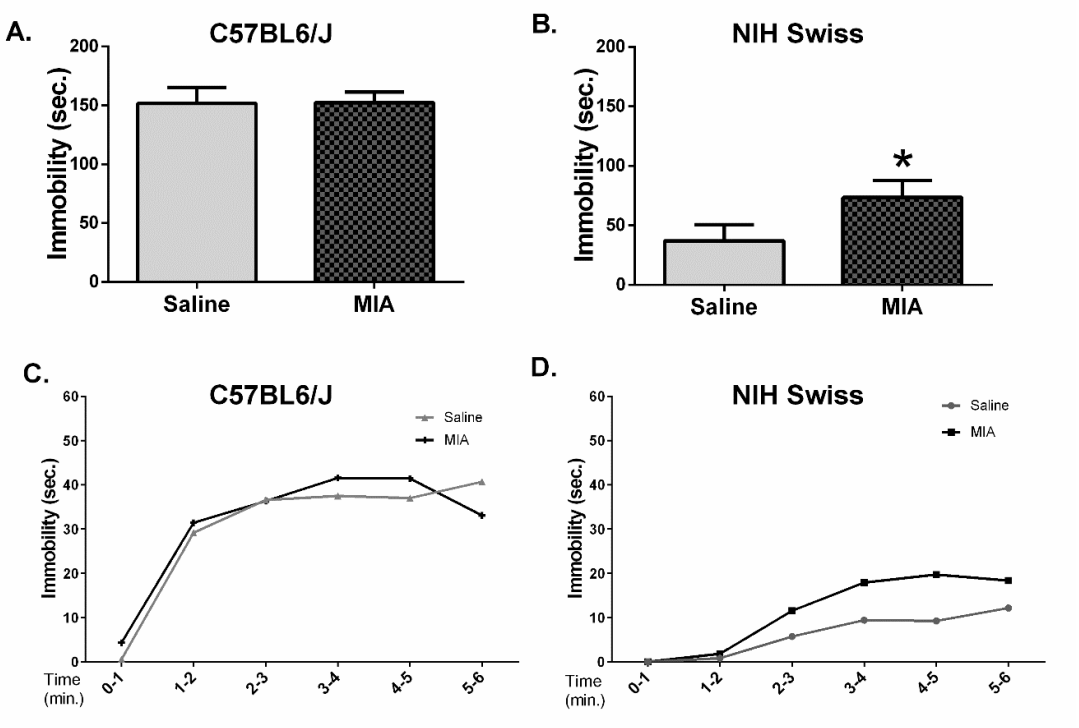



***Figure 4. 6- Maternal immune activation (MIA) effects on depression-like behaviour.***

**(A)** No significant effect of MIA was noted on the immobility time in C57BL6/J mice. **(B)** MIA significantly increased immobility time in NIH Swiss mice. **(C-D)** There was no significant effect of MIA across the 6-min test in both **(C)** C57BL6/J and **(D)** NIH Swiss mice. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n=11, MIA NIH Swiss n= 11]) * indicates $p<0.05$ compared with the respective control group. $^{\#}$indicates $p<0.05$ compared to the C57BL6/J control.

### 4.4.9 Maternal Immune Activation Increases HPA Axis Response to Acute Stress in the NIH Swiss Strain

To examine whether MIA alters HPA axis response in the offspring, corticosterone production was measured before and after an acute stress (FST). Mixed-design ANOVA revealed a significant effect of time ($F_{(4,31)}=92.063$, $p<0.0001$) and a significant interaction between time and strain ($F_{(4, 31)}=7.852$, $p<0.0001$). Additionally, there was a significant effect of the strain and treatment in respect with the time ($F_{(4,31)}=40.769$, $p<0.046$). However, no interaction between time and treatment was found ($F_{(4,31)}=7.852$, $p=0.749$). Overall, in both strains MIA offspring had similar baseline corticosterone levels in comparison to control group (**Figure 4. 7a-b**). Interestingly, a restricted ANOVA of the 15 min post stress time-point revealed a significant interaction between treatment and strain ($F_{(4,35)}=5.74$, $p<0.022$) with MIA resulting in a higher corticosterone response in the NIH Swiss strain as revealed by LSD post hoc analyses (**Figure 4. 7a**). However, there was no significant effect of treatment ($F_{(4,35)}=0.688$, $p=0.412$) or strain ($F_{(4,35)}=1.602$, $p<0.214$) *per se*. Overall, no differences were found between C57BL6/J groups at any time point (**Figure 4. 7b**).

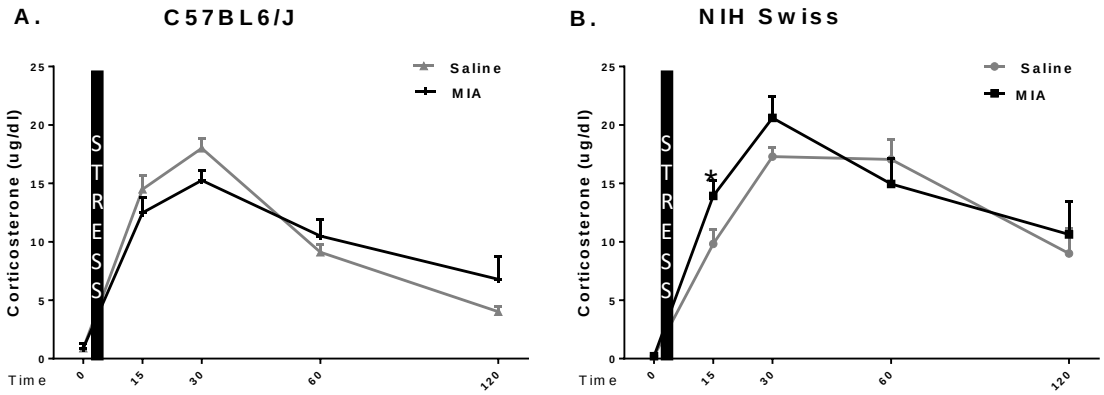



***Figure 4. 7- HPA axis response is altered in NIH Swiss maternal immune activation (MIA) offspring.*** **(A)** No differences in corticosterone levels were observed in C57BL6/J offspring. **(B)**MIA significantly increased the corticosterone response 15 min after swim stress in NIH Swiss offspring. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 8, Sal NIH Swiss n=8, MIA NIH Swiss n= 9]). * indicates $p<0.05$ compared with the respective control group.

### 4.4.10 Effects of Maternal Immune Activation on Anxiety and Locomotion

Offspring anxiety-like behaviour and locomotion were tested in the EPM and in the OF arena. In the EPM, two-way ANOVA revealed a significant effect of strain on the time spent in the open arms ($F_{(1,37)}=32.505$, $p<0.0001$). There was no significant effect of treatment ($F_{(1,37)}=2.799$, $p=0.103$) or the interaction between treatment and strain ($F_{(1,37)}=2.119$, $p=0.154$). Interestingly, further post hoc analyses showed a significant increase in the time spent in the open arms in the MIA NIH Swiss group when compared to its control group (**Figure 4. 8b**). However, no significant differences were found across C57BL6/J groups (**Figure 4. 8a**). In regards to the time spent in the closed arms, two-way ANOVA revealed a significant effect of strain ($F_{(1,37)}=27.715$, $p<0.0001$). Post-hoc analyses further showed that MIA NIH Swiss mice spent significant less time in the closed arms in comparison to saline controls (**Figure 4. 8d**). As was the case for the open arms, no differences between MIA and saline C57BL6/J were found for time spent in the closed arms (**Figure 4. 8c**). Further LSD post hoc showed that C57BL6/J mice spent significantly more time in the closed arms when compared to NIH Swiss mice.

When mice were tested in the OF arena, two-way ANOVA revealed significant strain effects in the time spent in the central zone ($F_{(1,37)}$=58.878, p<0.0001), such that NIH Swiss spent significantly less time in the central zone than C57BL6/J (**Figure 4. 8e-f**). However, there was no effects of MIA treatment ($F_{(1,37)}$=1.823, p=0.185) or interaction between treatment and strain ($F_{(1,37)}$=0.436, p=0.153). . Similarly, when general locomotion was assessed a significant effect of the strain was detected ($F_{(1,37)}$=72.781, p<0.0001) but no effects of treatment ($F_{(1,37)}$=0.438, p=0.512) and no treatment x strain interaction ($F_{(1,37)}$=0.1427, p=0.240). Locomotion was significantly increased in NIH Swiss mice compared to C57BL6/J mice (**Figure 4. 8g-h**).

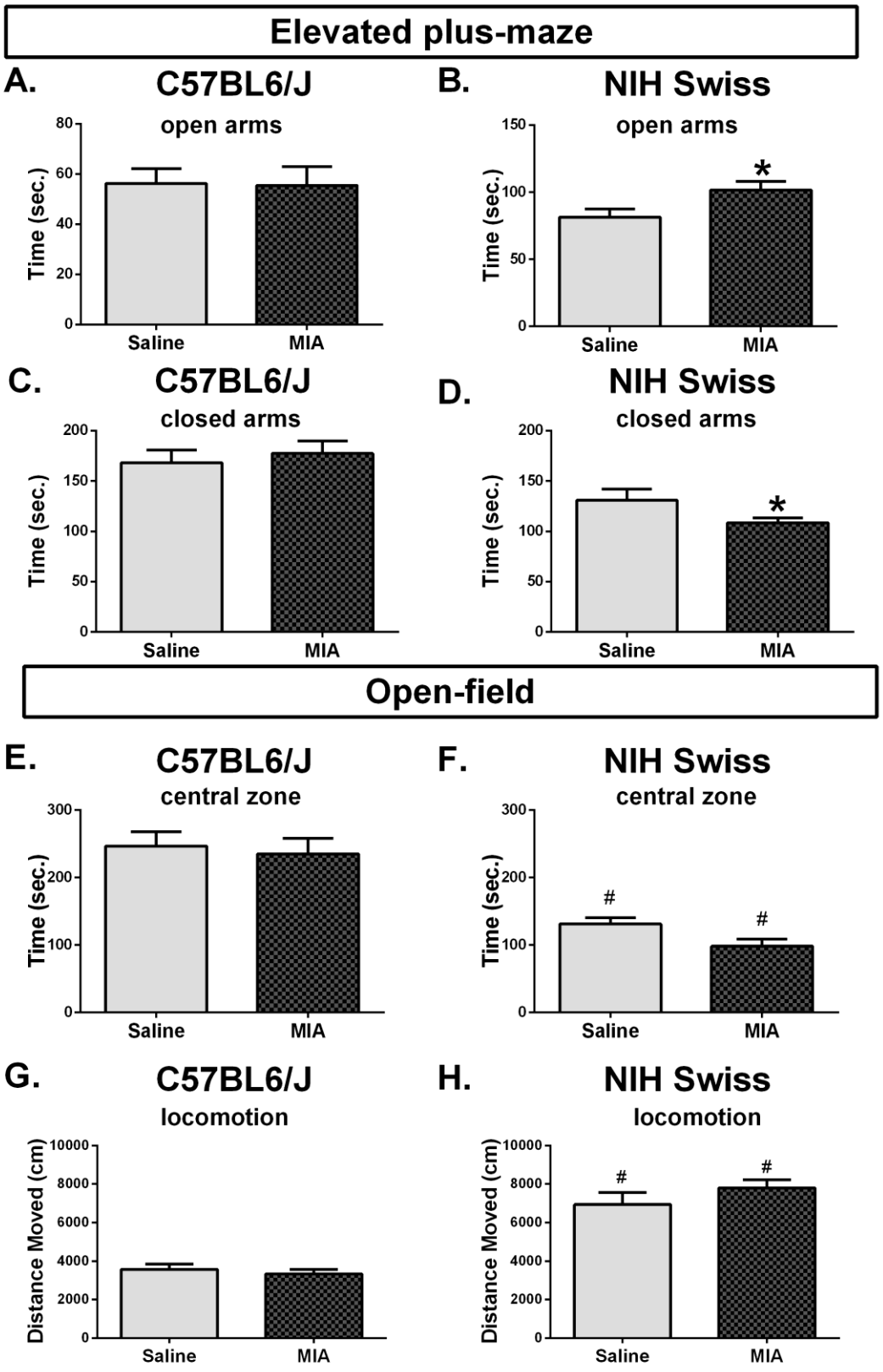



***Figure 4. 8- Effects of maternal immune activation on anxiety-like behaviour and locomotion.***

**(A)** No changes in C57BL6/J offspring were found. **(B)**Maternal immune activation significantly increased time spent in the open arms of the EPM in NIH Swiss offspring. **(C)** In C57BL6/J strain no differences were found between groups for the time spent in closed arms. **(D)** MIA NIH Swiss offspring

displayed less time in the closed arms of EPM. **(E,F)** In the OF no there was no treatment effect in either strain in the time spent in the central zone and in **(G,H)** the total distance moved (cm). Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n=11, MIA NIH Swiss n= 11]). * indicates $p<0.05$ compared with the respective control group. #indicates $p<0.05$ compared to the C57BL6/J control and MIA groups.

### 4.4.11 Maternal Immune Activation Did Not Impact Working Memory in the Novel Object Recognition Task

To examine if MIA affected working memory, mice were tested in the novel object recognition task. Two-way ANOVA revealed no significant effect of treatment ($F_{(1,36)}=0.004$, $p=0.948$), strain ($F_{(1,36)}=0.144$, $p=0.707$), or the strain x treatment interaction ($F_{(1,36)}=0.615$, $p=0.438$) (**Table 4.4**). One-sample *t*-test comparing the discrimination index of each group against the chance level (zero) revealed no significant differences for C57BL6/J control ($t=2.08$, $df=9$, $p=0.066$), C57BL6/J MIA ($t=0.337$, $df=6$, $p=0.748$), NIH Swiss control ( $t=1.301$, $df=11$, $p=0.220$) and for NIH Swiss MIA ($t=1.712$, $df=10$, $p=0.18$) groups.

| **ovel object recognition** | **57BL6/J** | | **IH Swiss** | |
|---|---|---|---|---|
| | line | IA | line | IA |
| | =10 | =7 | =12 | =11 |
| **iscrimination index** | 1432±0.068 | 056±0.16 | 10±0.07 | 175±0.102 |

***Table 4. 4- Maternal immune activation (MIA) did not affect working memory.***

[Discrimination index = (novel object exploration time - familiar object exploration time)/(novel object exploration time + familiar object exploration time)]. Data is represented as mean ± S.E.M

### 4.4.12 Maternal Immune Activation Reduces Vasopressin Receptor 1a (AVP1aR) Expression but Does Not Affect Oxytocin Receptor Expression in a Strain Specific Manner

Given that the oxytocin and vasopressin systems are strongly involved in regulating social behaviour, stress and anxiety (Neumann and Landgraf, 2012), the hypothalamus was dissected and AVP1aR and OXTR mRNA expression was investigated by quantitative RT-PCR (**Figure 4. 9**). With regards to AVP1aR mRNA expression,

Two-way ANOVA of relative fold change values revealed a trend towards the treatment having a significant effect ($F(1,32)=3.739$, $p=0.062$). There were no significant effects of strain ($F(1,32)=1.067$, $p=0.309$) or treatment x strain interaction ($F(1,32)=1.067$, $p=0.309$). An explorative LSD multiple comparison test revealed that MIA decreased AVP1aR expression in NIH Swiss mice compared to saline controls from the same strain (**Figure 4. 9a**). For OXTR mRNA expression, Two-way ANOVA of relative fold change values revealed no significant effects of treatment ($F(1,31)=0.122$, $p=0.729$), strain ($F(1,31)=1.317$, $p=0.260$) or treatment x strain interaction ($F(1,31)=0.489$, $p=0.489$).

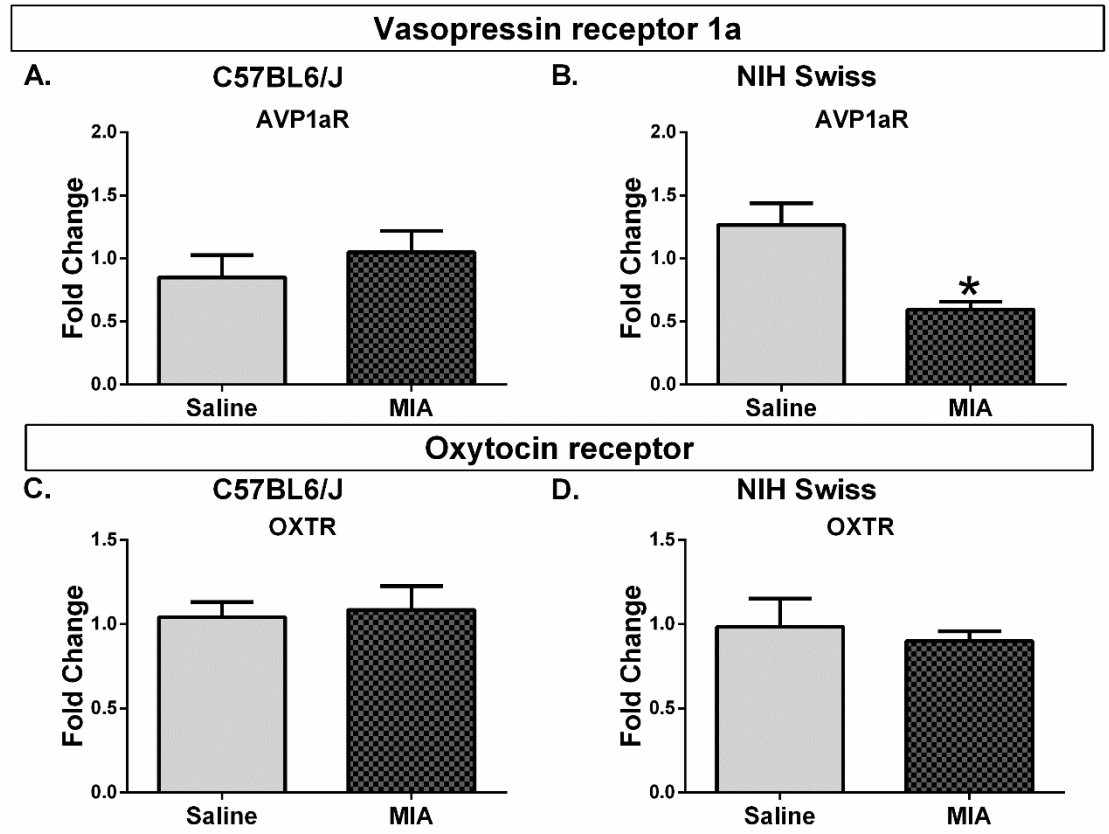



***Figure 4. 9- Maternal immune activation (MIA) decreased Vasopressin receptor 1a expression in adult NIH Swiss mice.***

**(A)** No differences in AVP1aR mRNA expression were observed in C57BL6/J offspring. **(B)** MIA significantly decreased AVP1aR mRNA expression in the hypothalamus of NIH Swiss offspring **(A,B)** ([Sal C57BL6/J n= 8, MIA C57BL6/J n= 8, Sal NIH Swiss n=10, MIA NIH Swiss n= 10]). **(C,D)** There were no significant differences in OXTR mRNA expression in both **(C)** C57BL6/J and **(D)** NIH Swiss mice. Data is represented as mean ± S.E.M ([Sal C57BL6/J n= 9, MIA C57BL6/J n= 8, Sal NIH Swiss n=10, MIA, NIH Swiss n= 8]). * indicates $p<0.05$ compared with the respective control group.

### 4.4.13 Maternal Immune Activation Did Not Affect Oxytocin Plasma Levels

We further investigated if MIA could lead to changes in plasma oxytocin levels. As revealed by a two-way ANOVA, there was a significant effect of strain ($F_{(1,38)}$=26.705, p<0.001) and a trend towards a treatment effect ($F_{(1,38)}$=3.819, p<0.058). However, no significant interaction between strain and treatment was found ($F_{(1,38)}$=0.231, p<0.634). Within strain post-hoc comparisons revealed higher oxytocin plasma concentrations in the NIH Swiss mice (**Table 4.5**).

| | **C57BL6/J** | | **NIH Swiss** | |
|---|---|---|---|---|
| | Saline | MIA | Saline | MIA |
| **Oxytocin (pg/mL)** | *n*=10 | *n*=9 | *n*=12 | *n*=11 |
| | 2.027±0.22 | 0.8996±0.39 | 4.353±0.59$^{\#}$ | 3.660±0.38$^{\#}$ |

***Table 4. 5- Maternal immune activation (MIA) did not affect oxytocin plasma levels.***
Oxytocin plasma levels (pg/mL) as measured by radioimmunoassay. Data is represented as mean ± S.E.M. $^{\#}$indicates p<0.05 compared to the C57BL6/J control and MIA groups.

### 4.4.14 Maternal Immune Activation Increased Intestinal Permeability but Normal Intestinal Motility in Adult NIH Swiss Offspring

Concentration of plasma FITC-dextran was taken as a measure of intestinal permeability. Two-way ANOVA showed a significant interaction between strain and treatment ($F_{(1,38)}$=4.695, p=0.037) but no significant strain ($F_{(1,38)}$=2.282, p=0.139) or treatment effect ($F_{(1,38)}$=1.73, p=0.196). Post hoc analyses revealed that MIA NIH Swiss mice exhibited increased intestinal permeability to FITC-dextran when compared to control mice from the same strain (**Figure 4. 10b**). Regarding intestinal motility, no effects of treatment ($F_{(1,38)}$=0.217, p=0.644) or strain x treatment interaction ($F_{(1,38)}$=0.479, p=0.493) were observed. However, a significant effect of strain was observed ($F_{(1,38)}$=16.365, p<0.0001), with faster intestinal motility in NIH Swiss groups compared to C57BL6/J groups (**Figure 4. 10c-d**).

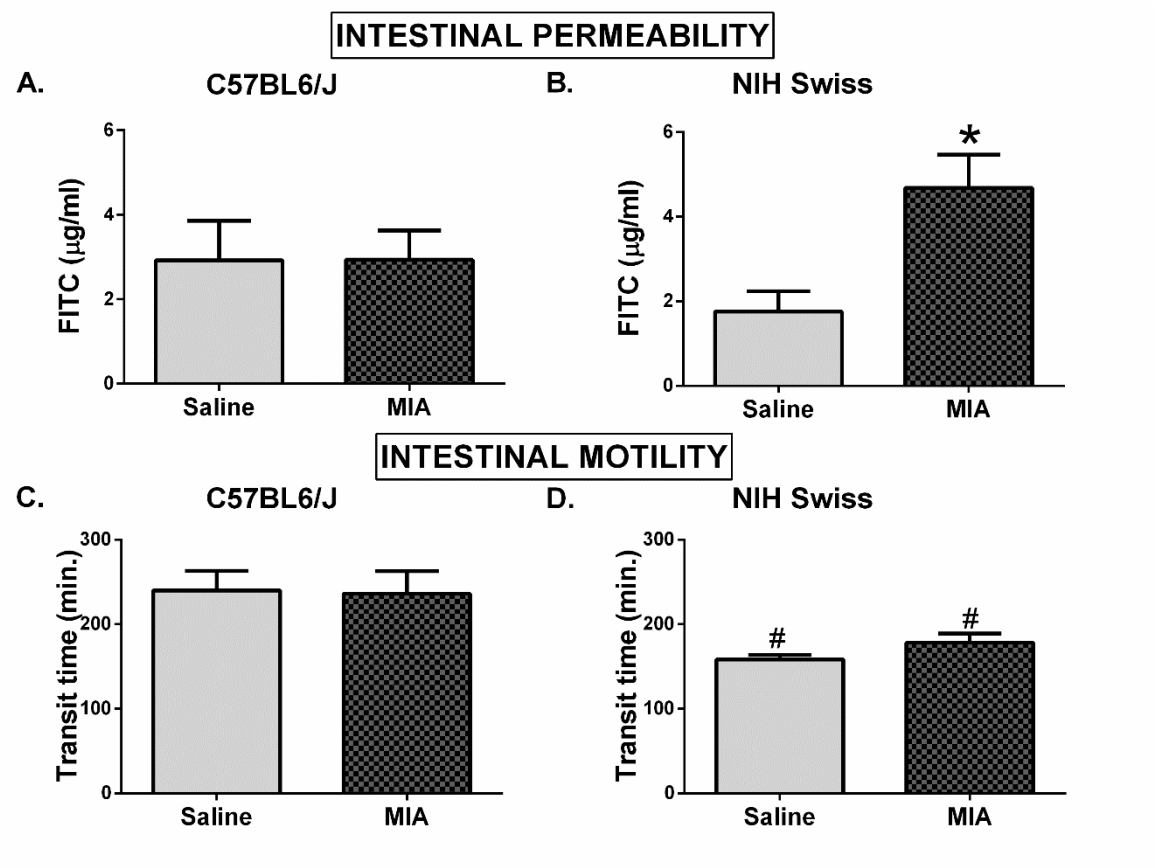



***Figure 4. 10- Maternal immune activation (MIA) affected intestinal permeability but does not alter intestinal motility.***

**(A)** No differences in FITC-dextran plasma concentration (µg/mL) were detected in the C57BL6/J offspring. **(B)** MIA NIH Swiss mice exhibit increased intestinal permeability to flurescein isothiocyanate (FITC)-dextran. **(A,B)** ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n= 12, MIA, NIH Swiss n= 11]) **(C,D)** Intestinal transit was unaffected by MIA in both **(C)** C57BL6/J and **(D)** NIH Swiss mice ([Sal C57BL6/J n= 10, MIA C57BL6/J n= 9, Sal NIH Swiss n=12, MIA, NIH Swiss n= 11]). Data is represented as mean ± S.E.M. * indicates $p<0.05$ compared to the respective control group (n- 9-12 per group). $^{\#}$indicates $p<0.05$ compared to the C57BL6/J control and MIA groups.

| Phenotype | Strain | | Two-way ANOVA effects | | |
|---|---|---|---|---|---|
| in response to MIA | C57BL6/J | NIH Swiss | Strain | Treatment | Strain x Treatment |
| Adolescent social interaction (Habituation) | ↓↓ | ↓↓ | F(1,36)=0.744, p=0.394 | F(1,36)=14.625, p=0.0001 | F(1,36)=0.004, p=0.952 |
| Adolescent social interaction (Test) | ↓↓ | ↓↓ | F(1,36)=3.531, p=0.068 | F(1,36)=3.025, p=0.091 | F(1,36)=4.824, p=0.035 |
| Sociability | ↓ | ↓ | F(1,37)=11.584, p<0.002 | F(1,37)=22.490, p<0.0001 | F(1,37)=0.361, p<0.552 |
| Preference for social novelty | ↓ | ↓ | F(1,37)=5.683, p=0.022 | F(1,37)=29.214, p<0.0001 | F(1,37)=7.132, p=0.011 |
| Social transmission of food preference (1h) | ↓↓ | ↓↓ | F(1,37)=0.433, p=0.515 | F(1,37)=1.033, p=0.316 | F(1,37)=6.863, p=0.013 |
| Social transmission of food preference (24h) | ↓↓ | ↓↓ | F(1,38)=1.203, p=0.280 | F(1,38)=2.341, p=0.134 | F(1,38)=6.376, p=0.016 |
| Repetitive behaviour (partially-buried marbles) | ↑ | ↑ | F(38)=0.026, p=0.872 | F(1,38)=1.713, p=0.199 | F(1,38)=7.041, p=0.012 |
| Depression | — | ↑ | F(1,37)=68.571, p<0.0001 | F(1,37)=3.580, p=0.066 | F(1,37)=3.384, p=0.074 |
| Anxiety (elevated plus-maze) | — | ↓ | F(1,37)=32.505, p<0.0001 | F(1,37)=2.799, p=0.103 | F(1,37)=2.119, p=0.154 |
| Stress response (T15) | — | ↑ | F(4,31)=1.602, p<0.214 | F(4,35)=0.688, p=0.412 | F(4,35)=5.74, p<0.022 |
| Intestinal permeability | — | ↑ | F(1,38)=2.282, p=0.139 | F(1,38)=4.695, p=0.037 | F(1,38)=4.695, p=0.037 |
| Vasopressin receptor expression | — | ↓ | F(1,32)=1.067, p=0.309 | F(1,32)=3.739, p=0.062 | F(1,32)=1.067, p=0.309 |

***Table 4. 6- Summary of maternal immune activation (MIA) effects in NIH Swiss and C57BL6/J offspring.***

The table summarize the main findings and specifies the statistical analyses output. Data was analysed by Two-way ANOVA. The table also specifies the corresponding p-values, degrees of freedom (DF). Significant effects ($P < 0.05$) are given in bold font.

## 4.5 Discussion

Several genetic and environmental factors have been linked to a higher risk of neurodevelopmental disorders (Schwartzer et al., 2013). Importantly, MIA has been proposed to act as a primer increasing individual susceptibility to genetic mutations and consequently playing a major role in the development of ASD and SZ symptoms (Careaga et al., 2017; Estes and McAllister, 2016). A growing body of literature links neurobehavioural symptoms of ASD and SZ with associated gut-brain axis dysfunction (Hill et al., 2017). Recently, some aspects of ASD and SZ associated gut-brain axis dysfunction were reproduced in the MIA poly (I:C) model in C57BL6/J mice (Hsiao et al., 2013). Here we replicate some of these previously reported findings for social and stereotyped behaviour induced by poly (I:C) in the C57BL6/J strain. In addition, we show, for what is to our knowledge the first time, that a single poly (I:C) injection on gestational day 12.5 induces a much wider array of neurobehavioural deficits in the NIH Swiss mice, including changes in anxiety and depression-like behaviours, stress responsivity and vasopressin receptor 1a mRNA expression in the hypothalamus. Notably, these strain-specific behavioural effects were associated with marked changes in gut permeability (Table 6). Thus, these findings highlight the importance of the interactions between MIA and genetic background on behavioural and molecular hallmarks of gut-brain axis dysfunction that may underpin important aspects of ASD and SZ phenotype.

Previous research in MIA animal models reproduced many of the core ASD and SZ symptoms demonstrating important construct, face and predictive validity to the model (Estes and McAllister, 2016; Meyer, 2014). Indeed, core social deficits and stereotyped behaviour were observed in the current study. For example, by adolescence, MIA offspring from both strains exhibited deficits in reciprocal social interaction. However, only C57BL6/J poly (I:C)-exposed mice showed a persistent deficit in social interaction at the test session. Juvenile social interaction deficits had been previously reported with LPS (Kirsten et al., 2010; Taylor et al., 2012) and valproic acid immune challenge in Long- Evans rats (Raza et al., 2015). Moreover, a subtle increase in the number of buried marbles was observed in MIA C57BL6/J in late puberty, replicating previous findings

that demonstrate repetitive behaviour in this strain (Choi et al., 2016; Schwartzer et al., 2013). The strain specificity on early behavioural phenotype may reflect differences in the postnatal neurodevelopmental trajectory and susceptibility to MIA insult of these two strains. Previous studies that attempted to investigate the effects of MIA induced by gestational poly (I:C) mainly focused on changes occurring after puberty (Patrich et al., 2016). It is important to note that, although we do not observe any significant changes in maternal care behaviour due to Poly (I:C) treatment, strain differences were noted in this study. Such strain differences have been studied previously and may play a role in programming epigenetic and behavioural differences later in life (Champagne et al., 2007). In this study, at P10, MIA pups from both strains have normal maternal attachment behaviour as suggested by their ability to recognise the familiar nest at P10. However, conflicting findings were previously reported in pups whose mother received gestational administration of LPS (Baharnoori et al., 2012) or valproate (Servadio et al., 2016). However, a better characterization of the early developmental period including differences in the neonatal environment are required to better target the diagnostic window of ASD and SZ (Careaga et al., 2017).

While C57BL6/J MIA offspring show a behavioural phenotype that reflects stereotyped and social core symptoms of ASD and SZ, they failed to show communication impairments in isolation-induced USV. Discrepancies from previous studies may reflect variations in the methodological approach to measure USVs. Here, USVs were measured at a single time point in order to avoid excessive stress due to separation whereas previous reports have measured USVs repeatedly at several developmental stages (Choi et al., 2016; Schwartzer et al., 2013).

As expected, MIA offspring from both strains presented with deficits in sociability and preference for social novelty in adulthood. These deficits have long been reported in C57BL6/J (Crawley, 2007; Moy et al., 2006) but, as far as we are aware, this is the first study to show these MIA-induced deficits in NIH Swiss mice. When mice social transmission of food preference was tested a decrease in the preference for the social cued food was observed in C57BL6/J. However, NIH Swiss control mice failed to show a baseline preference in this test. Social transmission of food preference is widely used as

a measure of communication deficits in animal models of autism and it also involves short-term and long-term memory (Greco et al., 2013; Silverman et al., 2010b; Wrenn et al., 2003). Thus, it is of interest that we observed no deficits in the novel object recognition after poly (I:C) treatment and we can conclude that cognitive impairments present in this study are specifically associated with social information processing. It is important to note that novel object recognition deficits have been previously reported in C57BL6/J poly (I:C) offspring in a short-term memory protocol (10 min.) (Ito et al., 2010) however, here we opted for a long-term memory protocol (24h).

In addition to the core behavioural symptoms, SZ and ASD patients are often diagnosed with mood disorders and stress (van Steensel and Heeman, 2017; Schwendener et al., 2009). One of the clearest behavioural findings of this study was that MIA induced differential effects on depression, stress response and anxiety-like behaviour in the two strains, with the outbred strain being more susceptible to MIA-induced changes in these measures. To our knowledge, this is the first time these effects have been observed in NIH Swiss mice. Within regard to C57BL6/J mice, our results showing an absence of changes in depression and anxiety–like behaviour in adulthood are in line with another strain comparison study by (Babri et al., 2014). They reported depression and anxiety-like behaviour, accompanied by an abnormal HPA axis response, in NMRI mice but not in the C57BL6/J. Surprisingly, in the present study we found that MIA increased time in the open arms of the EPM in the NIH Swiss strain. An increase in open-arm activity could be interpreted as reduced anxiety behaviour (Walf and Frye, 2007) or increased risk assessment (Blanchard and Blanchard, 1989). Although unexpected, increased time spent in the open arms of the EPM has been positively correlated with higher corticosterone levels (Mikics et al., 2005). Interestingly, the MIA-induced reductions in anxiety behaviour does not generalise to the open-field test cautioning over-interpretation of this aspect of the MIA phenotype. Indeed, it is important to note that to date the literature has reported contradictory results for the effects of MIA on depression and anxiety phenotypes (Giovanoli et al., 2013; Hava et al., 2006; Khan et al., 2014; Schneider and Przewłocki, 2005; Schwendener et al., 2009).

Early-life events such as MIA and stress are well known to contribute to HPA-axis programming during foetal development (Bale et al., 2010). In addition, a depressive phenotype has been associated with abnormal HPA axis response in MIA offspring in NMRI mice (Babri et al., 2014). In agreement with these previous findings, NIH Swiss mice exhibited increased corticosterone in response to an acute stress, thus evidencing that genetic background may play an important role in the development of the HPA-axis response and neuroanatomical substrates associated with mediation of stress response.

Oxytocin and AVP neuropeptides are produced by the paraventricular nucleus of the hypothalamus, which controls the HPA axis response by the regulation of corticotrophin releasing hormone. Thus, Oxytocin and AVP systems plays a modulatory role in neuroendocrine and behavioural functions including depression, anxiety and social behaviour (Insel, 2010,Neumann and Landgraf, 2012,Sarnyai and Kovács, 2014). Importantly, changes in OXT, AVP, *Oxtr* or *Avpr1a* have been shown to influence ASD and SZ phenotype (Carter, 2007; Demeter et al., 2016; Patterson, 2011). Notably, the strain-specific effects on HPA axis and behaviour in this study also tally with the reduction in the expression of *Avpr1a* (but not in *Oxtr*) in the hypothalamus of MIA NIH Swiss mice. It has been demonstrated previously that exposure to LPS during pregnancy could alter Avpr1a expression in the PVN but surprisingly this did not correlate with HPA axis responsiveness deficits (Shanks et al., 2000). Recently, decreased Oxtr binding in the amygdala was reported in the offspring of VPA-injected mothers (Bertelsen et al., 2017). It is tempting to speculate that changes in hypothalamic *Avpr1a* may at least in part mediate some of the strain differential effects induced by poly (I:C) administration. Given the emerging role of AVP and OXT as potential biomarkers and treatment targets for ASD (Carson et al., 2015), future studies should consider a more detailed investigation of AVP signalling pathway in the poly (I:C) MIA model.

Previous studies have highlighted the importance of gut-brain axis pathways on the intensity of behavioural symptoms in ASD and SZ (Hill et al., 2017). Several routes of communication between the gut and the brain have been studied including neuroendocrine and metabolic pathways, neuronal signalling, intestinal barrier function (“leaky gut”) and the immune system (Cryan and Dinan, 2012). It is well established that stress can affect

the developmental trajectory of the intestinal barrier and in has been associated with a "leaky gut" (Kelly et al., 2015; Smith et al., 2010). Importantly, a disrupted barrier function has been associated to alterations in microbiota in the poly (I:C) (Hsiao et al., 2013) and in the BTBR mouse model of autism (Coretti et al., 2017; Golubeva et al., 2017). A critical question addressed in this study was whether the strain dependent effects on behaviour, stress response and neuronal signalling could underlie GI dysfunction and compromise gut-brain axis signalling. Here, for the first time, we show an increase of intestinal permeability to FITC-dextran in MIA NIH Swiss, but not in MIA C57BL6/J offspring. Importantly, intestinal motility was not affected across the groups excluding the possibility of bias due to intestinal transit time. Conflicting results with previously reported findings in poly (I:C) injected C57BL6/J offspring may be due to the difference in the fasting time between this and the previous study (Hsiao et al., 2013).

The present study suggests that neuroendocrine and intestinal barrier pathways of the gut-brain axis are likely to be playing a role on strain differences in the vulnerability to MIA-induced behavioural abnormalities common to ASD and SZ. However, a better characterization of strain differences in the behavioural tests more closely related to SZ is also warranted in the future and will complement these findings. Recently (Kim et al., 2017) demonstrated that differences in the susceptibility to poly (I:C) insults of two C57BL6/J linage (from Taconic bioscience and Jackson laboratories) are dependent on the activation of TH17 cells and the presence of particular gut bacteria population. Since both the immune system and the microbiota are important pathways of the gut-brain axis communication, further mechanistic studies comparing the vulnerability of C57BL6/J and NIH Swiss mice should consider that. A limitation of the current study is that the influence of litter-to-litter variation has not been taken into account and may contribute to subject to subject variation (Vorhees et al., 2015). In conclusion, the findings of the current study are important for a better understanding of gut-brain connections in a widely used model of neurodevelopmental disorders in order to support future investigation on new effective treatments for neurodevelopmental disorders. However, the sequence of events that explain the presence of specific behavioural, stress and intestinal barrier changes in one strain but not another remains unknown.

# Chapter 5. Discussion

## 5.1 Summary of the Findings

The data generated in this thesis highlight the importance of early-life on shaping gut-brain axis development and function with major implications for mental health. We focus on the role of perinatal gut-brain axis disruption and its neurobehavioural consequences in two different scenarios: following birth by Caesarean-section *(*C-section) and after MIA with polyinosinic:polycytidylic acid (poly I:C) during pregnancy. To this end, firstly, we developed a mouse model of C-section and demonstrated for the first time that the mode of delivery at birth can induce anxiety-like behaviour, social and cognition deficits that is apparent in early life and persists into adulthood as observed in several different behavioural paradigms: pup ultrasonic vocalization (USV), maternal attachment, elevated-plus maze (EPM), open-field (OF), marble burying (MB) and 3-chamber test. In addition to an anxiety-like phenotype, mice born by C-section demonstrate impairments in maternal attachment, and deficits in the social recognition and novel object recognition tests. These neurobehavioral deficits were associated with an exaggerated response to acute stress, increased oxytocin receptor (OXTR) mRNA expression in the amygdala and increased *Corticotropin-releasing hormone receptor 1* (Crhr1) mRNA expression in the pituitary gland of CS born offspring, and an abnormal immune response in adulthood. One of the most striking results of this thesis is that C-section leads to marked changes in gut microbiota composition and diversity in early-life and adolescence, with decrease in *Bifidobacterium* spp. at P21 (**Chapter 2**), similar to what has been reported in humans born by C-section (Dominguez-Bello et al., 2010, 2016). Interestingly, some of the deficits induced by C-section, including impaired recognition, are reversed by co-housing C-section with VB mice in adolescence, which effectively transfers microbiota due to coprophagia, and highlights the importance of the microbiota in neurobehavioural development (**Chapter 2**). By targeting the gut microbiota from birth with bifidogenic diets (prebiotics and probiotics) we were able to reverse anxiety-like behaviour in early-

life and recognition deficits both in early-life and in adulthood (**Chapter 2**). We also observed that increased OXTR expression in amygdala of CS mice is reversed by promoting growth of bifidobacteria. In a complementary experiment in Chapter 2, we investigated whether aspects of the core C-section phenotype might be observed in humans. Young volunteers of known birth modality underwent a Trier Social Stress Test (TSST) to investigate hypothalamic pituitary adrenal (HPA) axis, inflammatory and psychological responses to acute psychosocial stress. Strikingly, stress-related behavioural and immune system alterations were also observed in a cohort of young-adult individuals born by C-section (**Chapter2**).

Given that data from **Chapter 2** strongly implicate C-section in social recognition deficits and associated changes in OXTR expression within the amygdala, in Chapter 3 we further investigated whether treatment with OXT from postnatal (P)1 to P5 (via s.c. injection) was able to prevent C-section born offspring from developing the associated phenotype. Interestingly, early postnatal administration of OXT reversed specific effects associated with mode of birth, including early-life deficits in maternal attachment, impaired social recognition and some aspects of anxiety-like behaviour in adulthood. OXT treatment also attenuated the splenic immune response in C-section born mice stimulated with Lipopolysaccharide (LPS) and prevented C-section-associated impairments in intestinal motility. Furthermore, we confirmed that postnatal OXT treatment has enduring effects on the OXT system, as elevated plasma OXT was observed in these mice into adulthood, although OXTR and vasopressin (AVPR) mRNA expression remained unchanged.

Since MIA has previously been reported to alter the microbiota composition of mouse offspring (Hsiao et al., 2013), we further interrogated the significance of gene x environment interactions in priming the gut-brain axis function using this paradigm. Chapter 4 investigated whether administration of poly I:C at gestational day 12.5 would lead to behavioural, physiological and molecular changes relevant to neurodevelopmental disorders in offspring of outbred (NIH Swiss) and inbred (C57BL6/J) mouse strains. By comparing these two different strains we were able assess the effects of genotype and environment on the susceptibility to develop a dysfunctional gut-brain axis. We demonstrated that these strains differ in anxiety and depression-like behaviours, with the

effects more pronounced in NIH Swiss mice. These strain-specific behavioural effects in the NIH Swiss mice were associated with marked changes in important components of gut-brain axis communication: stress responsivity and gut permeability.

## 5.2 The Stress of Being Born Without a Maternal Microbial Signature

That birth is a stressful experience for both mother and newborn is well established (Lagercrantz, 2016). Freud considered being born as the very first stressful experience in life and thus a source of anxiety. In fact, the passage through the birth canal involves a combination of events that trigger the stress response in the newborn and is referred to as "the stress of being born" (Lagercrantz and Slotkin, 1986b). As a matter of fact, infants born by vaginal delivery have higher levels of stress hormones right after birth in comparison to CS babies (Chiș et al., 2017; Taylor et al., 2000; Vogl et al., 2006). This activation of HPA-axis with a peak in corticosterone right after birth is considered an adaptive response to environmental challenge (Lagercrantz, 2016).

The latest research showed that microbiota is required for a proper HPA-axis development, activation, and response (Foster and McVey Neufeld, 2013; Foster et al., 2017; Golubeva et al., 2015; Neufeld et al., 2011; O'Mahony et al., 2014; Sudo et al., 2004). Early life disruption of the microbiota is implicated in stress- and anxiety-related disorders later in life (O' Mahony et al., 2015). This impact extends beyond the HPA-axis, influencing many aspects of brain development, function and behaviour (Cryan and Dinan, 2012; Foster et al., 2017; Sharon et al., 2016). The assembly of an individual's gut microbial community is determined by maternal–offspring exchanges that primarily occur during, and shortly after, birth (Mueller et al., 2017). As discussed in the **Chapter 1**, these processes are influenced by several prenatal and postnatal factors, with the mode of delivery at birth being critical for shaping microbial composition and diversity of the newborn gut (Tamburini et al., 2016). This concept underlies the main hypothesis of **Chapter 2**, that birth by C-section impacts the wiring of the entire microbiota-gut-brain axis, with consequences to stress reactivity and behaviour. By using an animal model, we

remarkably demonstrated that birth by C-section induces long-term effects to stress and anxiety-like behaviour in early-life and in adulthood with long-term consequences for the HPA-axis response and stress-associated gene expression of Crhr1 and OXTR in corticolimbic structures. These changes occurred in parallel with marked alterations in the gut microbiota in early-life and adolescence. By transferring VB microbiota to CS mice by co-housing in adolescence or restoring Bifidobacteria through dietary interventions from birth, we were able to attenuate some aspects of the anxiety-like behavioural phenotype induced by C-section. Furthermore, in **Chapter 2** we took a further step, to assess whether these findings translated to a human cohort of young adults. We demonstrate that healthy young adults delivered by C-section also exhibit an increased psychological vulnerability to either acute or prolonged stress. Although differences in gut microbiota are resolved by adulthood in both mice and humans, C-section circumvents natural exposure to the vaginal microbiota during labor and birth, altering HPA-axis development, with enduring consequences to behaviour.

The precise mechanisms whereby early-life microbes determine the set point of the HPA-axis are unknown, but it is suggested that microbial signaling molecules interact with neuro-immuno-endocrine pathways (Lyte and Ernst, 1992; Sudo, 2014). Early-life microbes derived from the mother are also important for setting the immune system response, preparing the newborn for host-microbial mutualism (Gomez de Agüero et al., 2016). Interestingly, the data obtained in **Chapter 2** also demonstrate that C-section exacerbates immune-system responses, with increases in pro- and anti-inflammatory cytokines, both in mice and in humans. These results are in line with several reports that associate C-section with higher risk of developing immune system disorders (Cho and Norman, 2013; Hansen et al., 2014). The gut microbiome is known to secrete luminal catecholamines including norepinephrine (NE), which is a key mediator of the HPA-axis (Sudo, 2014). Both corticosterone (CORT) and NE can modulate immune system response through sympathetic innervation of immune system tissues, including spleen and thymus, and immune cells, such as lymphocytes and monocytes (Kraneveld et al., 2008). The C-section animal model developed in this thesis offers a valuable means to study the mechanisms underlying microbiota and neuro-immuno-endocrine interactions in a more

natural human condition, as opposed to the use of germ-free (GF) animals. Using a combination of pharmacological approaches, optogenetics, circuit tracing, and behavioral analysis will be crucial for elucidating these pathways.

Recently, it was revealed that gut microbiota is necessary for microglia maturation and activation (Erny et al., 2015). Interestingly, glucocorticoid receptors are highly expressed on microglia throughout the brain (Bellavance and Rivest, 2014). Whether microglia activation is affected by C-section in unclear, but currently under investigation. Microbiota-HPA-axis-microglia cross-talk could potentially be investigated by use of sequencing methods, including single-cell genomic analysis of microglia. The use of single cell transcriptomics enables researchers to identify specific subsets of microglia in neuroinflammatory conditions (Boza-Serrano et al., 2018). A whole-transcriptome resource of microglia gene expression across different stages of central nervous system (CNS) and microbiota development will be helpful to understand this relationship.

Although activation of the HPA-axis and stress responses enable organisms to cope with situations and promote adaptation, sustained stress exposure and HPA activity can have adverse effects on brain function and behaviour (van Bodegom et al., 2017; Faravelli et al., 2012). Therefore, these findings have significant implications, due to increases in C-section deliveries and the potential for stress-associated disorders later in life. Future studies should also consider sex differences on C-section's effects, both in mice and humans.

## 5.3 The Neurobiology of Attachment and Neuronal Substrates of Early-life Disruption of the Gut Microbiota

Stress can critically impact social behaviour and brain circuits that are engaged in the processing of social information and expression of social behaviour (Sandi and Haller, 2015). An interesting finding of this thesis is that microbial disruption in early-life alters the expression of genes associated with stress responses and social behaviour in adulthood (**Chapter 2**). Of note, birth by C-section impairs maternal attachment, social recognition

and increases the number of distress calls early in life (**Chapter 2**). Social recognition deficits persist into adulthood (**Chapter 2**). Offspring born to MIA mothers also demonstrate strong social behaviour deficits in adulthood (**Chapter 4**). Indeed, the finding that gut microbiota can influence social behaviour is one of the most consistent in the field (Needham et al., 2018; Stilling et al., 2014). GF mice exhibit abnormal social interactions (Desbonnet et al., 2014) and microbiota alterations often are reported in association with social disorders and are also present in animal models of autism spectrum disorders (ASD) (de Theije et al., 2014; Buffington et al., 2016; Golubeva et al., 2017; Hsiao et al., 2013). Microbiota, attachment and social behaviour may be closely correlated, but the neuronal mechanisms mediating these interactions are still an important and yet unanswered question in the field.

"Attachment is a psychological construct reflecting a strong emotional bond between two individuals, such as relationship between a mother and her child" (Porges, 2003). In mammals, attachment is facilitated by the social engagement system which involves the combination of somatomotor and visceromotor components which initially coordinate suckling-swallowing-breathing-vocalizing in a newborn. Some researchers contend that it is difficult to think of any behavioural process that is more intrinsically important to humans than attachment (Carter and Porges, 2011). Indeed, for mammals, social behavioural interactions allow for survival and increase fitness of the species. As humans are social animals, we live for social attachments. Social attachment also involves neural circuits mediating motivation and reward (Insel, 2003; Insel and Young, 2001). Disruptions of the social behavioural system early in life can lead to difficulties in sociability and emotional processing later in life (Carter and Porges, 2011). Deficits in social interactions seem to be a common facet of neurodevelopmental disorders. Thus, the findings of this thesis can inform about the complex relationships between early-life, microbiota, attachment and social behaviour relevant to neurodevelopmental disorders.

AVP and OXT systems mediate attachment and social bond formation in mammals (Donaldson and Young, 2008). In recent years, OXT and AVP have received growing attention due to evidence that OXT may have important clinical applications for disorders such as ASD, anxiety and addiction (Neumann and Slattery, 2016; Peters et al., 2013;

Shen, 2015; Young and Flanagan-Cato, 2012). An noteworthy finding of this thesis is that early life disruption of the microbiome leads to alterations in the OXT and AVP systems, in C-section born and MIA offspring, respectively (**Chapter 2** and **Chapter 4**). While birth by C-section decreases OXTR expression in the amygdala, MIA decreases AVPR expression in the paraventricular nucleous of the hypothalamus (PVN), in a strain-specific manner. When C-section born mice received OXT treatment for the first 5 days of life, some of the C-section-mediated effects on attachment, social behaviour and some aspects of anxiety-like behaviour were attenuated. OXT reversed gastrointestinal deficits and blunted immunological response to CS (**Chapter 3**). Whether OXT has the potential to ameliorate MIA-associated effects remains to be addressed.

That early-life OXT administration acts on the immune system and regulates gastrointestinal motility in mice born by C-section is intriguing and suggests a potential role for OXT actions on the gut-brain axis, perhaps directly attenuating the effects of birth in the absence of a maternal microbial signature. Intriguing, treating CS offspring with a bifidogenic diet from birth was able to restore OXTR expression in the amygdala of adult mice, and restore normal attachment and social behaviours. Recently, supplementation with *L. reuteri* was shown to increase plasma levels of OXT, with beneficial effects to the host and improvements in general health (Erdman and Poutahidis, 2014). Moreover, *L. reuteri* modulates immune response, increases maternal care, modulates social behaviour, and attenuates stress responses through OXT production (Buffington et al., 2016; Erdman and Poutahidis, 2014; Poutahidis et al., 2013; Varian et al., 2017). Since the microbiota-gut brain axis involves bidirectional pathways, the findings of this thesis open an avenue for exploration of the effects of the neuropeptide OXT on the microbiome in a top-down perspective, as well as it supports the search for microbial interventions that modulate OXT system in the brain from the bottom-up. The timing of our pharmacological intervention indicates an organizational effect of OXT on priming behaviour, immune and gastrointestinal function.

There are other issues of OXT pharmacology that should be considered. For example, there is significant cross-talk between OXT, AVP and other neurotransmitter systems such as gamma-aminobutyric acid, dopamine and opioid receptors (Hung et al., 2017;

Khazipov et al., 2008; Neumann et al., 2006; Panksepp, 1992). Thus, some of the effects of exogenously administered OXT could be due to interactions with these other neurotransmitter systems. Moreover, the positive effects of OXT seem to be dose-dependent, varying across mouse developmental window; while high-dose (HD) OXT reversed social recognition deficits induced by C-section in early-life, only low-dose (LD) OXT was effective in ameliorating social recognition deficits by adulthood (**Chapter 3**). Such dose-dependent effects of OXT are not surprising, given the dynamic plasticity of the OXTR system across the life-span (Bales and Perkeybile, 2012; Kosaka et al., 2016); however it adds complexity from a pharmacological treatment perspective. Another relevant aspect of OXT is its sexually dimorphic nature, with different functions and receptor distributions between males and females (Bales et al., 2007). Thus, further studies should evaluate sex differences in susceptibility to behavioural deficits induced by early life gut-brain axis perturbations and with respect to OXT treatment. Although OXT is shown to increase trust, stimulate social engagement and attachment in humans (Johnson and Young, 2017), very little is known about OXTR distribution in the human brain. Thus, caution should be taken when extrapolating these findings in OXT systems of mice to humans (Andari et al., 2017).

The fact that early-life disruption of the microbiota induces attachment and long-lasting social recognition deficits, with possible role for OXT and AVP system, is one of strongest findings of this thesis and should be explored in depth, both in terms of mechanisms and from an intervention perspective.

## 5.4 New Tools for Investigating Mechanisms Underlying Microbial Modulation of Oxytocin System and Behaviour

Our findings indicate a pharmacological mechanism by which a disrupted microbiota could modulate behaviour. Because early-life is a very sensitive period for brain and for OXT system, some of the effects of the mode of delivery observed in the **Chapter 2** were blunted by the injections in early-life in **Chapter 3**. These conflicting results may be

explained by handling effects on maternal care and on OXT system development (Bales and Perkeybile, 2012).

An interesting perspective study could include the use of OXTR, OXT, AVP or AVPR knockout animals colonised with the microbiota of MIA or CS mice, to test the role of gene and environmental interactions in relevant behavioural tests. Instead of inducing a global knockout of OXT or OXTR it is also possible to manipulate regional expression of receptors through the use of viral vectors. Moreover, the use of optogenetics technology and Cre-lines expressed from the OXTR and AVPR promoter would be also helpful in unraveling neurocircuits involved. Sequencing methods such as single-cell sequencing of PVN, supraoptical nucleous, medial amygdala would also bolster our understanding of the role of microbiota on the OXT system. The use of these tools are crucial for addressing mechanisms of interaction between gut microbiota and the brain with application to psychiatric disorders.

## 5.5 Gene x Environment Interactions and Vulnerability to Microbiota-gut brain axis insults

Neurodevelopmental disorders such as ASD and schizophrenia (SZ) have multifactorial origins. Recent discoveries have advanced our understanding of the genetic and environmental factors that may trigger their manifestation. Numerous environmental risk factors contribute to the increased risk for neurodevelopmental disorders, including: birth mode, MIA and maternal infection, season of birth, maternal diet and medication, early-life stress, drug abuse and microbiota (Buffington et al., 2016; Kelly et al., 2017; Kraneveld et al., 2016; O' Mahony et al., 2015; Patterson, 2002; Sampson and Mazmanian, 2015). Despite the progress in the identification of risk factors, we still have a poor understanding of how these factors interact with genes in neurodevelopmental disorders. In the **Chapter 4** of this thesis, we aimed to investigate one such interaction by using a very well-established model of neurodevelopmental disorders, MIA induced by poly I:C administration. The link between maternal infection and neurodevelopmental disorder had been appreciated for many years (Meyer et al., 2007; Patterson, 2002). More

recently, the Zika virus outbreak and the surge in cases of microcephaly have finally returned public attention to the detrimental effects of maternal infection (Estes and McAllister, 2016; Jurado et al., 2017), reinforcing the urgency for understanding these associations.

As described in the **Chapter 1**, in addition to the core social symptoms associated with ASD, gastrointestinal symptoms are frequently reported. Recently, causal associations between alterations in the microbiota-gut-brain axis and ASD have been demonstrated in animal models (de Theije et al., 2014; Buffington et al., 2016; Golubeva et al., 2017; Hsiao et al., 2013; Kim et al., 2017). Here we investigated whether administration of poly I:C on gestational day 12.5 could lead to behavioural, physiological and molecular aspects relevant to neurodevelopmental disorders, in a strain-dependent manner. Although MIA induced social behavioural deficits in both strains, the effects of MIA on anxiety- and depression-like behaviours, stress reactivity and gut permeability were more pronounced in NIH Swiss mice (**Chapter 4**). The findings in this thesis complement existing literature on the MIA model and indicate the possible role for gene x environment interactions in modulating gut-brain axis programming during gestation, which may contribute to neurodevelopmental disorders. A very pertinent study recently demonstrated that the susceptibility to poly (I:C) insults of two C57BL6/J lineages (from Taconic bioscience and Jackson laboratories) may be dependent on the presence of particular gut bacteria populations which activate TH17 immune responses (Kim et al., 2017). These reports support the findings of this thesis, indicating that the differential effects observed in NIH Swiss mice and C57BL6/J may reflect differences in gut microbiota composition between the two strains.

The bidirectional interaction of the microbiome and immune system is evident from multiple mouse studies using GF mice, which demonstrate that microbiota is required for development of a proper innate immune system, in both the periphery and in the CNS (El Aidy et al., 2015). Identification of individual genetics with environmental factors and the microbiome is needed for the development of personalized microbiota targeting treatment (Kurilshikov et al., 2017). Going forward, this will require detailed metagenomic analyses, more complete bacterial and brain sequencing, testing proof of concept ideas in

more than one mouse lineage and sex differences. These studies will provide important information that can used to manipulate this system to support neurodevelopmental disorder treatment and/or management.

## 5.6 Early-life: a Critical Period of Unique Sensitivity

For more than a century, clinical research has demonstrated that early life can influence the development of adult psychopathology (Bale et al., 2010). The emergence of the gut-brain axis has driven a paradigm shift in the way we view mental health and neuropsychiatric disorders (Dinan and Cryan, 2017b). The early life period is marked by the dynamic development of multiple physiological systems and domains, wherein key elements of the gut-brain axis, such as immune system, CNS, gastrointestinal tract and HPA-axis develop and mature in parallel with the microbiota (O' Mahony et al., 2015). Strong evidence points to the presence of a window of opportunity or vulnerability in early-life, during which changes in gut microbial colonisation can result in immune and HPA-axis dysregulation, and perturbation of the complex processes of brain and behavioural development *(Borre et al., 2014)*. The overall goal of this thesis was to investigate the impact of early-life disruption of the gut-brain axis and its neurobehavioural consequences. The findings presented in this thesis add relevant information as to the effects of early-life insults to gut-microbiota colonisation with public health and mental health implications.

C-section is a lifesaving procedure that is deemed necessary in approximately 10 to 15% of births to avoid risking the life of mother and/or child (Deshpande and Oxford, 2012). However, the number of infants delivered by C-section has rapidly increased worldwide over recent years with many regions far exceeding the World Health Organization guidelines (Betran et al., 2016). Currently, the emphasis of research and discussion around mode of delivery is on the benefits and potential harms for the pregnant woman herself (Cho and Norman, 2013). C-section birth has been associated to a greater risk of developing immune and metabolic-related disorders More recently, studies using an animal model of C-section support a causal relationship between microbial dysbiosis induced by C-section and increased body weight (Martinez et al., 2017) and innate-

immune dysfunction (Hansen et al., 2014). The findings of this thesis suggest that birth by C-section affects priming of the entire gut-brain axis, leading to stress hyperresponsivity, anxiety-like behaviour, and recognition deficits. Although we are only just starting to understand the mechanisms underlying these associations, our findings raise concerns regarding the overuse of C-section in modern medicine and provide new information on the importance of early-life microbial colonisation for neuro- and behavioural development, its potential consequences on mental health, and ultimately the introduction of an animal model for investigating microbiota-gut brain axis interactions.

MIA has long been associated with an increased risk for neurodevelopmental disorders. The poly(I:C) MIA mouse model has both face and construct validity for ASD and SZ, and has largely contributed to our understanding neuro-immune interactions during foetal brain development and the manifestation of psychiatric disorders (Patterson, 2002). However, the microbial components of these interactions are only beginning to be explored (Estes and McAllister, 2016). The findings of this thesis reinforce the contribution of gene-environment interactions in gut-brain axis dysfunction. Unravelling these interactions will be important for identifying individuals at-risk for neurodevelopmental disorders.

## 5.7 Early-life: a Window of Opportunities

As discussed in **Chapter 1**, pregnancy, lactation and weaning are critical periods for microbiota maturation. The fact that gut microbiota is modifiable at these stages, presents windows of opportunity. Thus, early-life also represents a preferred stage of life for targeting the gut microbiota, in order to prevent or ameliorate the effects of pathologies, including neuropsychiatric conditions associated with microbiota-gut-brain axis dysfunction. In fact, data from numerous studies has shown that the use of probiotics to support therapeutic agents is a safe, more economically affordable and less invasive treatment. In this thesis we first targeted the microbiota in adolescence, by using a common approach for microbial transfer: co-housing. In a proof of concept experiment, we demonstrated that transferring VB microbiota to CS mice by co-housing can reverse recognition deficits induced by C-section and some aspects of anxiety phenotype (**Chapter 2**). These findings highlight the

importance of environment, including individuals with whom we interact, on shaping the gut microbiota (Song et al., 2013; Thevaranjan et al., 2017). Our data are in line with a recent study which demonstrated that microbiota transfer by co-housing mice exhibiting autism-like features with controls partially restores behavioural phenotype, improves sociability and modulates the OXT system (Buffington et al., 2016).

Recently, partial restoration of the gut microbiota of infants born by C-section was successfully demonstrated via vaginal microbial transfer. Vaginal seeding, performed by swabbing babies with vaginal fluid over their entire bodies, successfully colonised the newborn gut with maternal vaginal microbes for up to 30 days post-birth and are now in clinical trials (Dominguez-bello 2016). However, this practice is controversial, due to the lack of sustained evidence and further cohorts are necessary to attest to the safety and efficacy of vaginal seeding. Here, we recapitulated the maternal-offspring vertical transmission by feeding offspring *Bifidobacterium breve* and a bifidogenic diet. The importance of vertical transmission of Bifidobacteria for priming the immune system in early-life has been well-characterised (Makino et al., 2013; Ménard et al., 2008). Moreover, *Bifidobacterium breve* have been used in pediatric practice for clinical conditions such as necrotizing enterocolitis (Anderson, 2015; Caplan et al., 1999; Kitajima et al., 1997; Patel and Underwood, 2018). In **Chapter 2** of this thesis, we report for the first time that restoration of Bifidobacteria in the neonatal gut affects brain and behavior, and the current probiotic and prebiotic presented here could potentially be explored as a coadjutant treatment for many neurodevelopmental conditions associated with microbiota-gut-brain axis dysregulation. Manipulating gut microbiota during the early-life period offers the potential for therapeutic interventions before deleterious symptoms appear, decreasing the incidence of, or even preventing, the emergence of psychiatric illnesses associated with microbiota-gut-brain axis dysfunction in adulthood.

A key environmental determinant of infant gut colonisation is breastfeeding. Breast milk is a direct route of maternal-offspring transmission of both microorganisms and prebiotics, influencing immune system development (Pannaraj et al., 2017; Walker and Iyengar, 2015). However, breast milk is not only a source of microbiota but also supplies the infant with important hormones, such as OXT (Higashida et al., 2017). In this thesis

we have demonstrated that early-life treatment with OXT prevented the development of gut-brain axis alterations in mice born by C-section. Although research has only just begun to unravel the interactions between microbes-oxytocin and gut-brain interactions, and the findings in animal models are limited, the results of this thesis have some clinical significance. C-section should not be discontinued as medical-indicated C-section can be truly lifesaving. However, there should be greater awareness and discussion about the associations between elective C-section and its potential adverse health effects on offspring. On the other hand, strategies that reestablish or rebalance the gut microbiota and increase oxytocin exposure in early-life may serve as a novel and worthy target for improving health outcomes associated with the procedure.

## 5.8 Conclusion

Taken together the findings of this thesis advanced our knowledge on neurobehavioural consequences of early life microbiota disturbances. Here, we suggested that targeting the microbiota-gut-brain axis from birth can have preventive effect on behavioural and physiological deficits. Understanding the connection between the gut microbiome and brain early life will support research towards development of a preventive therapy based on prebiotic, probiotic and pharmacological therapies for neurodevelopmental disorders. Elucidating this mechanisms and development of safe and effective therapeutics are needed to address the modern changes in lifestyle, including burden of maternal infection, improved sanitization, C-section, indiscriminate antibiotic use that can shift the microbiota composition, and are being studied as potential drivers of increase in psychiatric, immune and metabolic disorders in the developed world.

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