THE INTERPLAY OF GUT MICROBIOTA AND THE IMMUNE SYSTEM IN BEHAVIOR

MICROBIOTA INDUCED IMMUNE SYSTEM MATURATION PLAYS A KEY ROLE IN DEVELOPMENT OF NORMAL BEHAVIOR

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ABSTRACT

Gut microbiota has been shown to regulate the growth and development of the central and enteric nervous systems (CNS and ENS) after birth. There is ample evidence to suggest that intestinal bacteria can influence behavior of the host through both immune and immune-independent mechanisms. Gut-microbiota-regulated CNS structural changes are focused in the limbic system, at centres associated with memory, social and emotional behaviour. Several studies using germ-free (GF) and specific pathogen free (SPF) mice demonstrated microbial influence on behaviour development accompanied by neurochemical changes in the brain. Higher exploratory and lower anxiety-like behavior was found in GF mice compared to SPF mice with lower central expression of neurotrophins, such as nerve growth factor and BDNF. The mechanisms by which the microbiota influences behavior are unknown but could be immune-mediated, neural, or humoral in origin.

In this study I investigated the role of immune system maturation on mouse behaviour after bacterial colonization. I showed that mono-colonization of GF mice with *E. coli* normalizes behaviour similar to colonization with complex microbiota (SPF and ASF) and the continuous presence of bacteria is not required to maintain this normal behaviour. I also showed that innate immunity through the MyD88/Ticam pathway is crucial for the development of normal behaviour and that multiple innate immunity and neuronal genes are involved in this process.

Together these results suggest that bacterial colonization primes and matures the innate immunity and development of the central nervous system ultimately leading to normal behaviour. I believe that bacterial colonization at birth is not only important for the epithelial barrier function, gut homeostasis, and immune functions, but also for the development of normal behaviour. Altered immune priming during the postnatal period due to abnormal microbial colonization may have wider implications when considering psychiatric disorders in humans.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACTH	Adrenocorticotrophic hormone
AHR	Aryl hydrocarbon receptor
ANS	Autonomic Nervous System
AP-1	Activator Protein-1
ASD	Autism spectrum disorder
ASF	Altered Schaedler flora
ATM	Antimicrobial
BBB	Blood-Brain-Barrier
BDNF	Brain-Derived Neurotrophic Factor
CBA	Cytometric Bead Array
CNS	Central Nervous System
CRH	Corticotrophin release hormone
cSPF	Conventionally raised SPF mice
D-Ala	D-isomer of alanine
DCs	Dendritic cells
DG	Dentate Gyrus
DNBS	Dinitrobenzene sulfonic acid
DRG	Dorsal Root Ganglia
DSS	Dextran sulfate sodium
ECs	Enterochromaffin cells
EGCs	Enteric glial cells
ENS	Enteric Nervous System
GABA	γ-aminobutyric acid
GBA	Gut-Brain Axis
G-CSF	Granulocyte colony stimulating factor
GDNF	Glial cell-derived neurotrophic factor
GF	Germ-free
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GPCRs	G protein–coupled receptors
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary Adrenal
IBD	Inflammatory Bowel Disease

IBS	Irritable Bowel Syndrome
IDO	Indoleamine-2,3-dioxygenase
IFN-γ	Interferon-γ
IL-10	Interleukin- 10
IL-12p70	Interleukin-12p70
IL-6	Interleukin-6
ILFs	Isolated lymphoid follicles
IPA	Ingenuity pathway analysis
LPS	Lipopolysaccharides
MAMPs	Microbial-associated Molecular Patterns
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
m-DAP	Meso-diaminopimelic acid
MLN	Mesenteric lymphnodes
MPO	Myeloperoxidase
NE	Norepinephrine
NMDA	N-methyl-D-aspartate
NOD	Nucleotide oligomerization domain
NR2B	N-methyl D-aspartate receptor subtype 2B
NTS	Nucleus of the Solitary Tract
Ocln	Occludin
PBS	Phosphate-buffered saline
PNS	Peripheral Nervous System
Poly I:C	Polyinosinic : polycytidylic acid
PPs	Peyer patches
PRRs	Pattern recognition receptors
PSNS	Parasympathetic Nervous System
PVN	Paraventricular nucleus
SCFAs	Short Chain Fatty Acids
SCID	Severe Combined Immunodeficiency
Ser32	Serine-32
SNS	Sympathetic Nervous System
SPF	Specific Pathogen-Free
TDO	Tryptophan-2,3-dioxygenase
TH	Tyrosine hydroxylase
Thr232	Threonine-232

TLRs	Toll-like receptors
TnaA	Tryptophanase enzyme
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	Tumor Necrosis Factor
TPH	Tryptophan hydroxylase
T-reg	Regulatory T-cells

CHAPTER 1: INTRODUCTION

Intestinal Microbiota

The human body coexists in a mutualistic relationship with a complex microbial ecosystem collectively referred to as the intestinal microbiome. There are unique microbiota profiles in specific niches such as the skin, mouth, respiratory tracts, and the gastrointestinal (GI) tract[1]. Bacterial colonization of the GI tract occurs during birth as well as shortly thereafter, and almost 100 trillion microorganisms colonize the intestines. More than 1,000 bacterial species have been identified in this microbiome using cultured and molecular-based approaches. Although there are considerable inter-subject variations in the microbial composition due to environmental and genetic factors, a 'core' gut microbiota composition exists from birth to old age[2]. During birth, the GI tract is colonized with vaginal or maternal skin bacteria depending on vaginal or caesarean birth, respectively. 16S rRNA sequencing has revealed that a healthy, vaginally delivered infant gut is colonized initially by *Bifidobacterium, Lactobacillus, Enterobacteriaceae* and *Staphylococcus*, with later increases in *Veillonella* and *Lachnospiraceae*[3]. The infant gut microbiota is changing in parallel with the maturation of the immune system until around two years of age, when solid foods are introduced. Around the third year of life the composition stabilises and begins to resemble an adult-like microbial composition[4]. The vast majority of gut bacteria in adults fall into the *Firmicutes* and *Bacteroidetes* phyla, with a much smaller proportion into the *Actinobacteria*, *Proteobacteria,* and *Verrucomicrobia* phyla[5]. The microbial diversity in an adult GI tract is resilient to

change and play an important role in the proper maturation of the immune system, enhancing nutrient acquisition, strengthening the integrity of the intestinal barrier and providing defence against pathogenic microorganisms[6].

The intestinal microbiota is commonly referred to as a metabolically active organ, as there exists a dynamic relationship between the host and its resident microbiota, particularly in relation to the health of the host[7]. The advent of metagenomic and metabolomic profiling has brought about a resurgence of interest in the intestinal microbiome and its impact on health and disease. The abundance of genes contained within this microbial organ far outweighs the genetic content of the host by an estimated factor of 50-200x and it is conceivable that for these reasons the microbiota is able to maintain a powerful influence on the host, particularly on GI tract and GI-related functions[8]. Alterations in the gut microbiota (dysbiosis) due to antibiotics, infections, medications, and long-term dietary changes can potentially lead to detrimental negative health outcomes including diarrhea, opportunistic infections, and obesity[9, 10]. Most chronic gut disorders such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), and celiac disease are associated with dysbiosis[11]. Future studies are needed in order to shed light on whether a particular microbial composition is associated with specific diseases.

Gut microbiota influences brain function and development

The last decade of research has shown that microbiota can influence not only the immune and metabolic systems, but also the nervous system[12]. While understanding the mechanisms by which gut bacteria control immunity and metabolism have led to a modern renaissance in biomedical research, regulation of the nervous system by the microbiota had remained relatively unexplored until very recently[13]. How could gut microbes influence a complex and distant organ such as the brain? The notion that microbiota can influence neurological functions was already known through parasites capable of altering behavior and environmental preferences to benefit the microbe. For example, *Toxoplasma gondii* can alter the host rodents' fear response causing it to be an easy prey for cats and allowing *Toxoplasma* to continue its lifecycle in the feline host[14]. The *Spinochordodes tellinii* parasite causes infected grasshopper hosts to seek aquatic environments where the parasite emerges to mate and produce eggs[15]. Temperature preference of the host can also be altered, as observed during infection of stickleback fish by *Schistocephalus solidus*, which changes the hosts' preference from cooler to warmer waters where the parasite can grow more readily. Microbes can influence the social behavior of their hosts, causing insects, such as ants, to become more or less social to the benefit of the parasite[16].

All of the above examples raise the possibility that microbes, which are in constant, life-long interaction with their human and animal hosts, could influence brain function and behavior. Perhaps the best known pieces of evidence for a

role of gut bacteria in human brain function are the beneficial effects of laxatives and oral antibiotics in patients with hepatic encephalopathy[17]. Antibiotics have also been anecdotally reported to induce acute psychosis in patients which resolved after withdrawal of the drug[18]. Dysbiosis has been shown to be present in patients with autism and interestingly, some patients with late-onset autism ameliorate their symptoms after antibiotic administration[19].

A number of approaches have been utilised in preclinical models to investigate how the gut microbiota influences brain function and behaviour, including the use of germ-free (GF) mice, faecal microbiota transplant, probiotics and antibiotic treatments. *Lyte et al* were the first to show that mice infected with *Campylobacter jejuni* displayed anxiety-like behaviour before the onset of any discernible inflammatory response to infection[20]. Subsequent studies from this group suggested that the neural pathways - specifically the vagus nerve - were involved in this altered behavior[21]. Several studies reported marked behavioral differences between GF and SPF colonized mice. Higher exploratory and lower anxiety-like behavior was found in GF compared to SPF mice using standard behavioral tests (elevated plus maze, open field and light/dark preference)[22]. *Heijtz et al* showed that compared to GF mice, SPF mice had higher central expression of neurotrophins, including nerve growth factor and BDNF, as well as altered expression of genes involved in the secondary messenger pathways and synaptic long-term potentiation in brain regions involved in motor control and anxiety-like behavior[22]. *Neufeld et al* demonstrated that the presence of

microbiota also affects expression of N-methyl-D-aspartate receptor subunit, NR2B in amygdala and serotonin receptor 1A in the hippocampus[23]. GF mice also exhibit altered spatial working memory and reference memory, which could be indicative of impaired hippocampal development[24]. The aforementioned GF studies suggest that the presence of microbiota influences the development of the neuronal circuitry that is relevant to anxiety-like behaviour, motor control, memory and learning. The observed difference between GF mice and those colonized with complex microbiota may relate, at least in part, to the ability of bacteria to affect host immunity, metabolism, and gut physiology. It has been shown that even mono-colonization with *B. thetaiotaomicron* alters the expression of a large array of host genes involved in multiple bodily functions[25].

The effect of bacteria on behavior and brain chemistry was also demonstrated in conventionally raised mice. Our group showed that perturbation of healthy intestinal microbiota using oral non-absorbable antimicrobials (ATM) in mice increased exploratory behavior and altered expression of BDNF in the amygdala and hippocampus[26]. These changes in brain chemistry and behaviour was not due to the direct effect of ATM since no difference was observed in SPF mice treated with ATM intraperitoneally or when ATM was administered orally in GF mice. Confirmation that the microbiota influences the brain comes from the observation that behavioural traits of donor mice can be adoptively transferred into adult GF mice of a different strain via the intestinal microbiota[26]. Our group showed that GF mice colonized with microbiota from their own strain (BALB/c or

NIH Swiss), displayed similar exploratory behaviour as their conventionally raised SPF counterparts. However, GF mice colonized with microbiota from the other strain displayed a behavioural profile similar to that of the donor strain[26]. Furthermore, *Bacteroides fragilis* has improved anxiety-like behavior, as well as social and repetitive behaviors in an animal model of autism with the underlying mechanisms being related to changes in gut microbiota composition and serum metabolomic profile[27].

Specific probiotic bacteria have been shown to affect the CNS. *Bifidobacterium longum NCC3001* normalized anxiety-like behaviour and BDNF expression in the hippocampus of mice with mild to moderate colitis that was induced by a chronic parasitic infection[28]. Similarly, in a chemical model of low-grade inflammation, *B. longum NCC3001* normalized anxiety-like behaviour, the beneficial effect being dependent on the integrity of the vagus nerve and independent of immunomodulatory activity[28, 29]. *Lactobacillus rhamnosus* (JB-1) reduced stress-induced corticosterone and anxiety- and depression-related behavior in mice[30]. These mice displayed altered GABA receptor expressions in the amygdala and hippocampus regions compared to control-fed mice. The neuro-chemical and behavioral effects of the probiotics were not found in vagotomized mice, identifying the vagus nerve to be responsible for these observed changes[30]. Two clinical studies have confirmed beneficial effects of specific bacteria on the CNS activity. *Tillish et al* has shown that consumption of a fermented milk product with several probiotics (*Bifidobacterium animalis subsp*

Lactis, Streptococcus thermophiles, Lactobacillus bulgaricus, and Lactococcus lactis subsp Lactis) by healthy women for four weeks affected activity of brain regions that control central processing of emotion and sensation[31]. In a pilot study, we have demonstrated that administration of *B. longum NCC3001* to patients with IBS and co-morbid depression and/or anxiety improves depression scores and alters activity patterns in multiple areas of the brain involved in mood control, including the amygdala[32].

Stress response and microbiota

Stress may be defined as a complex dynamic condition in which the homeostasis or the steady state internal milieu of an organism is disturbed or threatened. Inevitably all organisms at some point are exposed to factors, such as physical, psychological or immunological in nature, that disturb this steady state resulting in a stress response. This disruption to homeostasis elicits physiological responses that are necessary to restore homeostatic balance once the stress or perceived threat has subsided. For example, activation of the stress response through the hypothalamic-pituitary-adrenal (HPA) axis can lead to changes in intestinal permeability, motility, and mucus production[33, 34]. The coordination of this stress response is governed by two neuroendocrine pathways, namely the HPA axis and the sympathetic nervous system (SNS). Activation of the HPA axis occurs through the release of corticotrophin release hormone (CRH) from neurosecretory cells found in the paraventricular nucleus (PVN) of the hypothalamus. CRH stimulates the release of adrenocorticotrophic

hormone (ACTH) from the anterior pituitary gland. The ACTH then travels through circulation and stimulates the release of behaviour-altering chemicals including glucocorticoids (cortisol in humans and corticosterone in animals), mineralocorticoids and catecholamines from the cortex of the adrenal glands[35]. Glucocorticoids released by the HPA axis have profound anti-inflammatory influences on innate and adaptive immune cells but can also stimulate pro-inflammatory responses under certain conditions[36]. The activity of the HPA axis is regulated by multiple afferent sympathetic, parasympathetic, and limbic circuits (amygdala, hippocampus) innervating either directly or indirectly the PVN of the hypothalamus. In addition to the HPA axis, the sympathetic nervous system (SNS) becomes activated during stress and is responsible for the release of catecholamine into systemic circulation and tissues[37]. The primary function of the HPA axis and SNS is to cope with and adapt to the stressful stimulus by increasing the bioavailability of glucose via gluconeogenesis, suppression of the immune system, and enhanced fat and protein metabolism[38]. Chronic exposure to stress and subsequent glucocorticoid production results in various adverse side effects such as osteoporosis, diabetes, and hypertension[39]. IBS patients have an altered microbiota-gut-brain signalling with altered gut microbial composition and irregular HPA axis activity[40].

There is a growing body of literature supporting the role of the gut microbiota in the regulation and development of the stress response system[12, 41]. *Sudo et al* showed that commensal bacteria can affect the postnatal development

of the HPA stress response in mice[42]. In this study, the plasma ACTH following restrain stress was elevated in GF mice compared to mice with SPF microbiota, suggesting that absence of bacteria renders mice more susceptible to stress. Interestingly, plasma ACTH was reduced in ex-GF mice mono-colonized with *Bifidobacterium infantis*, although this was observed only in very young mice indicating a critical period in which the neural regulation of the stress response is sensitive to input from the microbiota[42]. Interestingly, mono-colonization with the enteropathogenic bacteria *Escherichia coli* induced even higher stress hormone release in GF mice[42]. These contrasting findings indicate that there are bacterial strain-specific effects on stress responses. There are conflicting evidences as to whether basal corticosterone levels are altered in GF mice, since both increased[23] and normal[43] levels have been documented. These contrasting findings are likely due to differences in mouse strain, sex, mode of killing, and acclimatization period between studies.

It has been reported that different psychological stressors, such as maternal separation and chronic social defeat, can alter the microbiota composition in rodents[27, 44, 45]. Exposure to a social disruption stressor affects the gut microbiota and circulating levels of cytokines, particularly interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1)[44]. In fact, social stress has been reported to increase the risk of inflammation-related diseases, promoting pro-inflammatory gene expression and monocyte differentiation[46]. Recently our group showed that behavioural abnormalities in maternally separated mice are

dependent on the microbiota and host factors[45]. The microbiota does not play a role in the altered HPA axis and colonic cholinergic neural regulation but is required for the induction of anxiety-like behaviour and behavioural despair in maternally separated mice[45].

A number of studies have investigated the effect of probiotics intervention in stress models. For example *Bifidobacterium infantis* normalized the behaviour phenotype in maternally separated mice without restoring HPA axis activity[47]. Probiotics treatment with *Lactobacillus* strain successfully reversed behavioural abnormalities without altering neuroendocrine output in restraint and food deprivation tests[48]. Another study demonstrated that the HPA axis response to acute stress was attenuated by *lactobacillus farciminis*[49]. All of these aforementioned studies suggest that a number of different pathways can interact with the neuroendocrine pathway to normalize behaviour in a stress induced model. The exact mechanisms warrant further investigation.

Serotonin synthesis and tryptophan metabolism

The precursor for serotonin (5-hydroxytryptamine, 5-HT) synthesis is the essential amino acid, tryptophan. Only 5% of the body's 5-HT is present in the CNS and the remaining 95% is located within the GI tract - mostly in the epithelial enterochromaffin cells (ECs) and some in the ENS[50]. 5-HT regulates diverse biological processes, including respiration, GI secretion and absorption, motility, and cardiovascular responses. In the CNS, 5-HT is involved in a range of mood, behavioural and cognitive functions, and is the main target of many psychiatric

medications[51]. 5-HT is synthesized by the rate-limiting enzyme tryptophan hydroxylase (TPH) which converts tryptophan to a short-lived intermediate product, 5-hydroxytryptophan (5-HTP).

The majority of tryptophan is metabolised along the kynurenine pathway. The rate of tryptophan metabolism through the kynurenine pathway is dependent on the expression of the tryptophan-2,3-dioxygenase (TDO) enzyme found in the liver or the indoleamine-2,3-dioxygenase (IDO1) enzyme found in all tissues. TDO can be induced by tryptophan or glucocortocoids, whereas IDO is influenced by the action of inflammatory cytokines particularly IFN-γ and TNF-α[52]. IDO1 expression has been proposed as a biomarker of GI diseases such as IBD, where it reflects mucosal inflammation, and colon cancer[53]. Once kynurenine is produced by TDO or IDO1, it is further metabolized into kynurenic and quinolinic acids which are NMDA antagonist and agonist, respectively. In the CNS, kynurenic acid has long been viewed as neuro-protective whereas quinolinic acid is considered excitotoxic[52]. Less is understood regarding the functions of kynurenic acid and quinolinic acid in the GI tract; however, both appear to be involved in immunoregulation[54]. Interestingly, kynurenic acid has anti-inflammatory properties in the GI tract[55].

MICROBIAL REGULATION OF SEROTONIN SYNTHESIS AND TRYPTOPHAN METABOLISM

It has been recently shown that microbes can facilitate serotonin production. Spore-forming bacteria within the gut can enhance EC production of serotonin through the release of SCFAs. These microbially stimulated ECs

increase expression of Tph1, the enzyme catalysing the rate-limiting step of serotonin biosynthesis from tryptophan[56]. Marked alterations in tryptophan and serotonin expression in GF mice support the gut microbial regulation of tryptophan metabolism and serotonin signalling. GF mice have increased levels of plasma tryptophan and hippocampal 5-HT concentrations. Microbial colonization of GF mice normalized plasma tryptophan levels and anxiety-like behaviours, but failed to normalize serotonin levels in the hippocampus[43]. Alteration of kynurenine:tryptophan ratios (used as an index of IDO or TDO activity) has been linked with depression and anxiety[57]. GF mice displayed a decrease in kynurenine:tryptophan ratio compared to conventional animals, which was normalized following bacterial colonization post-weaning[43]. An increase in this ratio was also apparent following infection with *Trichuris muris* in conventional mice[28]. These studies have spurred interest in whether targeting microbiota might be a viable strategy to influence circulating tryptophan availability for kynurenine metabolism. For example, administration of *Lactobacillus johnsonii* in rats decreased serum kynurenine concentration possibly via the inhibition of IDO activity by increasing hydrogen peroxide production[58]. A subset of Irritable bowel syndrome (IBS) patients have an increase IDO activity,[59] and low-grade inflammation has been reported in IBS patients which could drive the IDO activity and increase production of kynurenine metabolites leading to altered brain function and behaviour in these patients.

The gut microbiota can also directly utilise tryptophan to produce indole. Indole production by certain bacteria is catalysed by tryptophanase, an enzyme not present in eukaryotic cells[60]. Certain bacteria that have the tryptophan synthase enzyme can directly synthesize tryptophan[61]. The effects of tryptophan-derived indoles on the host include impact on oxidative stress, intestinal inflammation, and hormone secretion[62]. Indoles produced by bacteria can also have a beneficial impact on intestinal epithelial cells by strengthening the mucosal barrier[63]. It has been demonstrated that indole metabolites can promote 5-HT synthesis in the GI tract[56]. Indoles can modulate immune responses and facilitate host-microbiota homeostasis by activating aryl hydrocarbon receptors[64]. Kynurenine can also activate aryl hydrocarbon receptors and regulate immune responses, and in turn aryl hydrocarbon receptors can regulate IDO and TDO expression[65]. This complex crosstalk is an important example of the interface between the gut microbiota, kynurenine pathway metabolism and the immune response. It is clear that the major influence on circulating tryptophan availability in the host is the utilization and metabolism of tryptophan by the microbiota, and that this is one of the mechanisms by which gut bacteria can influence the gut and brain function.

TRYPTOPHAN METABOLITES INFLUENCE BRAIN DEVELOPMENT AND BEHAVIOUR

The influence kynurenine metabolites have on behaviour, including depression and anxiety[28], as well as cognitive performance, social behaviours and visceral pain perception[66, 67], is becoming clearer from recent works[68]. There

are now strong indications that altered kynurenine pathway metabolism during the perinatal period could have important neuro-developmental implications. Prenatal inhibition of the kynurenine pathway in rats produced changes in hippocampal neuron morphology as well as differences in neocortical and cerebellar protein expression which persisted into adulthood[69]. Conversely, increases in brain kynurenic acid in rats following dietary exposure to kynurenine during gestation and postnatal development resulted in neurochemical and cognitive deficits in adulthood[70]. It is plausible that many of the detrimental effects of altered postnatal microbiota could be partially due to a defective microbial regulated tryptophan availability and kynurenine metabolism in the periphery and CNS.

The limbic system and microbiota

The brain is an immensely complex organ with vast networks of neurons, connections and chemicals that facilitate communication to other organs. The limbic circuit of the brain is capable of producing a wide range of emotional and behavioural responses based on previous experiences and memories. It is composed of a number of intimately connected brain nuclei, particularly the amygdala and hippocampus, which contain an array of important biochemical signalling molecules. One such molecule, brain-derived neurotrophic factor (BDNF), promotes the growth and development of new neurons and the survival of existing neurons[71]. Since the chemical basis of learning and memory is thought to involve the formation of new neurons and synaptic connections, BDNF is

largely implicated in this process[72]. Several studies have described the therapeutic potential of BDNF, as BDNF administration provided broad neuroprotective effects against Alzheimer's and Parkinson's disease in animal models[73, 74].

In addition to genetic and epigenetic control of BDNF[75], prebiotics and diet regulate BDNF levels[76], which suggests a potential microbial-directed mechanism. *Trichuris muris* infected mice displayed increased anxiety-like behaviour associated with decreased BDNF mRNA expression within the hippocampus[28]. Treatment with anti-inflammatory agents mitigated anxiety responses but did not change BDNF expression. However, treatment with *B. longum* normalized behaviours and BDNF expression highlighting the varied pathways involved in microbiota gut-brain interactions[28]. Perturbation of the microbiota using non-absorbable antimicrobials neomycin, bacitracin and primaricin not only changed host behavior but is also associated with transient alterations in the expression of BDNF in the hippocampus and amygdala. Specifically, perturbation resulted in an increase in hippocampal BDNF and a decrease in amygdala BDNF expression[26].

GF mice are an excellent tool to probe the role of microbiota in behaviour and corresponding changes in the limbic circuitry of the brain. An increased turnover of dopaminergic and 5‑hydroxytryptaminergic neurotransmitters, as well as increases in markers of synaptogenesis, have been observed in the striatum

of GF mice, contributing to changes in the locomotive and exploratory behaviour of these animals[22]. The hippocampus is primarily involved in memory and spatial navigation, and a decrease in 5-HT levels and BDNF expression in the hippocampus contributes to working-memory impairment in GF mice[23, 24]. Adult hippocampal neurogenesis could be influenced by the gut microbiota; specifically cell survival is increased in the subgranular zone of the hippocampus[77]. The mammalian amygdala is a key emotional brain region for eliciting social behaviour, critically involved in anxiety and fear-related behaviours. In GF mice, changes in the levels of NMDA receptors, 5HT1 and BDNF in the amygdala, as well as the changes in the striatum described above, contribute to the increase in risk-taking behaviour that characterizes these mice[24, 42]. This brain region provides input to the hypothalamus, which regulates stress responses. The regional specificity of aforementioned findings either suggests that the mechanisms underlying these changes are selective for certain regions of the brain, or reflects the time point at which experiments were conducted, as the window of opportunity for bacterial influences might differ among brain regions.

γ-Aminobutyric acid (GABA) and microbiota

GABA is the primary inhibitory neurotransmitter in the CNS and low levels are linked to anxiety, depression and Alzheimer's disease[78, 79]. In the brain GABA biosynthesis occurs via the glutamine–glutamate–GABA cycle, but it is also found in the GI tract and ENS. *Lactobacillus* and *Bifidobacterium* strains from the human microbiota are capable of synthesizing GABA when grown on glutamate-

containing media[80]. GF mice have decreased levels of GABA in the colon, suggesting that GABA in the GI tract is produced by gut microbes[81]. Even though there is no evidence to suggest that GABA produced in the gut enters the brain it seems that this neurotransmitter can influence the CNS via ENS or vagal nerve activation. As mentioned earlier, mice treated with *Lactobacillus rhamnosus*, a probiotic with anti-inflammatory properties, exhibited altered GABA receptor expression in the brain accompanied by reduced anxiety and depressive-like behaviours.

C-fos transcription factor

The expression of critical transcription factors has been a popular approach for studying functional activation pathways, particularly in the CNS. C-fos is a cellular oncoprotein that is 314 amino acids in length, which is transcribed from the FOS gene [a member of the Immediate Early Gene (IEG) family]. It is rapidly and transiently expressed in response to various stimuli, such as growth factors, cytokines, neurotransmitters and stress[82]. C-fos proteins dimerize with additional IEG products, such as c-Jun, to form the Activator Protein-1 (AP-1) complex that activates downstream effects related to cell proliferation, differentiation and plasticity[83]. Although immediate, the expression of c-fos is transient and may fall below detectable levels within 15 minutes following translation, making it a challenging candidate for detection.

Phosphorylation of c-fos by extracellular-signal-regulated kinases (Erk) in response to extracellular stimuli further enhances its transcriptional activity.

Phosphorylation of Serine-32 (Ser32) and Threonine-232 (Thr232) residues by Erk1/2 increases protein stability and nuclear localization, respectively[84]. Phosphorylation of the Thr232 residue on c-fos also perpetuates the transcriptional activity of the protein, making it a useful candidate when attempting to identify functional c-fos proteins isolated in its area of action, the nucleus. This also alleviates some of the concern regarding the longevity of c-fos expression, allowing for a more functional and stable target. The nuclear export mechanism of c-fos is reliant on the nuclear export system ($NES_{221-233}$) of amino acids 221-233 of the peptide chain[83]. Therefore, if phosphorylated at the Thr232 residue, the protein is spared from nuclear export and from degradation by cytoplasmic ubiquitin-protein ligase E3 component n-recognin (UBR).

Blood-Brain-Barrier (BBB) and microbiota

The BBB serves as the main gatekeeper of the brain, regulating the passage of oxygen and nutrients from the circulatory system and guarding the nervous system from toxins and pathogens. This barrier maintains brain homeostasis, regulating the passage of peptides, neurotransmitters, and cytokines into the brain[85]. The BBB is formed early in fetal life and made up of epithelial cells linked by tight junction proteins to protect against harmful toxins during this sensitive period of brain development[86]. Breach of the BBB has been linked to inflammatory and neurodegenerative diseases, including stroke[87], multiple sclerosis[88], Alzheimer's and Parkinson's disease[89]. Even though the exact aetiology of BBB deterioration is multifactorial, recent work highlights

potential microbial induced mechanisms. Lipoprotein A from gram-positive bacteria induces strong pro-inflammatory cytokine responses, weakening the BBB *in vitro*[90]. One report showed rats fed a high-fat diet exhibited weakened BBB integrity and decreased hippocampal-dependent cognitive functioning[91]. It is tempting to speculate this could be microbial-dependent since diet can influence microbiota composition. In a seminal study it was reported that fetal GF mice exhibited increased BBB permeability compared to SPF and this continued throughout adulthood[92]. Colonization of adult GF mice with SPF flora increased expression of tight junction proteins and strengthened BBB integrity. The group went on to show that the effect of colonization on the BBB was due to SCFAs or metabolites produced by gut bacteria since mono-colonization with a butyrate-producing bacterial strain or sodium butyrate administration alone strengthened the BBB integrity[92]. This study highlighted the importance of the microbiota in BBB development. The abnormal behavioural and physiological profile of GF mice could be partially due to a leaky BBB.

Enteric Nervous System (ENS)

The ENS is a self-reinforcing neural network that has the ability to function autonomously and govern most of the GI function bi-directionally with the CNS. It encompasses the intrinsic neuroglial network of the gut and is organized into three major plexuses, the myenteric, submucosal and mucosal plexuses, which controls virtually all aspects of gastrointestinal physiology[93]. The autonomic nervous system (ANS), with the sympathetic and parasympathetic limbs, drives

both afferent signals, arising from the lumen and transmitted through enteric, spinal and vagal pathways to CNS, and efferent signals from CNS to the intestinal wall. With respect to the GI tract, sympathetic, parasympathetic, and spinal afferent nerve fibers are considered to be the extrinsic innervations with the ENS and afford the necessary conduit to maintain bi-directional communication with the CNS through intimate connections with the spinal cord. The vast innervations of the GI tract and the connections between intrinsic and extrinsic fibers allow the CNS to monitor a number of gut parameters, from chemical sensing in the lumen, to sensing mechanical stress along the gut wall[94]. Along the GI tract, the vagus nerve has three afferent endings within the gut wall: intraganglionic laminar endings within the myenteric plexus, intramuscular arrays within the smooth muscle layers and mucosal fibres within the mucosa. The stomach has the highest density of the vagal afferent ending and the density deceases towards the distal regions of the GI[95].

The sympathetic neurons (effector branch of the ANS) have axons that extend along the mesenteric nerves deep into the gut wall to the myenteric, submucosal and mucosal plexuses of the ENS[96]. The terminals of these axons are composed of numerous neurotransmitters and their associated enzymes, mainly norepinephrine (NE) and tyrosine hydroxylase (TH). New enteric neurons and glia continue to integrate themselves to pre-existing neuronal circuits several weeks after birth[97]. This suggests that changes in the environment associated

with diet, luminal microflora, and maturation of the mucosal immune system may likely affect postnatal development of the ENS.

Inflammatory signals have a broad effect on the CNS and peripheral nervous system. For example, pro-inflammatory cytokines IL-1β and TNF-α promoted neurogenesis in a co-culture model of enteric neurons and smooth muscle cells in a NF-κB–dependent manner to up-regulate expression of GDNF[98]. The potential effects of cytokines or other immune products during normal ENS development are unknown, but the ability of enteric neurons to respond to inflammatory cytokines and leukotrienes[99] raises the possibility that such signaling molecules could play an important role in the development and maturation of the mammalian ENS. This could be more pronounced in the postnatal gut during the establishment of the microbiota and the maturation of the innate and adaptive immune systems.

MICROBIOTA AND THE ENS

Studies in GF animals have shed light into the importance of bacterial colonization on proper development of the ENS[100]. During embryogenesis, the ENS develops within a largely sterile environment, but the postnatal stages of ENS development and maturation take place under strikingly different conditions due to the ingestion of food and the establishment of microbiota, which result in a multitude of changes to the maturation of the immune system[101]. The absence of microbiota is associated with alterations in the gut sensory-motor functions, resulting in delayed gastric emptying and intestinal transit, reduced migrating

motor complex cyclic recurrence and distal propagation, and enlarged cecal size[102]. GF mice showed altered spontaneous muscle contractions, decreased nerve density in the small intestine[103], and reduced sensory neuron excitability which was restored following bacterial colonization[104].

There is ample evidence that bacteria can affect the function of the ENS also in conventional mice. Chronic infection with *H. pylori* increased the density of substance P, calcitonin gene-related peptide, and vasoactive intestinal polypeptide immunoreactivity nerves in the gastric myenteric plexus and spinal cord[105] and altered response to gastric distension which only partially improved after bacterial eradication[106].

As mentioned earlier, the anxiolytic effect of *B. longum* requires vagal integrity but does not involve gut immuno-modulation[28]. *B. longum* has been shown to alter excitability of the enteric sensory neurons and thus it may signal to the brain by interacting through vagal pathways at the level of the enteric nervous system[28, 107]. Another well characterized probiotic, *Lactobacillus reuteri* effects gut motility and pain perception in rats by increasing excitability of the enteric neurons through the inhibition of the calcium-dependent potassium channel opening[108]. In an *ex vivo* perfusion model, *L. reuteri* was shown to moderate mouse jejunal motor patterns within minutes, but this effect was absent when administering heat-killed *L. reuteri* or another live commensal (*Lactobacillus salivarius*)[109]. *Verdu et al* showed that antibiotic induced perturbation in the gut microbiota produced changes in inflammatory cell infiltrate and substance P

levels in the gut resulting in an increase in visceral sensitivity to colorectal distension[110]. These changes were prevented by administration of *Lactobacillus paracasei* suspended in spent culture medium. *Lactobacillus paracasei* also prevented partial restraint stress-mediated visceral hyperalgesia in maternally deprived rats[111]. *Lactobacillus farciminis* treatment prevented hyperalgesia to colorectal distension induced by acute stress[112] via a decrease in the stress-induced sensitization of sensory neurons at the spinal and supraspinal level[113].

A number of molecules have been identified through which the microbiota can communicate with the ENS. Metabolites produced by gut microbes include SCFAs, metabolites of bile acids, and neuroactive substances such as GABA, tryptophan precursors, serotonin, biologically active forms of catecholamines[114], and cytokines released during the immune response to microbes,[44] can all signal to the host via receptors on nerve endings within the gut. These factors can also signal via neurocrine (through afferent vagal and possibly spinal) pathways to targets well beyond the GI tract, including vagal afferents in the portal vein and receptors in the brain. *Lactobacilli* can produce hydrogen sulfide that modulates gut motility by interacting with the vanilloid receptor on capsaicin-sensitive nerve fibers[115]. Most metabolites identified in the circulation of the host are of gut microbial origin[116], providing the theoretical basis for the microbiota-gut-brain axis signaling system. Short-chain fatty acids (SCFAs), such as butyric acid, propionic acid and acetic acid, are able to stimulate sympathetic nervous system[117] and mucosal serotonin release[118]. Signaling through GPCRs and SCFAs transported

by SLC5A8 and the resulting physiological effects can occur due to the dietary intake of fermentable fiber[119]. Different types of SCFA receptors have been identified on enteroendocrine cells as well as on neurons of the submucosal and myenteric ganglia[120].

Role of microbiota on immune system

Intestinal microbiota contributes to the development and regulation of the gut immune system. GF animal studies demonstrate that the microbiota is necessary for the development of gut mucosal immunity[121]. In addition, microbiota-driven immune response can prevent the development of inappropriate inflammatory response to commensal microbiota and establish a host-microbial homeostasis. A break-down of this gut homeostasis due to microbial dysbiosis could cause immune-related disorders,[122] diabetes,[123] allergies,[124] and obesity[125].

Gut-associated lymphoid tissues (GALTs) are lymphoid structures that include the peyer patches (PPs), isolated lymphoid follicles (ILFs), and mesenteric lymphnodes (MLNs). PPs are present in the small intestine and composed of 3 or more lymphoid aggregates with an overlying follicle associated epithelium, a T-cell zone, and a subepithelial dome containing dendritic cells (DCs)[126]. The follicle associated epithelium contains specialized epithelial cells called the M cells which are important for the uptake of antigen and microbes from the gut lumen and its delivery to underlying lymphoid tissue[127]. ILFs are similar to the PPs but lack T-cell zones and are also present in the large intestine.

The intestinal epithelium is a single layer of cells derived from the epithelial stem cells within the crypt. It provides a physical and biochemical barrier separating the intestinal lumen from the inside of the body. Epithelial cells are highly responsive to both microbial and immune-mediated signals. Globlet cells, which are specialized epithelial cells, produce mucus to inhibit pathogen invasion by separating the gut lumen from the surface of the intestinal epithelium[128]. Epithelial cells are potent producers of antimicrobial peptides, such as REGIIIβ and REGIIIγ that function as natural antibiotics by either directly killing or inactivating bacteria[129]. Epithelial cells can express numerous pattern recognition receptors (PRRs), including toll-like receptors (TLRs) and nucleotide oligomerization domain-like (NOD) receptors. PRRs are germline encoded receptors in the epithelial and innate immune cells which recognize microbial particles, such as DNA, lipopolysaccharides (LPS), peptidoglycans, flagellin, and metabolites. PRRs have crucial roles in innate immunity because they can sense pathogen-associated molecular patterns and initiate signaling cascades that lead to innate immune response[130].

The composition of intestinal microbiota has an important role in shaping host immunity, including cytokine expression, development of GALT and mucosal barrier. GF mice have numerous immune abnormalities, including failure of secondary lymphoid development, lower levels of antimicrobial peptides and smaller numbers of intraepithelial lymphocytes [131]. Colonization of GF mice can restore the proper organization of the intestinal immune system[132]. GF mice have

a significantly thinner colonic adherent mucous layer, but when exposed to bacterial products, such as LPS and peptidoglycan, the thickness of the adherent mucous layer is restored to levels observed in conventional mice[133]. They also have fewer villi, PP, and enterochromaffin cells, which are responsible for the production of serotonin in the GI tract (a key regulator of GI motility and secretion)[134]. Production of antimicrobial peptides REGIIIβ and REGIIIγ is impaired in MyD88 knockout mice, resulting in increased susceptibility to infection by enteric pathogens[135]. Thus microbiota can enhance innate immunity through mucous secretion and production of antimicrobial peptides. Moreover, microbiota can enhance mucosal barrier function through the production of SCFAs. For example, *Bifidobacteria* can inhibit the translocation of the *E. coli O157:H7* Shiga toxin from the gut lumen to the blood through the production of acetate[135]. Butyrate is shown to reduce T-cell–mediated immune reaction by modulating antigen-presenting cell function[136]. SCFA binds to G-protein-coupled receptor 43 (GPR43) to regulate inflammatory responses, and mice deficient in GPR43 develop severe inflammation in the models of colitis[137].

Selective bacterial species can shape specific aspects of adaptive and innate immunity, including the differentiation of certain effector T-cell lineages. For example, only certain bacteria that can adhere to intestinal epithelial cells, such as segmented filamentous bacteria, *Citrobacter rodentium* or *E. coli 0157* can effectively mount a T_H17 response[138]. Other bacteria, including *Listeria*, are permissive for host TNF, IL-1β and TH1 responses[139]. Conversely, some

bacteria promote immunosuppressive cytokines, such as IL-10 by Treg cells, which can be dependent on the production of SCFAs by clusters of *Clostridia*[140], or polysaccharide A derived from *Bacteroides fragilis*[141]. Together, these data show that the precise composition of the intestinal microbiota can qualitatively and quantitatively alter host immune responses, which in turn have the capacity to affect brain function.

Microbiota can influence neurological functions via the immune system

Growing data indicate that the peripheral immune system can influence neurological function and behavior. In fact, immune signaling has been shown to cause or allow progression of certain neurological disorders, including neurodegenerative diseases and psychological illnesses such as anxiety and depression. Patients with chronic inflammatory diseases such as cardiovascular disease, rheumatoid arthritis, chronic obstructive pulmonary disease, and type 1 diabetes, exhibit high rates of depression[142, 143].

Clinical studies have demonstrated a clear link between plasma cytokine levels (or inflammatory markers) and depression. For example, pro-inflammatory cytokines (IL-1β, IL-6, TNFα) in women between the ages of 20-40 with major depressive disorder were elevated compared to control[144]. In another study, significant improvement in depression was found among patients with psoriasis treated with etanercept (anti-inflammatory drug that prevents TNF-α mediated cellular response)[145]. Also, patients undergoing cytokine therapy for cancer or

hepatitis C, exhibit frequent neurobehavioral symptoms like anxiety and major depression[57].

One of the better known examples of immune-mediated effects on neurological function is sickness behavior. Cytokine-induced sickness behavior is a well described phenomenon with both neurovegetative and psychological components[57]. This behavior is characterized by appetite suppression, decreased motor activity, loss of social interaction, and reduced cognition. Sickness behaviour may overlap with depression-like behaviour in mice; in other words, sickness behaviour is observed at an early time point (2-6 hours post-cytokine administration), while chronic exposure to cytokines (more than 24 hours) induces depression-like behaviour[57]. Microbial-associated molecular patterns (MAMPs), such as LPS, bacterial lipoprotein, flagellin, and CpG DNA, activate innate immune cells such as macrophages, neutrophils, and dendritic cells. Once activated, these cells produce numerous pro-inflammatory cytokines, such as IL-1α, IL-1β, TNFα, and IL-6, which can induce sickness behaviour by crossing BBB and acting on receptors expressed by neurons and microglia in the brain[146]. In the periphery, these pro-inflammatory cytokines are able to bind to receptors in the afferent nerves and send direct signals from the peripheral body sites to the CNS. In further support of pro-inflammatory cytokines mediating the onset of this behavior, treatment with anti-inflammatory mediators, such as IGF-1 and IL-10, prevents sickness behavior[147].

Microbiota can modulate neuronal function via non-inflammatory cytokine pathways. For example, GF mice exhibit lower plasma concentration of the cytokine granulocyte colony stimulating factor (G-CSF)[148] which has been shown to cross the BBB and stimulate neurogenesis in the brain[149]. G-CSF is a protective factor following ischemic injury,[150] as well as protective and therapeutic in certain models of both Parkinson's and Alzheimer's diseases[151]. Thus microbiota has the potential to influence normal neuronal development via G-CSF signalling.

Very recent reports have suggested that the intestinal microbiota influences brain injury and have identified the mucosal immune system as the key driver of this phenomenon. By perturbing the microbial diversity with antibiotics, treatment for brain infarct volume was markedly reduced in mice following transient middle cerebral artery occlusion[152]. A defective differentiation of microbially induced pro-inflammatory intestinal γδ T cells protected mice from ischaemic injury. In conventional mice, diverse bacterial communities lead to a robust differentiation of IL-17 producing γδ T cells in the gut, and following an ischaemic injury these cells migrate to the brain and induce a pro-inflammatory response that exacerbates tissue damage. However, in mice with reduced bacterial diversity due to antibiotic treatment, impaired differentiation of IL-17 producing γδ T cells in the gut was found, with fewer of these cells migrating to the meninges or cervical lymph nodes, thereby limiting post-ischaemic inflammatory tissue damage[152]. Moreover, it seems ischaemic or traumatic brain

injury itself can drive changes in the intestinal microbiota in mice through the noradrenergic stress responses[153].

GUT MICROBIOTA INFLUENCES ENS DEVELOPMENT VIA INNATE IMMUNITY

The host senses gut bacteria via the pattern-recognition receptors (PRRs), a large family of proteins that have the ability to recognize unique microbial components and play a crucial role in innate immunity and protection against pathogenic microorganisms. Since intestinal epithelial cells and the innate immune response are the first line of defence against commensal and pathogens, their products are likely to influence the development and homeostasis of the ENS[154]. Gene expression analyses show that TLR-3 and TLR-7 (receptors for viral RNA) and TLR-4 (LPS receptor) are expressed by enteric neurons and glia, which suggest that ENS lineages can directly sense microbial microbiota[155]. *In vitro* studies with isolated rat myenteric plexus demonstrated a direct effect of LPS on enteric glial cells (EGCs), evidenced by the increased production of the pro-inflammatory factors IL-1 and prostaglandin E2. *Anitha, et al* showed that germ-free and antibiotic-treated mice exhibited reduced motility and fewer nNOS+ neurons[156]. This effect was mediated, at least partly, via TLR-4, as $Tlr4^{-/-}$ mice exhibited similar deficits in intestinal motility and a reduced number of nitrenergic neurons as GF mice. This phenotype was reproduced in mice with ENS specific MyD88 knockout, suggesting that TLR-4 signaling is critical for the nitrergic neurons within ENS lineages. The same study demonstrated that LPS promoted the survival of cultured enteric neurons in an NF-κB–dependent

manner[156]. Enteric neurons and glia also express TLR2 and the myenteric ganglia of Tlr2$^{-/-}$ mice contained fewer neurons compared with their wild-type mice, with reduction in inhibitory nNOS+ neurons being the most notable phenotype[157]. The reduction in nNOS+ neurons is accompanied by intestinal dysmotility and impaired chloride secretion in ileum. Administration of GDNF can correct many of the ENS deficiencies in Tlr2$^{-/-}$ mice and in antibiotic-treated animals, suggesting that one of the roles of the microbiota-TLR-2 axis is to promote the expression of neurotrophic factors that are required to maintain the functional organization of the mammalian ENS[157]. Even though there remains much to discover regarding the mechanism by which microbiota can influence ENS development and function, the significance of these observations have significant implications. For example, over-stimulation of the TLRs by recurrent infection or through early life dysbiosis caused by antibiotic use could hamper the proper development and maturation of the ENS[158]. Data suggest that the developing ENS is capable of responding to environmental cues and adjusts to the volatile milieu of the gastrointestinal lumen. Reverse regulation, in which the ENS contributes to the shaping of the microbiota is also possible, as suggested by a study in which alterations in the composition of colonic and fecal microbiota were observed in a mouse model of congenital aganglionosis[159].

ADAPTIVE IMMUNITY AND BRAIN FUNCTION

It is well established that the crosstalk between the CNS and immune system involves active participation of microglia, astrocytes and neurons[160].

While older literature supported the notion of T cells being innocent victims or bystanders of neuropathophysiology, new data is demonstrating that T cells play a pivotal role in brain development and behaviour. Immunocompromised mice have impaired cognitive and emotional behaviors that have been attributed specifically to $CD4^+$ T cell. An increase in $CD4^+$ T cell numbers in the meninges was observed in mice after training for a cognitive task and blocking it was sufficient to cause cognitive impairment[161]. Severe combined immunodeficiency (SCID), $Rag1^{-/-}$, $Rag2^{-/-}$ (which lack T cells and B cells), and nude mice (which lack mature T cells) all display impaired learning and memory[162]. Adoptive transfer of $CD4^+$ T cell was sufficient to reverse these cognitive deficits except in $IL4^{-/-}$ mice, suggesting that T cells via IL4 production are important for proper brain function[161]. Defective $CD4^+$ T cell are not only involved in cognitive deficit, since $Rag1^{-/-}$ displayed compulsive behaviour and a deficit in nest building that was reversed after repopulating with $CD4^+$ T cells. Another study showed that antigen specific T cells, most likely effector-memory T cells, were needed for proper cognition[162].

A number of tissues from autism spectrum disorder (ASD) patients exhibit a pro-inflammatory phenotype with the severity of symptoms correlating with abnormal immune function[163]. In the brain, microglia have an activated morphology in several regions, most notably the frontal cortex and cerebellum, discovered by postmortem analysis of brains from ASD patients[164]. This pro-inflammatory milieu is not limited to the CNS but it is also present in the

periphery. T cells isolated from ASD patients were hyper-excitable, having an exaggerated response when stimulated *ex vivo* with the mitogen phytohaemagglutinin[165]. ASD patients had also elevated pro-inflammatory molecules, such as IL-6 and decreased anti-inflammatory molecules, such as TGF-β in the plasma, suggesting an overall pro-inflammatory state[166].

Kipnis et al, suggested that communication between the peripheral immune system and the brain occurs at the BBB interface, specifically at the meningeal spaces[167]. To test whether cognitive deficit in SCID mice was due to a malfunction of IL-4 producing T cells leading to a pro-inflammatory state of CNS myeloid cells, macrophages treated with IL-4 *ex vivo* were injected into SCID mice. This ameliorated the pro-inflammatory state of the meningeal myeloid cells and improved cognitive function in the absence of functional T cells[168]. Thus, it is plausible that T cell malfunction or scarcity may underlie some of the cognitive impairment associated with ASD. Astrocytes and microglia also express IL4 receptors and may respond to the pro-inflammatory state of the meningeal myeloid cells in the absence of functional T cells[161, 169]. Recently it was shown that meningeal immunity is important for social behaviour, and mice with impaired adaptive immunity displayed social deficits and hyper-connectivity of fronto-cortical brain regions[170]. The study went on to show that IFN-γ is the key molecular link between meningeal immunity and neural circuits recruited for social behaviour.

Development and function of brain resident immune cells

MICROGLIA: are the key immune cells of the CNS. They are innate sentinel immune cells that can detect subtle changes in their local environment and regulate neuro-inflammatory processes. These cells are distributed throughout the brain and spinal cord, but are especially abundant in the grey matter, where they extensively branch and communicate with neurons and other cells of the CNS[171]. Derived from yolk sac erythromyeloid progenitors, microglia perform canonical functions of myeloid cells, including cytokine/chemokine production, phagocytosis, antigen presentation, and regulation of neurotransmitters. Their effective surveillance capabilities allow them to survey the entire brain in a couple of hours with limited physical migration and to clear debris/infection agents rapidly[172]. During neurodevelopment, microglia tag and clear synapses for pruning, promote neuronal circuit wiring and produce cytokines and chemokines that guide neuronal differentiation[173].

ASTROCYTES: are the most abundant glial cells in the brain and they are crucial for regulating the BBB integrity, ion gradient balance, neurotransmitter turnover, cerebral blood flow and nutrient transport. Astrocytes are also important for glycogen storage in the brain and to provide support for neurons during hypoglycemic states[174]. They integrate information from adjacent glial, neuronal, vascular and immune cells to regulate neural excitability and synapse formation[175]. Although astrocytes are not considered CNS resident immune cells, they perform immune related processes, such as expressing pattern recognition

receptors for detection of microbe-associated molecular patterns and modulating neuro-inflammatory responses through cytokine production and antigen presentation via MHC II[176].

Besides microglia, there are other immune resident cells such as *PERIVASCULAR MACROPHAGES, CD4$^+$ T and CD8$^+$ T cells,* and *mast cells* that reside in the CNS. Although the effect of microbiota on these brain-resident immune cells has not yet been described, it is known that microbiota can modulate peripheral myeloid cells, T cells and mast cells[177], which share common hematopoietic progenitors with the brain subsets. Determining whether microbial effects on peripheral hematopoietic progenitors result in alterations in brain meningeal and/or tissue-resident immune cells would be important. Perivascular and choroid plexus macrophages would be of particular interest given their close localization to the vasculature and potential exposure to circulating factors that are regulated by the microbiota.

Microglial function and development under the control of microbiota

Despite the microglia being secluded in the CNS by the BBB, it is evident that microbiota influences the maturation and function of the microglial cells. GF mice have increased numbers of immature microglia with defects in maturation, activation, and differentiation. They also have altered microglia morphology and a compromised immune response to bacterial or viral infection, including impaired induction of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α[178]. Interestingly, these altered microglial phenotypes were reversed after bacterial

colonization via co-habitation for 6 weeks. Consistent with this, gene expression differs between GF adult and newborn microglia when compared to their age-matched conventionally colonized controls. Many of these genes showed a decrease in expression in GF microglia, but more genes are down-regulated in microglia from adult GF mice than in those from newborns[179]. This suggests that the maternal microbiota may contribute to early life programming of microglial development and reinforces the important relationship between our intestinal bacteria and the immune system for proper brain development and function.

Although the exact mechanism by which microbiota influences microglia is unknown, there seems to be a specificity of microglial modulation to particular bacterial taxa. GF mice colonized with three strains of the altered Schaedler flora (ASF): *Bacteroides distasonis* (strain ASF 519), *Lactobacillus salivarius* (strain ASF 361) and *Clostridium cluster XIV* (ASF 356) maintained abnormalities in microglia[178]. This suggests that microbial effects on microglia are not dependent on just the presence of bacteria but require either great bacterial diversity or particular microbial species not represented by the minimal community tested. Notably GF associated abnormal microglial morphology, abundance, and gene expression were normalized by postnatal supplementation with SCFAs, suggesting SCFA-producing bacteria are required for the proper function and development of microglia[178]. *Clostridium cluster XIV* is known to produce SCFAs propionate and acetate[140], despite its failure to correct microglial abnormalities after colonization in the three-member community. This suggests a need to

examine levels of SCFAs achieved by microbial colonization relative to dosages that were supplemented exogenously. Conventionally raised mice with GPR43 (SCFA receptor) knockout exhibited microglial abnormalities similar to GF mice[178]. However, microglia does not express GPR43 directly[178], so whether the effects of the microbiota on microglia are mediated by direct signaling of SCFAs is unknown. It is plausible that SCFAs can indirectly affect microglia since they have a broad array of functions that influence the peripheral immune system, liver metabolism, and blood-brain barrier integrity. Whether microbiota affects microglial function during postnatal period is not known but is worth investigating due to implications in brain development and function. During early postnatal life, microglia are integral for synaptic pruning, tagging synapses for clearance with complement proteins on the basis of neural activity[180].

CHAPTER 2: THESIS OBJECTIVES

Thesis scope

Our knowledge and understanding of the influence intestinal microbiota has on host development and function has come a long way, however, we are still far from understanding how gut bacteria influence these systems throughout life. It is evident that gut microbiota and the CNS are able to communicate with each other to maintain a homeostatic relationship. Although the exact pathways by which bacteria communicate with the CNS have not been fully elucidated, immune, neural, or humoral pathways have been proposed. It is likely that these systems work in concert with each other to foster normal brain development and behaviour which ultimately benefits both the host and the microbiota. Immune-dependent pathways could play a major role during the initial colonization by activating and maturing the mucosal immune system. Demonstrating that the perturbation of intestinal microbiota using oral antimicrobial changed behavior, and GF mice colonized with SPF flora developed anxiety-like behavior,[26] suggests that the host's normal behaviour receives an input from the microbiota. Also studies have shown differences in brain chemistry and behaviour between GF and SPF mice[22, 23]. Given the symbiotic evolution that has occurred between mammals and environmental bacteria, it is not surprising such a relationship exists, since the intestinal microbiota has no less a stake in their survival in the relationship than does the host. Highlighting the particular regions involved in this microbiota-gut-brain axis, as well as the mechanism(s) involved, is extremely important in our understanding of how the microbiota and CNS interact.

Aims/hypothesis

The overall goal of my thesis is to investigate the role of immune system maturation on mouse behaviour after bacterial colonization. My hypothesis is that bacterial colonization of germ-free mice leads to changes in brain chemistry and normalizes behaviour through the maturation of the immune system. This study was designed to shed light into the immune mechanism underlying the microbiota-gut-brain communication using a reductionist approach with bacterial mono-colonization. We focused on the immune-mediated pathway, however other pathways such as neuronal or humoral, including microbial metabolites, are likely involved in the gut-brain communication. Major findings of this thesis are organized into five aims or chapters:

1. *Bacterial colonization normalizes behavior in ex-germ free mice independently of the microbial complexity*
2. *The innate arm of the immune system through the MyD88/Ticam pathway is crucial for the development of normal behaviour*
3. *Continuous presence of bacteria is not required to maintain normal behaviour*
4. *Bacterial stimulation through TLR-4 is necessary for the development of normal behaviour*
5. *Innate immunity and neuronal genes are significantly altered after bacterial colonization*

CHAPTER 3: MATERIAL AND METHODS

Animals

Ten week old Swiss-Webster specific pathogen-free (SPF) mice were purchased from Taconic, USA, and housed in a conventional specific pathogen-free unit at McMaster University Central Animal Facility. Ten to fourteen week old GF Swiss-Webster, C57BL/6, SCID, MyD88$^{-/-}$ Ticam1$^{-/-}$ mice were maintained axenic in sterile isolators at the Farncombe Family Axenic Gnotobiotic Unit of Central Animal Facility, McMaster University, Canada. To ensure sterility, handling of GF mice were carried out under the most scrupulously clean conditions using previously published protocol[181]. All mice were maintained on a 12-hour day/night cycle with unrestricted access to food and water. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and received approval from the McMaster University Animal Research Ethics Board.

Bacterial colonization of germ-free (GF) mice

GF mice (10 to 14 week old) were mono-colonized with 10^9 CFU of *E. coli* JM83 (*rpsL ara Δ(lac-proAB) φ80 dlacΔ(lacZ)M15*) or *E.coli* HA107 via intragastric gavage. *E.coli* HA107 (*Δasd Δalr ΔdadX::kan*^{R}) strain is a mutant form of the parental strain *E. coli* JM83, which lacks the ability to synthesize meso-diaminopimelic acid (m-DAP) and D-isomer of alanine (D-Ala) required in the peptidoglycan crosslink of the cell wall and thus only transiently colonizes (12-48 hours) mouse intestine[182]. The permanent colonizer *E. coli* JM83 was gavaged once, and then the mice were gavaged three times weekly for two weeks with normal saline. The transient colonizer *E.coli* HA107 was gavaged three times

weekly for two weeks. Mono-colonized mice were maintained in sterile isolators within the Axenic Gnotobiotic Unit. Additional groups of GF mice were colonized with Altered Schaedler Flora (ASF, consisting of 8 bacterial strains) or complex Specific Pathogen Free (SPF) microbiota via oral gavage and housed in ultraclean conditions using ventilated racks.

Bacterial preparation

E. coli JM83 was grown in LB broth and *E. coli* HA107 was grown in D-Ala (200µg/ml)/m-DAP (50µg/ml)-supplemented LB broth and incubated with shaking at 160 rpm at 37°C for 12 hours. Bacteria were harvested by centrifugation (15 min, 3500g) in a 400ml sterile flask, washed in sterile PBS and concentrated to a density of about 10^9 CFU/ml in PBS, all performed aseptically under a sterile laminar flow hood. The bacterial suspensions were sealed in sterile tubes, with the outside surface kept sterile, and imported into sterile isolators, where 200 µl (10^9 CFU) were gavaged into germ-free mice. Cecal contents from ASF and SPF mice were extracted and resuspended in sterile PBS and 200ul was gavaged into GF mice.

Gnotobiotic husbandry

Isolators that house GF mice undergo meticulous protocols to prevent introduction of microbes from animal handlers or the environment. Samples of the imported materials are taken for aerobic and anaerobic bacterial culture regularly. Feces and bedding are taken from the isolator for direct bacteriology, microscopy and 16S PCR testing of intestinal contents to test for culturable and unculturable

organisms. Behavioural experiments on GF mice colonized with single or multiple bacteria are carried out in smaller 'surgical' isolators separate from the breeding isolator to minimize risk of bacterial contamination of the stock during manipulations.

Behaviour assessment

Anxiety-like behavior was assessed using light/dark preference test as previously described[183] using a custom modified version of an automated detection system (Med Associates Inc, St. Albans, Vermont, USA). Each mouse was placed in the center of the "aversive" zone lit by ambient light that was attached via a small doorway to a smaller "safe" dark box in the same arena and animal behavior was assessed for 10 minutes. Parameters that were measured included total time spent in light region, number of transitions between light and dark regions (zone entries), total vertical/rearing counts, total distance, total resting time, and average velocity. Quantification was performed by an automated computer program (Med Associates Inc, St. Albans, Vermont, USA).

Exploratory behavior was assessed using the step-down latency test[184]. Each mouse was placed on the center of a circular elevated platform (diameter, 10cm; height, 4cm) and the time it took for each mouse to step down and vacate the platform using both two paws and all four paws was measured (maximum time, 4 minutes). Time to step down was recorded and analyzed according to the statistical analysis section.

Depression-like behavior was assessed using the tail suspension test as previously described[185]. Briefly, each mouse was suspended by their tails for 5 min and the time they spend immobile was recorded. An animal was considered immobile when it does not show any body movement and hanged passively.

Microbial analysis

Cecal contents from mono-colonized mice were suspended and serially diluted in sterile 1xPBS and plated on LB agar plate and incubated aerobically at 37°C overnight. To confirm *de novo* GF status after colonizing with *E.coli* HA107, cecal contents were plated on m-DAP and D-Ala supplemented LB agar plate 2 and 4 weeks after the last gavage. Also cecal contents from *E.coli* HA107 treated mice were plated on non-supplemented LB agar plate to rule out bacterial contamination. Since *E.coli* HA107 strain has genetically engineered kanamycin resistant gene cecal contents were also plated on kanamycin LB agar plate to ensure the original bacterial strains are recovered. Cecal contents from *E.coli* JM83 treated mice were plated on LB agar to ensure proper colonization. Bacterial immunofluorescence staining of the cecal contents was carried out to ensure the sterility of the GF mice.

Tissue processing

Upon sacrifice of mice, colon and cecum contents were flash frozen in liquid nitrogen and stored in -80°C. Brains were immediately snap frozen in 2-Methylbutane (isopentane) over dry ice and stored at -80°C. All brains were cut into 5µm sections on a Microm HM 550 cryostat (Thermo Scientific, WI, USA)

and mounted onto pre-cleaned double frost Apex coated slides (Surgipath, Ontario, Canada) and stored at -20°C until processing. Region of interest in the brain included hippocampus (sections between bregma -1.22mm and -2.70mm) and amygdala (sections between bregma -1.22 and -2.18).

Immunofluorescence staining

Brain sections were allowed to thaw at room temperature for one hour and then washed in phosphate-buffered saline (PBS, pH 7.4) for 5 minutes to remove any residual OCT. Tissue sections were fixed in methanol for 10 minutes and permeabilized in TBS + 0.0025% Tween-20 for 10 minutes. Then proteins were blocked using 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. Primary antibodies to BDNF (1:500, Novus, Canada) and c-fos (1:100, Abcam, Canada) were applied and incubated overnight in a humidity chamber for 12-18 hours at 4°C, and then detected with Alexa Fluor secondary antibody (1:1000, Invitrogen, Canada) for 1 hour room temperature in dark.

Image Analysis

Brain tissue sections were viewed with a Leica DMLS microscope (Leica Microsystems, Wetzlar, Germany), and images were captured using an Olympus QColor 3 camera (Olympus, Canada) and Adobe Photoshop CS2 imaging software (Adobe System, USA). Positively stained cells were measured using ImageJ analysis software (NIH). Briefly, particle measurements, in tandem with set color thresholds, were used to quantify the number of c-fos and BDNF

immuno-positive cells. Positive cell counts were analyzed using Prism 4 software (GraphPad Software, Inc, CA, USA).

Myeloperoxidase (MPO) activity

Frozen colon tissues were assayed using a previously described method[186]. 50mg of colon tissues were homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer and centrifuged for 5 min. 7uL of the supernatant were transferred to a 96 well microplate in triplicates. Activate 50 mL of O-dianisidine dihydrochloride solution by adding 25uL of 1% H_2O_2 and multipipette 200uL of the solution into each well. Place the microplate into the reader and take three readings at 460nm at 30 second intervals.

MPO is a granule-associated enzyme primarily contained in neutrophils, and its measurement has been widely used as a marker of acute intestinal inflammation. MPO activity is expressed in units per mg of tissue, where 1 unit of MPO is defined as a quantity of the enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 minute at room temperature.

Intestinal tissue cytokines

Colon samples were homogenized in Tris-HCl buffer containing protease inhibitors, centrifuged, and the supernatants were kept on ice. Cytometric Bead Array (CBA) kit was used to quantitatively measure Interleukin-6 (IL-6), Interleukin- 10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein

levels in a single sample. CBA assays provide a method of capturing soluble analyte with beads (conjugated with a specific antibody) of known size and fluorescence, making it possible to detect analytes using flow cytometry. The protein concentration in each sample was measured using a BCA protein assay kit (Bio-Rad).

Tryptophan and kynurenine metabolites assessment

Plasma kynurenine and tryptophan were analyzed by ultra performance liquid chromatography using a previously described method[28]. Briefly, plasma mixed with sulfosalicylic precipitation reagent and the supernatant was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. 1uL of derivatized sample (50 nL of plasma) were injected onto ultra performance liquid chromatography column. In our apparatus the retention time for kynurenine and tryptophan was 27.2 and 30.8 minutes, respectively.

LPS and Poly I:C administration

E.coli 0111:B4 LPS (Sigma, Canada) and poly I:C (Sigma, Canada) were diluted in sterile PBS to a concentration of 2 mg/kg and 180 µg/ml respectively, all performed aseptically under a sterile laminar flow hood. Both were sealed in sterile tubes with the outside surface kept sterile and imported into sterile isolators where 200 µl were gavaged into germ-free mice C57BL/6 mice. In order to keep the protocol consistent with the mono-colonization experiment, *E.coli* 0111:B4 LPS and poly I:C were gavaged three times weekly for two weeks and behaviour was assessed before and after treatment.

NanoString gene expression assay

RNA was extracted from colon and brain (hippocampus and amygdala regions) tissues using the RNeasy Mini Kit (Qiagen, Toronto, Canada) and DNase digestion was carried out using the RNase-free DNase (Qiagen). NanoString nCounter® Gene Expression CodeSet for mouse inflammation genes was run on colon tissues and a custom Nanostring gene expression codeset for selected genes was run on brain sections according to the manufacturer's instructions (NanoString Technologies, Inc., Seattle, WA). The results obtained were analyzed with the analysis software nSolver 2.5 (NanoString Technologies). The Log2 ratios built from the data obtained were then uploaded into Ingenuity Pathway analysis software (Qiagen) for further analysis. The computationally networks and gene interactions were predicted based on the evidence stored in the Ingenuity Knowledge Base (genes and endogenous chemicals). The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's exact test. Only experimentally observed relationships were used to generate the summary of the top canonical pathways.

TLR-3 and TLR-4 gene expression study

The mRNA expression of mouse intestinal TLR-3 (toll-like receptor 3) and TLR-4 (toll-like receptor 4) was analyzed by RT-PCR in colonic and small intestine sections. Total RNA extractions were conducted with the RNeasy Mini Kit (Qiagen, Toronto, Canada) and DNase digestion during purification was carried out using the RNase-free DNase (Qiagen) according to the manufacture's

instructions. The RNA was reversely transcribed to cDNA with M-MLV Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. cDNA was amplified using the following primers from QuantiTect Primer (Qiagen, Toronto, Ontario) accession number NM_021297 for mouse TLR-4 and accession number NM_126166 for mouse TLR-3 gene. The amplification was carried out with an iCycler iQ5 (Bio-Rad, Hercules, CA) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Data were normalized in reference to TATA-box-binding protein 1 (TBP1) and analyzed with the 2 ΔCt method. To confirm amplification specificity, a melting curve analysis was carried out after the last cycle of each amplification. For each gene (TLR-3, TLR-4, and TBP1) a standard curve with efficiency between 90 and 103% was run together with the samples.

Statistical analysis

Statistical analysis was performed using Prism 6 software. Statistical comparisons were performed using student t-test, one-way ANOVA, Kruskal-Wallis test or multiple t-test, as appropriate. One-way ANOVA Dunn's multiple comparison was used to compare GF and colonized (*E. coli*, ASF, SPF) mice, whereas an unpaired student t-test was used to compare GF and cSPF mice. Immunofluorescence was analyzed using an unpaired student t-test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

CHAPTER 4

BACTERIAL COLONIZATION NORMALIZES BEHAVIOR IN EX-GERM FREE MICE INDEPENDENTLY OF THE MICROBIAL COMPLEXITY

COLONIZED EX-GF MICE DISPLAYED BEHAVIOURAL CHANGES SIMILAR TO CONVENTIONAL MICE

Several previous studies demonstrated differences in behaviour and brain chemistry between GF and conventional mice[22, 23, 26]. To confirm these findings, we first assessed behavior in GF and conventionally raised Swiss-Webster mice (cSPF) using light preference, step-down and tail suspension tests. Compared to GF mice, cSPF mice spent less time in the light compartment, stepped down faster from the elevated platform and exhibited more immobility – behavioural despair (**Fig 1A**). To determine whether the change in behaviour after bacterial colonization is dependent on the complexity of the microbiota, GF Swiss Webster mice were colonized with ASF (simple, 8 bacterial strains) and SPF (complex) microbiota and behaviour was accessed before, 2 and 4 weeks post colonization. A subset of mice colonized with ASF flora was euthanized 2 and 4 weeks post colonization for colonic tissues and blood to analyze their immune activation (cytokines, MPO, kynurenin/tryptophan ratio).

There was a significant change in behaviour in ASF and SPF colonized mice compared to GF mice. Both groups spent less time in the light compartment and displayed increased immobility at 2 and 4 weeks post colonization (**Fig 1A**). There was no difference between 2 and 4 week time points. In the step-down test, SPF colonized mice tended to step down faster, similar to cSPF mice, while the opposite trend was observed in ASF colonized mice (**Fig 1A**). This suggests that this aspect of behavior is dependent on the composition of the gut

microbiota. There was no change in the total distance traveled between ASF and SPF colonized mice compared to GF mice, suggesting the change in behaviour was not due to sickness behaviour (**Fig 1B**). Additional experiments in GF mice suggested that repeated handling with saline gavage does not affect behavior (**Fig 1C**).

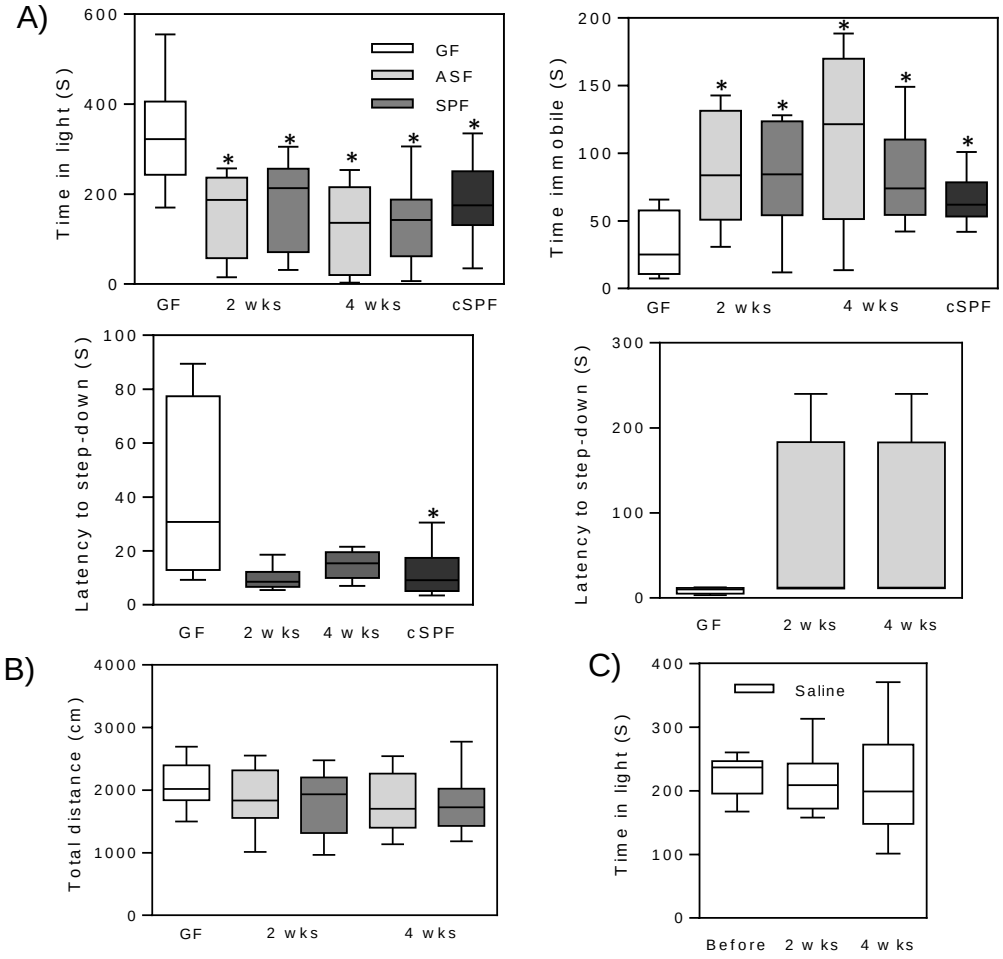


Figure 1: Behaviour tests in GF mice before and after colonization with ASF and SPF microbiota, and conventionally raised mice (cSPF). A) Light preference (n=20), tail suspension, and step-down tests (n=10) (B) total locomotor activities. (C) Light preference test on GF mice treated with saline (n=12). (*p<0.05 vs paired GF, Kruskal-Wallis test)

ABSENCE OF OVERT INFLAMMATION IN THE COLON OF ASF COLONIZED MICE

To access immune activation, MPO and cytokine levels were measured in colon tissues at 2 and 4 weeks post ASF colonization. The MPO values were low before and after colonization, indicating a lack of overt inflammation and no significant difference was observed even though there was a slight decrease at the 4 weeks time point (**Fig 2**). Cytometric Bead Array kit was used to quantitatively measure various inflammatory cytokines and chemokines such as IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70 protein levels in colonic tissues. All of the cytokine protein levels were below the level of detection indicating an absence of inflammation. Chemokine MCP-1 was detectable but no statistical differences were found before and after ASF colonization (**Fig 2**). It was previously reported that colonization of GF mice with ASF establishes an immune homeostasis in the colon through the activation of regulatory T (Treg) cells[187]. This explains the lack of overt inflammation in the colonized mice since a host-intestinal microbial immune homeostasis was established at 2 and 4 weeks. Moreover, ASF is comprised of eight commensal intestinal bacterial species and

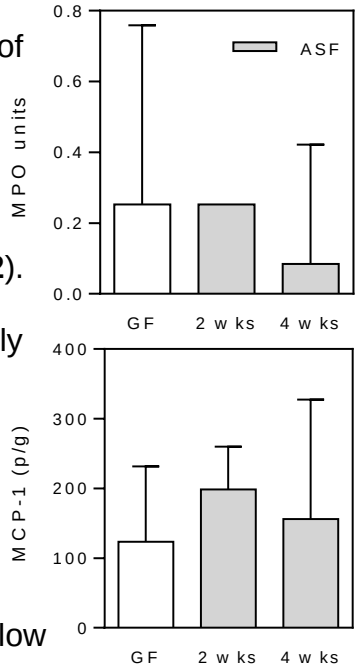


Figure 2: MPO and MCP-1 levels in colon tissues from GF mice before and after colonization with ASF (n=9)

would not induce an overt intestinal inflammation compared to colitis models (such DSS and DNBS) or bacterial infections.

KYNURENINE METABOLITES WERE LOWER IN ASF COLONIZED MICE

The precursor for serotonine (5HT) and downstream metabolites of the kynurenine pathway is the essential amino acid, tryptophan. The rate of tryptophan metabolism through the kynurenine pathway is dependent on the TDO or IDO1 enzyme leading to the production of kynurenine and kynurenic acid. Kynurenine itself can cross the BBB to participate in CNS synthesis of these neuroactive metabolites leading to changes in brain chemistry and behaviour[188]. The majority of the kynurenine in CNS is derived from the periphery, and plasma kynurenine increases are thought to be reliably reflected in the CNS[52].

In light of this we accessed the plasma kynurenine, kynurinic acid, tryptophan, and the kynurenine:tryptophan ratio (used as an index of IDO or TDO activity) in GF mice colonized with ASF. The kynurenine:tryptophan ratio was significantly lower at 2 weeks post colonization compared to GF mice suggesting that the TDO/IDO enzyme activity was reduced. Similarly kynurenine, kynurenic acid, and tryptophan levels were lower in colonized mice compared to GF mice (**Fig 3**). This suggests that the decrease in these neuroactive metabolites maybe involved in the change in behaviour.

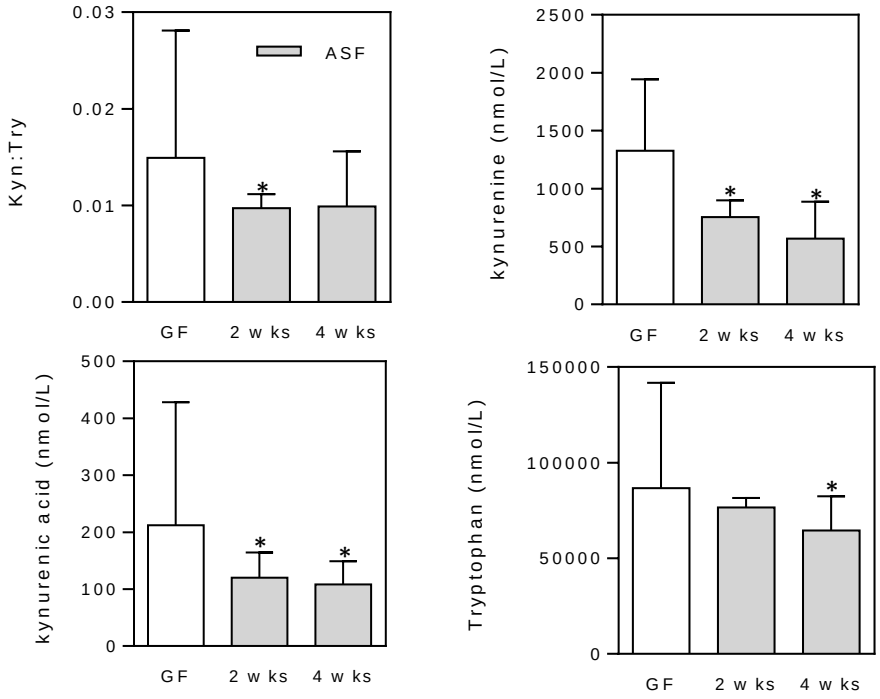


Figure 3: Plasma kynurenine metabolites and tryptophan levels in GF mice before and after colonization with ASF (n=12, *p<0.05 vs unpaired GF, Kruskal-Wallis test)

MONO-COLONIZATION NORMALIZES BEHAVIOUR SIMILAR TO COMPLEX MICROBIOTA

To determine whether a single bacterial strain, *E.coli* JM83, can change behaviour similar to ASF and SPF microbiota, GF Swiss Webster mice were mono-colonized with *E.coli* JM83 and behaviour was accessed before, 2, 4, and 6 weeks post colonization. A subset of mice were euthanized at 2 and 4 weeks post colonization for colonic tissues and blood to analyze their immune activation (cytokines, MPO, kynurenine/tryptophan ratio).

GF mice mono-colonized with *E.coli* JM83 displayed similar changes in behaviour as those colonized with ASF and SPF microbiota. They spent less time in the light and step-down faster at 2, 4, and 6 weeks post-colonization (**Fig 4A**). There was no change in the total distance traveled between GF and mono-colonized mice, suggesting that the behavioural change was not due to sickness behaviour (**Fig 4B**). This suggests that bacterial colonization normalizes behavior in ex-GF mice independently of the microbial complexity. Thus GF mice display abnormal behaviour compared to cSPF mice and bacterial colonization normalizes the behaviour. That being said, whether mono-colonization with a different bacterium (such as *Lactobacillus*) results in different behavioural changes remains unknown and requires further investigation.

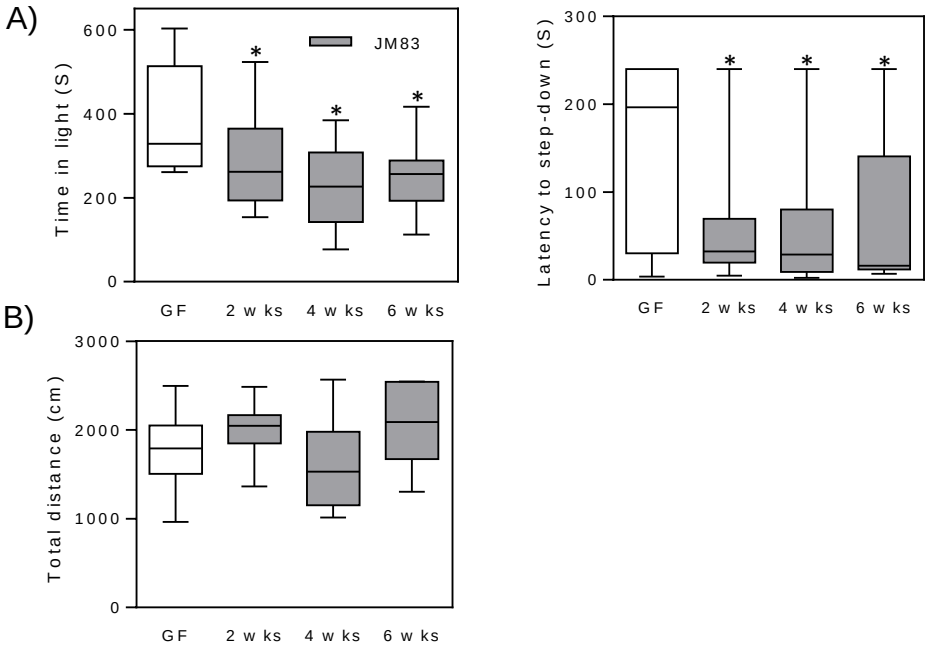


Figure 4: Behaviour tests in Swiss Webster GF mice before and after mono-colonization with E. coli JM83. A) Light preference, step-down test, and (B) total locomotor activity. (n=20, *p<0.05 vs paired GF, Kruskal-Wallis test)

ABSENCE OF OVERT INFLAMMATION IN *E.COLI JM83* MONO-COLONIZED MICE

To access immune activation, MPO and cytokine levels were measured in the colon tissues at 2 and 4 weeks post *E. coli* JM83 colonization. The MPO values were low before and after mono-colonization indicating a lack of overt inflammation, and at 4 weeks it was below the level of detection (**Fig 5**). CBA quantitative analysis of inflammatory cytokines and chemokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70) in colonic tissues showed all levels were below detection except MCP-1 chemokine, which showed similar levels before and after mono-colonization (**Fig 5**). In general, no overt inflammation was found in the colon of *E.coli* JM83 colonized mice similar to the ASF colonized mice. This could be due to the host-intestinal microbial immune homeostasis established following colonization through the activation of Treg cells as previously described[187]. It is also important to note that *E.coli* JM83 is a derivative of *E.coli* K12 commensal bacteria and therefore is unlikely to induce overt inflammation as does pathogenic bacteria.

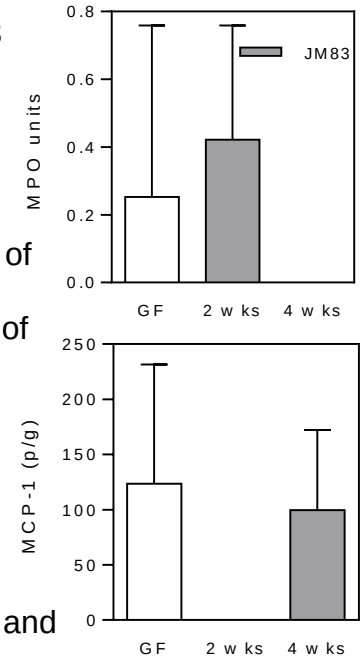
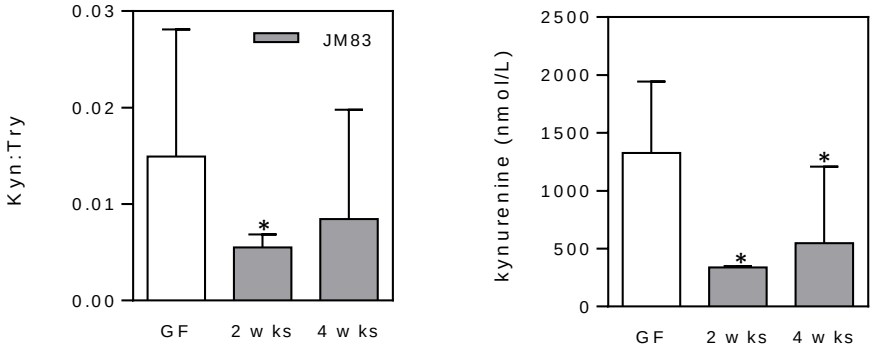


Figure 5: MPO and MCP-1 levels in colon tissues from GF mice before and after colonization with *E. coli* JM83 *(n=9)*

KYNURENINE METABOLITES WERE LOWER IN *E. COLI JM83* MONO-COLONIZED MICE

We accessed the plasma kynurenine, kynurinic acid, tryptophan, and the kynurenine:tryptophan ratio (used as an index of IDO or TDO activity) in GF mice mono-colonized with *E.coli* JM83 since these metabolites have been shown to influence host brain chemistry and behaviour as mentioned earlier. The kynurenine:tryptophan ratio was significantly lower at 2 weeks post colonization compared to GF mice, suggesting that the TDO/IDO enzyme activity was reduced. Also kynurenine metabolites, specifically kynurenic acid and kynurenine, were significantly lower in colonized mice (**Fig 6**). It is plausible that these neuroactive metabolites maybe involved in the normalization of behaviour following bacterial colonization.



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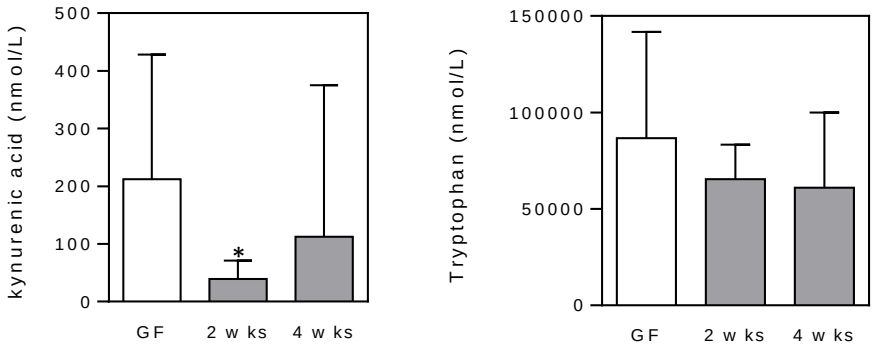


Figure 6: Plasma kynurenine metabolites and tryptophan levels in Swiss Webster GF mice before and after mono-colonization with *E. coli* JM83 (n=12, *p<0.05 vs unpaired GF, Kruskal-Wallis test)

BDNF AND C-FOS LEVELS LOWER IN *E. COLI JM83* MONO-COLONIZED MICE

The amygdala and hippocampus regions of the brain contain a number of important biochemical signalling molecules. One such molecule, brain-derived neurotrophic factor (BDNF), helps to keep neurons functional and to direct the birth of new ones. Perturbation of the microbiota is associated with an increase in hippocampal BDNF and a decrease in amygdala BDNF expression[26]. In the hippocampus, increased BDNF plays an anxiolytic and antidepressant role through alterations in brain serotonin[189]. The amygdala is involved in memory and mood disorders, and increases in BDNF in the amygdala have been linked to fear learning[190]. *C-fos* is a cellular oncoprotein that is 314 amino acids in length and transiently expressed in response to various stimuli, such as growth factors, cytokines, neurotransmitters and stress. It is important for the activation of various neuronal functions such as proliferation, differentiation and plasticity[83].

To correlate changes in behaviour with brain chemistry, we assessed levels of BDNF and c-fos expression as markers of brain plasticity and neural activation, respectively. These markers were measured in the hippocampus and amygdala regions before and 6 weeks after *E. coli* JM83 mono-colonization using immunofluorescence staining. BDNF and c-fos levels were significantly reduced in the hippocampus and amygdala regions, two important regions involved in regulation of exploratory/anxiety-like behavior, compared to GF controls (**Fig 7**). These results suggest that a decrease in BDNF and c-fos levels is associated with changes in behaviour following mono-colonization.

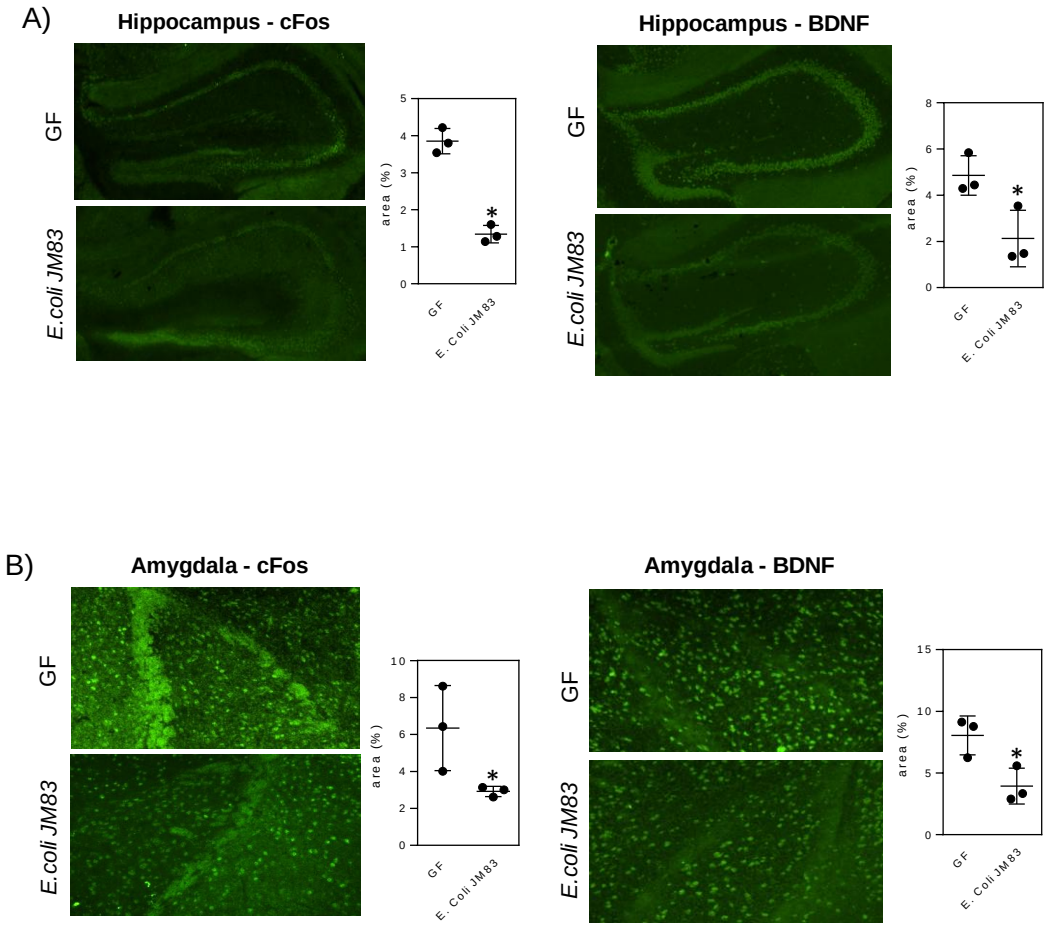


Figure 7: ***Immunofluorescence staining of Swiss Webster GF mice brains before and 6 weeks after mono-colonization with E. coli JM83*****.** BDNF and c-fos expression in the (A) hippocampus and B) amygdala regions of the brain (*p<0.05 vs unpaired GF, t-test)

CHAPTER 5

THE INNATE ARM OF THE IMMUNE SYSTEM THROUGH THE MYD88/TICAM PATHWAY IS CRUCIAL FOR THE DEVELOPMENT OF NORMAL BEHAVIOUR

ADAPTIVE IMMUNITY IS NOT INVOLVED IN THE NORMALIZATION OF BEHAVIOUR

Strong evidences suggest that the adaptive immune responses, specifically T lymphocytes, play an important role in cognition and behaviour. Significant impairment in acquisition of cognitive tasks is observed in severe combined immune deficient (SCID) mice compared to wild-type. Passive transfer of autologous T cells into wild-type mice after ablation of adaptive immunity restored previously impaired cognitive function[191]. Other adaptive immune deficient mice such as Rag $2^{-/-}$ (lack T and B cells) and nude mice (which lack mature T cells) all show impaired learning and memory[161, 192]. Adoptive transfer of $CD4^{+}$ T cells was sufficient to reverse these cognitive deficits demonstrating that functional T cells are necessary for normal brain function[161].

To test whether adaptive immunity is necessary for the development of normal behaviour in mono-colonized mice, behaviour was assessed in GF SCID mice before, 1 and 3 weeks post-colonization with *E. coli* JM83. Since the SCID mice are in a C57BL/6 genetic mouse background, behaviour was also assessed in wild-type C57BL/6 mice before, 1 and 3 weeks post-colonization to verify changes in behaviour. Similar to the Swiss Webster mice, behaviour in C57BL/6 mice was normalized after mono-colonization with *E. coli* JM83. They spent less time in the light compartment, step-down faster, and displayed increased immobility at 1 and 3 weeks post-colonization (**Fig 8A**). In SCID mice, mono-colonization normalized behaviour since they displayed a similar behavioural profile as the wild-type C57BL/6 mice (**Fig 8B**). This suggests that the adaptive

arm of the immune system does not play a role in the development of normal behaviour. Previous studies have shown the importance of adaptive immunity in social behaviour and cognitive functions[161, 191, 192].

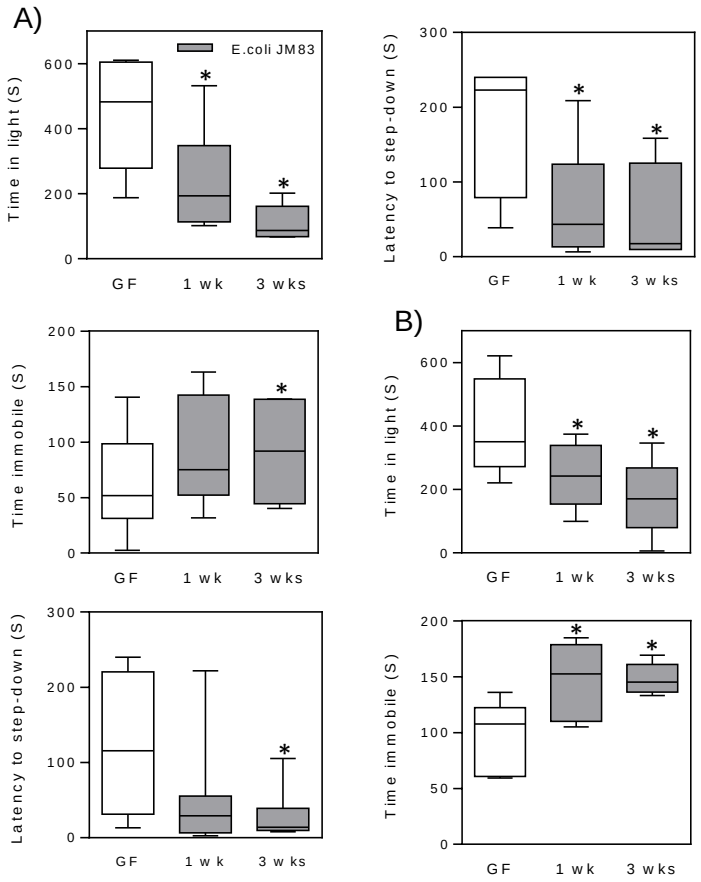


Figure 8: Behaviour tests in GF C57BL/6 and SCID mice before and after mono-colonization with E. coli JM83. Light preference, step-down and tail suspension test in (A) C57BL/6 (n=14) and (B) SCID (n=12) mice (*p<0.05 vs paired GF, Kruskal-Wallis test)

INNATE IMMUNITY IS CRUCIAL FOR THE NORMALIZATION OF BEHAVIOUR

Studies have shown the innate immune system plays an important role in the pathogenesis of psychiatric disorders[193]. The activation of MyD88 dependent Toll-like-receptor (TLR) by pathogen-associated molecular patterns initiates an intracellular kinase cascade by inducing the translocation of transcription factor, NF-κB, which leads to the production of a variety of inflammatory mediators and cytokines[194]. In addition to the involvement in inflammatory processes, TLRs are also known to play important roles in neurodevelopment, adult neurogenesis, and neuroplasticity in the absence of any underlying immune activation[195]. Furthermore, TLR-4 has been shown to be involved in higher cognition function and behavior[196].

To test whether the innate immune system is necessary for the development of normal behaviour in mono-colonized mice, behaviour was assessed in GF MyD88$^{-/-}$Ticam$^{-/-}$ mice before, 1 week and 3 weeks post-colonization with *E.coli* JM83. Unlike C57BL/6 mice, no behavioural change was observed in the light preference and tail-suspension tests at 1 and 3 weeks post-colonization with *E.coli* JM83. A slight but significant difference was found in the step-down test after mono-colonization (**Fig 9**). This suggests that innate immunity is necessary for the normalization of behaviour since two of the three behavioral tests showed no changes compared to wild-type C57BL/6 mice. The fact the step-down test showed behavioural changes suggests that different behavioural tests may be measuring different areas of the brain. Perhaps MyD88-

Ticam pathway may not be necessary to induce exploratory behaviour following bacterial colonization but may play a role in depression-like behaviour measured by the tail-suspension test. There is a possibility that different types of behaviour may be governed by different areas of the brain and these tests do not have to complement each other depending on the regions of the brain they each measure. Further research is required to understand which regions of the brains are being measured by the different behavioral tests. Nevertheless, the behavioural data suggest that the innate immune system via the MyD88/Ticam pathway plays a key role in the development of normal behaviour.

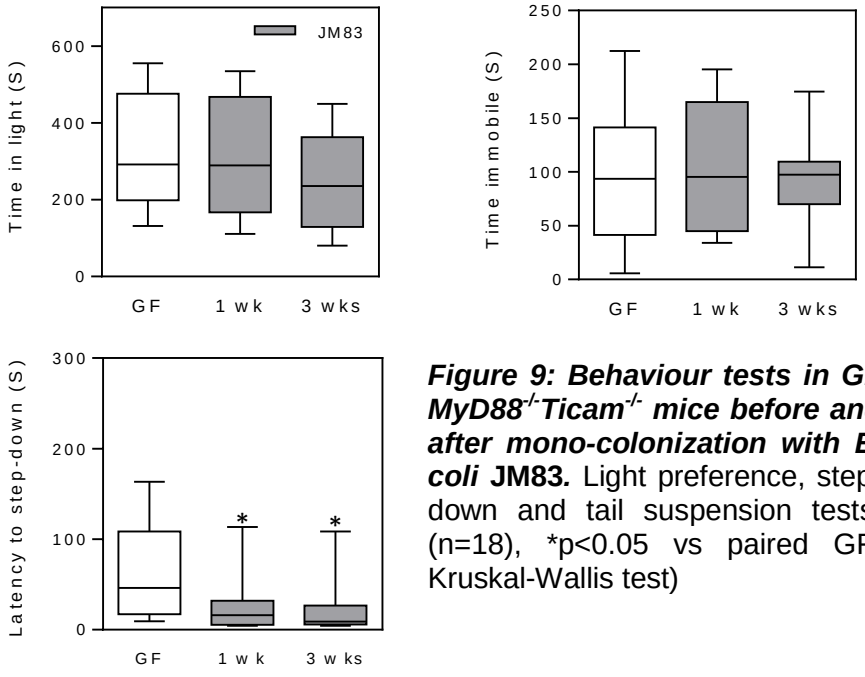


***Figure 9: Behaviour tests in GF MyD88$^{-/-}$Ticam$^{-/-}$ mice before and after mono-colonization with E. coli* JM83.** Light preference, step-down and tail suspension tests. (n=18), *p<0.05 vs paired GF, Kruskal-Wallis test)

NO CHANGES IN BDNF AND C-FOS LEVELS IN MYD88$^{-/-}$TICAM$^{-/-}$ MICE

To correlate lack of behavioural changes with brain chemistry, BDNF and cfos levels in the hippocampus and amygdala regions of the brain were measured before and 1 week after *E.coli* JM83 mono-colonization using immunofluorescence staining. Both in the hippocampus and the amygdala no changes in BDNF and c-fos levels were observed following mono-colonization (**Fig 10 A,B**). This suggests that the MyD88/Ticam pathway is crucial for communicating to the brain following bacterial colonization.

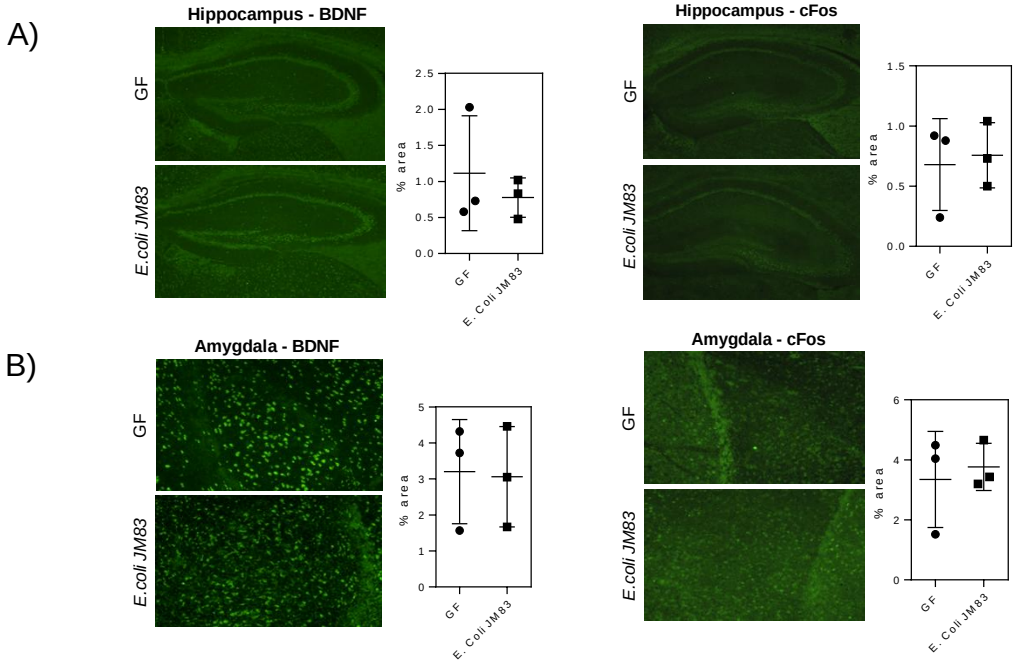


Figure 10: Immunofluorescence staining of GF MyD88$^{-/-}$Ticam$^{-/-}$ mice brains before and 1 week after mono-colonization with E. coli JM83. BDNF and c-fos expression in the (A) hippocampus and B) amygdala regions of the brain

CHAPTER 6

CONTINUOUS PRESENCE OF BACTERIA NOT REQUIRED TO MAINTAIN NORMAL BEHAVIOUR

TRANSIENTLY COLONIZING BACTERIUM NORMALIZES BEHAVIOUR

The lower intestine of adult mammals is densely colonized with commensal microbes. Bacterial colonization is essential for the maturation of the immune system either directly or indirectly leading to host-microbial mutualism. Analysis of GF mice following bacterial colonization revealed that major immune responses could be detected starting 4 days after bacterial colonization with a strong induction of innate immune functions followed by stimulation of adaptive immune responses[197].

It is evident that bacterial colonization regardless of the complexity of the microbiota normalizes behaviour in GF mice. However, due to the continuous presence of bacteria in the gut it is difficult to assess whether a constant bacterial stimulation is required to maintain normal behaviour after colonization. Therefore we used a transient colonizing bacterium, *E. coli* HA107, to uncouple bacterial stimulation from the mucosal immune response *in vivo*. *E. coli* HA107 (mutant form of the parental strain *E. coli* JM83) colonizes mouse intestine for 12-48 hours before reverting back to GF conditions[182]. To determine whether the continuous presence of gut bacteria is required to maintain normal behaviour after colonization, GF Swiss-Webster mice were gavaged with *E.coli* HA107 six times over two weeks and behaviour was assessed at 2, 4, and 6 weeks post-colonization. A similar change in behaviour after colonization as with ASF, SPF, and *E. coli* JM83 was observed after mono-colonization with *E. coli* HA107. The mono-colonized mice spent less time in the light and step-down faster at 2, 4,

and 6 weeks post-colonization compared to the GF control group (**Fig 11A**). Interestingly, transiently mono-colonized mice maintained normal behaviour at 4 and 6 weeks post-colonization when the gut had reverted to germ-free conditions. The reversal to germ-free status was confirmed by culture as no bacterial growth in stool and cecum was observed at 4 and 6 weeks after mono-colonized with *E.coli* HA107. Again, no difference was found in the total distance traveled suggesting that the change in behaviour was not due to sickness behaviour (**Fig 11B**). Thus our data show that a constant presence of gut bacteria post colonization is not required to maintain normal behaviour. This suggests that the accompanying activation/maturation of the immune system following bacterial colonization permanently changes the brain chemistry leading to normal behaviour and does not require continuous bacterial stimulation from the gut.

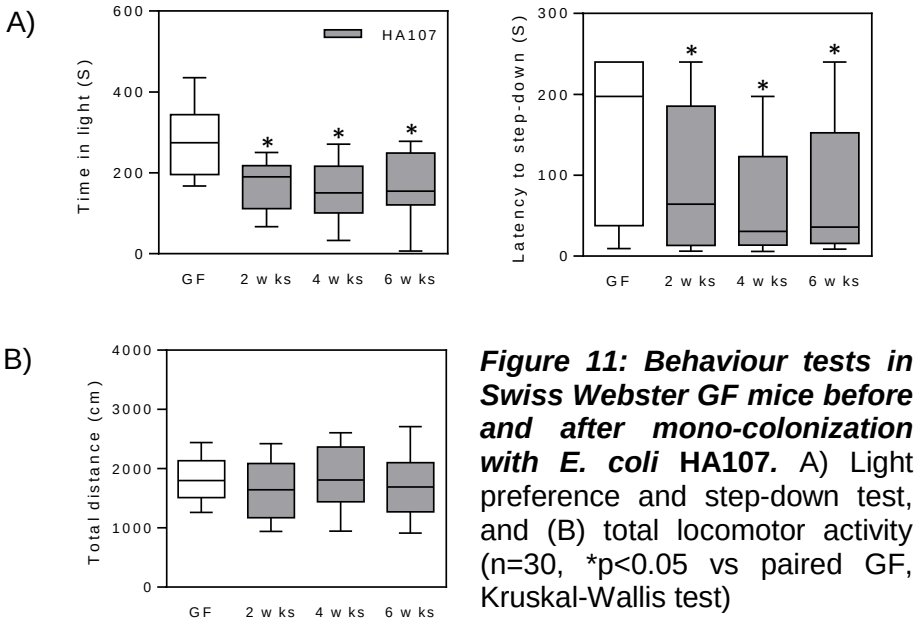


***Figure 11: Behaviour tests in Swiss Webster GF mice before and after mono-colonization with E. coli* HA107.** A) Light preference and step-down test, and (B) total locomotor activity (n=30, *p<0.05 vs paired GF, Kruskal-Wallis test)

ABSENCE OF OVERT INFLAMMATION IN *E. COLI HA107* MONO-COLONIZED MICE

To access immune activation, MPO and cytokine levels were measured in the colon tissues at 2 and 4 weeks post *E. coli* HA107 colonization. The MPO values were low before and after mono-colonization and at 4 weeks they were below the level of detection (**Fig 12**). Analysis of inflammatory cytokines and chemokines (IL-6, IL-10, MCP-1, IFN-γ, TNF, and IL-12p70) in colonic tissues showed all levels were below detection except MCP-1 chemokine, which was slightly higher before compared to 2 and 4 weeks after mono-colonization, but this did not reach statistical significance (**Fig 12**). In general, no overt inflammation was found in the colon of *E. coli* HA107 colonized mice, similar to the ASF colonized mice.

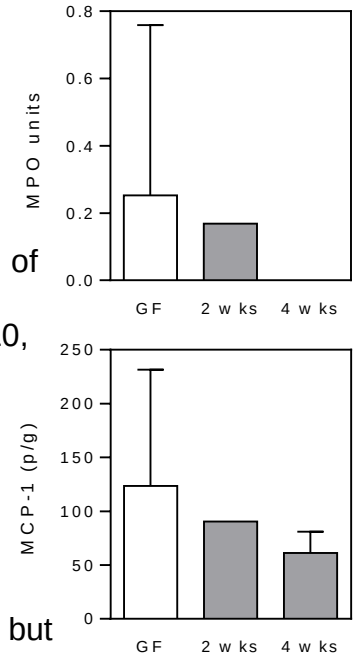


Figure 12: MPO and MCP-1 levels in colon tissues from GF mice before and after colonization with E. coli* HA107 *(n=9)

KYNURENINE METABOLITES WERE LOWER IN *E. COLI HA107* MONO-COLONIZED MICE

We accessed the plasma kynurenine, kynurinic acid, tryptophan, and kynurenine:tryptophan ratio (IDO or TDO activity) in GF mice mono-colonized with *E.coli* HA107*,* since these metabolites have been shown to influence host brain chemistry and behaviour as mentioned earlier. The kynurenine:tryptophan ratio was significantly lower at 2 and 4 weeks post colonization, compared to GF mice, suggesting that TDO/IDO enzyme activity was reduced. Also, kynurenine

metabolites, specifically kynurenic acid and kynurenine, were significantly lower in colonized mice (**Fig 13**). This suggests a correlation between the decrease in tryptophan metabolites and bacteria induced changes in behaviour even in the absence of gut bacteria.

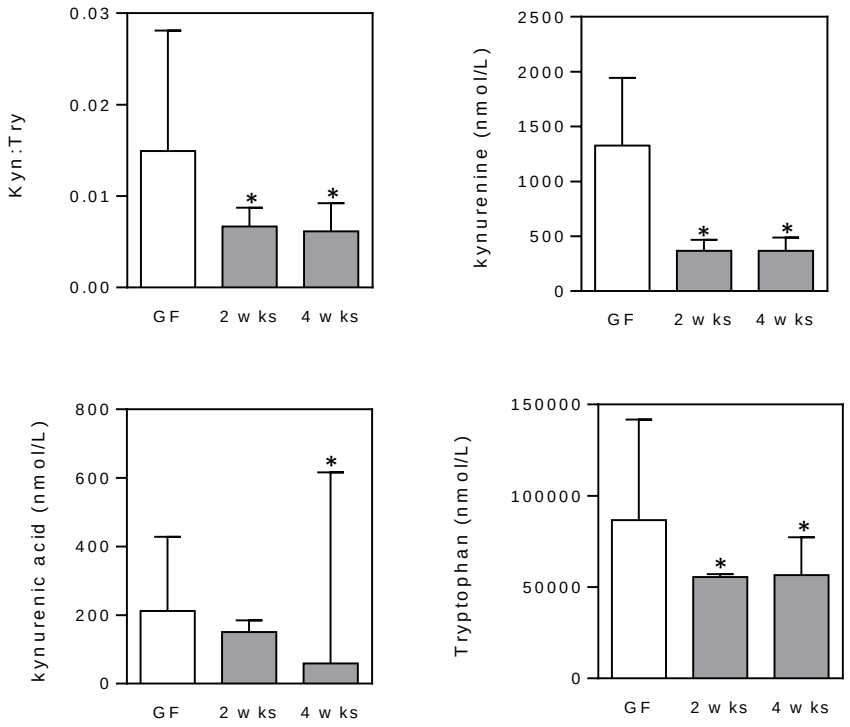


***Figure 13: Plasma kynurenine metabolites and tryptophan levels in Swiss Webster GF mice before and after mono-colonization with E. coli* HA107** (n=12, *p<0.05 vs unpaired GF, Kruskal-Wallis test)

CHAPTER 7

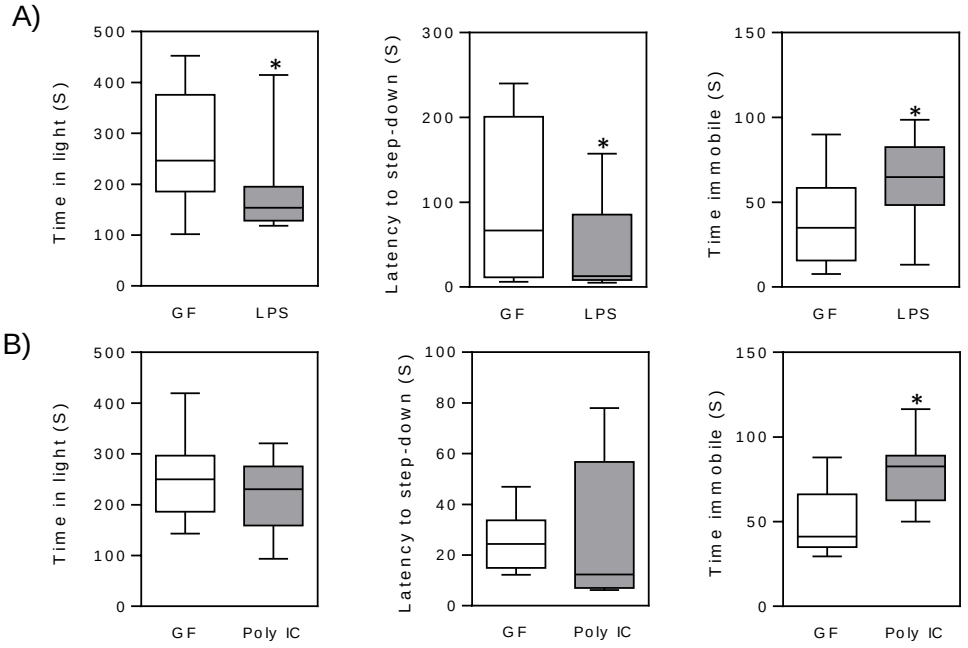
BACTERIAL STIMULATION THROUGH TLR-4 IS NECESSARY FOR THE DEVELOPMENT OF NORMAL BEHAVIOUR

E. COLI LPS NORMALIZED BEHAVIOUR SIMILAR TO COLONIZED MICE

TLRs 1–9 are highly conserved across humans and rodents during evolution and deletion of various TLRs in mice is associated with a wide range of neurocognitive and/or physiological sequelae[198]. For example, mice deficient in TLR-2 exhibit schizophrenia-like neuropsychiatric disorders, including hyperactivity, reduced anxiety, social withdrawal, prepulse inhibition deficit and cognitive dysfunction[199]. TLR-4 was shown to play a developmental role in regulating hippocampus-dependent spatial and contextual cognition[196], while enhanced hippocampal neurogenesis and improved hippocampus-dependent cognition was found in TLR-3 deficient mice[200]. TLR-4 recognizes lipopolysaccharide (LPS) present in Gram-negative bacteria and a variety of endogenous proteins such as low-density lipoprotein, beta-defensins, and heat shock protein. Mammalian TLR-3 recognizes viral double stranded (ds) RNA and that activation of the receptor induces the activation of NFκB and the production of type I interferons. Polyinosinic:polycytidylic acid (poly I:C) is a synthetic analogue of dsRNA used to mimic the intermediates of replication present in cells infected with RNA viruses and some DNA viruses[201].

Based on the results so far, it is evident that signalling through the MyD88/Ticam pathway is crucial for the normalization of behaviour following bacterial induced innate immune activation in GF mice. Next we wanted to investigate whether a normal behaviour can be achieved in GF mice by activating innate immunity in the absence of bacterial stimulation. To determine this, we

gavaged poly I:C (viral RNA) or *E. coli* LPS three times weekly for 2 weeks to activate TLR-3 or TLR-4 receptors, respectively. Behaviour was assessed in GF C57BL/6 mice before and after poly I:C or *E. coli* LPS administration. LPS treated mice displayed normal behaviour, similar to mice mono-colonized with *E. coli*. They spent less time in light, stepped down faster from an elevated platform, and remained immobile longer during the tail-suspension test (**Fig 14A**). In contrast, GF mice treated with poly I:C showed no significant change in the light preference and step-down test, but they displayed longer immobility in the tail-suspension test (**Fig 14B**). There was no difference in total locomotor activities in LPS and poly I:C treated mice compared to before treatment, confirming the change in behaviour was not a result of sickness behaviour (**Fig 14C**). This suggests that bacterial stimulation via TLRs, mainly the TLR-4 pathway, is required for the development of normal behaviour.



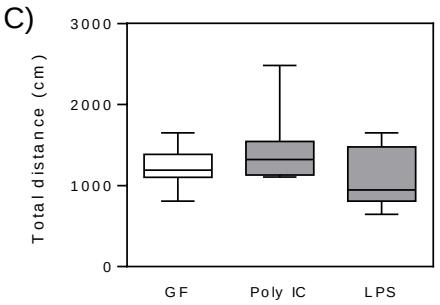


Figure 14: Behaviour tests in GF C57BL/6 mice before and after LPS and poly I:C treatment. Light preference, step-down, and tail suspension test in (A) LPS and (B) poly I:C treated mice. (C) Total locomotor activities. (n=8, *p<0.05 vs paired GF, t-test)

TLR-4 AND TLR-3 GENE EXPRESSION UNREGULATED

It has been reported that poly I:C can be rapidly degraded after oral administration because of the nuclease activity from pancreatic enzymes[202] and therefore it may not reach the lower intestinal tract. LPS, on the other hand, resists the various enzymatic activities of the stomach and pancreas. In light of this, we measured colonic and small intestinal TLR-3 and TLR-4 gene expression before and after poly I:C and LPS administration, respectively, to ensure that both ligands are reaching the lower gastrointestinal tract. We found an increase in TLR-4 expression in the colon and small intestine in LPS treated mice (**Fig 15A**) and an increase in TLR-3 expression in the small intestine of poly I:C treated mice (**Fig 15B**). This suggests that the difference in behavior profiles between LPS and poly I:C are not likely due to a lack of immune stimulation in the proximal gut. However, LPS-treated mice, but not poly I:C treated mice, had increased TLR-4 expression in the colon suggesting that LPS was sensed by the immune system. Our data suggests that activation of TLR-4 pathway may be important for the development of normal behaviour.

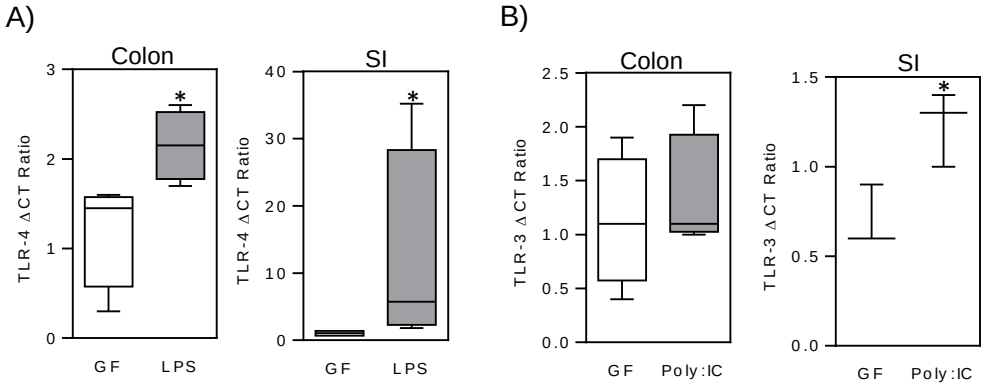


Figure 15: (A) TLR-4 and (B) TLR-3 gene expression from colon and small intestine (SI) in LPS and poly I:C treated mice. (n=4, *p<0.05 vs paired GF, t-test)

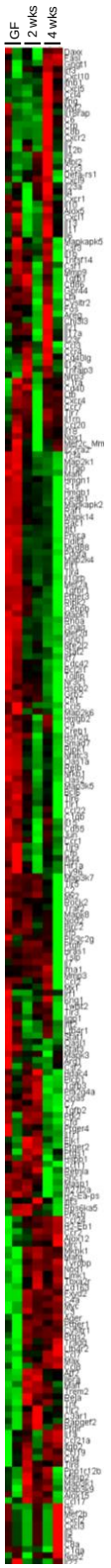
CHAPTER 8

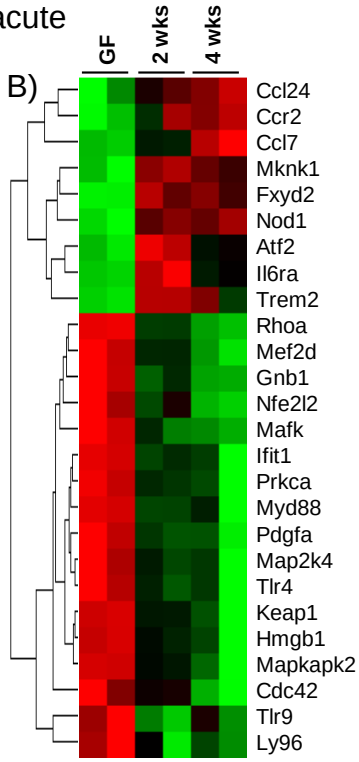
INNATE IMMUNITY AND NEURONAL GENES SIGNIFICANTLY ALTERED AFTER BACTERIAL COLONIZATION

INNATE IMMUNE RELATED GENES IN COLON WERE ALTERED AFTER MONO-COLONIZATION

To further investigate the underlying mechanisms, we carried out transcriptome analysis to evaluate the colonic genes that play a significant role in the development of normal behaviour after colonization. We hypothesized that there are a core set of immune genes in the colon that are critical for the behavioral changes following mono-colonization with *E. coli*. Two hundred and fifty inflammatory genes were analyzed in colon tissues from GF and *E. coli* mono-colonized mice. Colonic RNA was extracted from Swiss Websters, and MyD88$^{-/-}$ Ticam1$^{-/-}$ mice and gene expression were analysed using the NanoString nCounter® CodeSet for mouse inflammation. Overall, the gene expression profile showed major effects of mono-colonization, as most of the 250 genes were either upregulated or downregulated (**Fig 16A**). This was expected since many of these genes play a vital role in the development of lymphoid structures and immune-microbial homeostasis[203]. To determine which genes may play a major role in normalized behaviour, we considered those genes whose expression was in agreement with the following three criteria: 1) changes in transcripts after colonization in wild-type mice (GF vs colonized mice), 2) no changes in transcripts between 2 and 4 weeks post-colonization in wild-type mice, 3) no changes in transcripts after colonization in MyD88$^{-/-}$ Ticam$^{-/-}$ mice.

In total, we identified 28 genes which fulfilled these criteria, most of which are related to innate immunity and play a role in inflammatory responses upon exposure to microbial factors (**Fig 16B**). Using the Ingenuity pathway analysis (IPA), we found that many of them were not only related to innate immunity such as leukocyte activation and myeloid cell recruitment, but also neural development and behaviour (**Fig 16C**). The main canonical pathways between GF and colonized mice were the acute phase response ($P=1.71*10^{-32}$), toll-like receptor ($P=8.11*10^{-26}$), pattern recognition receptors ($P=4.38*10^{-25}$), and glucocorticoid receptor ($P=1.26*10^{-19}$).





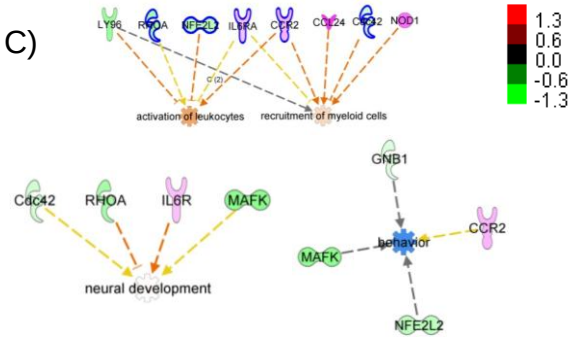


Figure 16: (A) 250 colonic inflammatory genes from GF and mono-colonized mice with E. coli HA107. B) Inflammatory genes from colon tissue that are important for normalized behaviour in *E. coli* HA107 mono-colonized mice (t-test, p<0.01, FDR = 1). (C) IPA network of colonic genes involved in innate immunity, neural development, and behaviour.

IMMUNE AND NEURAL GENES WERE ALTERED IN THE BRAIN AFTER MONO-COLONIZATION

Similar to the colon tissues, we evaluated transcriptional signatures in the brain to link the immune profile in the gut with the brain. We hypothesized that there are a core set of immune and neural genes in the brain that are critical for the behavioral change following mono-colonization. RNA was extracted from the hippocampus and amygdala regions of the brain from GF and *E.coli* colonized mice, and gene expression was analysed using the custom NanoString gene expression codeset. In total, 10 genes were significantly changed after bacterial colonization that belongs to neural, immune, and endothelial barrier function (**Fig 17 A,B**). Ingenuity Pathway Analysis revealed many of these genes play a key role in neural cell proliferation, inflammatory response, and BBB function (**Fig 17C**). The canonical pathways that were significantly different between GF and colonized mice were the toll-like receptor ($P=4.34*10^{-9}$) and axonal guidance signaling ($P=5.25*10^{-4}$).

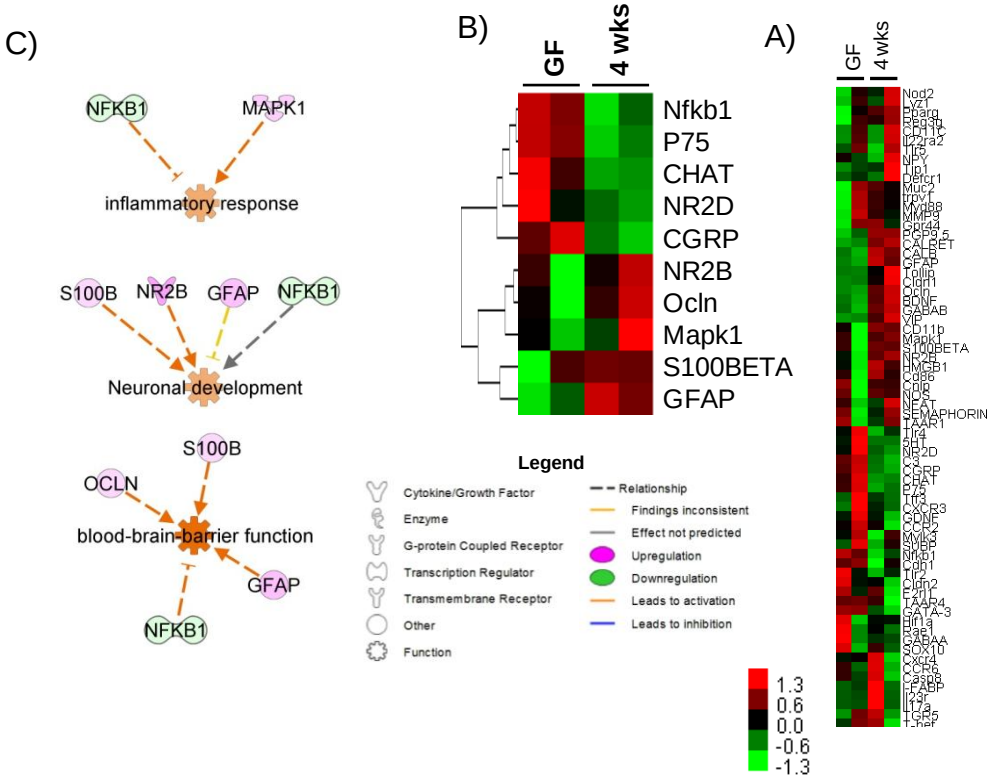


Figure 17: (A) 70 immune and neuronal genes from brain tissues in GF and E. coli HA107 mono-colonized mice. B) Genes that are significantly different between GF and mono-colonized mice from hippocampus and amygdala regions of the brain (t-test, $p<0.05$, FDR = 1). (C) IPA network of brain genes involved in inflammatory response, proliferation of neural cells, and blood-brain-barrier function

CHAPTER 9

DISCUSSION

Gut microbiota is a key mediator of gut-brain signalling. It can influence beyond the gut and is pivotal in many aspects of brain development, function and behaviour. Microbiota can signal to the brain through various inter-related pathways that include the neuro-immune-endocrine pathway, afferent sensory neurons of the vagus nerve, microbial metabolites, and tryptophan metabolism.

In this study we investigated the role of immune system maturation on mouse behaviour after bacterial colonization. We tested the hypothesis that bacterial colonization of GF mice leads to changes in brain chemistry and normalizes behaviour through the maturation of the immune system. The results of this study provided strong evidence for the importance of gut microbiota in the development of normal behaviour through an immune-dependent pathway. This is supported by several lines of evidence. First, bacterial colonization of GF mice normalizes behaviour independent of the complexity of the microbiota. Secondly, the signal to the brain is dependent on the innate arm of the immune system, specifically the MyD88/Ticam pathway. Thirdly, the continuous presence of bacteria is not required to maintain normal behaviour post bacterial colonization. Fourthly, normal behaviour can be induced in GF mice by stimulating the TLR-4 pathway through LPS administration. Lastly, transcriptome analysis of colon and brain tissues revealed that multiple innate immunity and neuronal genes were altered after mono-colonization.

Mono-colonization normalizes behaviour

Sudo and colleagues first reported that the hypothalamic pituitary to mild stress is exaggerated in GF mice and is partially normalized following bacterial colonization[42]. Subsequently, a wealth of microbiota-related effects have been reported in relation to behaviour, nociceptive responses, feeding behavior, and metabolic consequences[13]. In this study we showed that mono-colonization of GF mice with commensal *E. coli* normalizes behaviour similar to colonization with complex (SPF) and simple (ASF) microbiota, and conventionally raised mice. This is consistent with previous results that showed changes in behaviour when GF mice were colonized with SPF microbiota[22, 26]. We are the first to report that colonization of GF mice with a single commensal bacterium is sufficient to normalize behaviour similar complex microbiota. It was reported that a critical period exists early in life during which the brain receives input from the microbiota to induce long-term behavioural changes[22]. However, our results showed long term behavioural changes following bacterial colonization in adult (10-14 weeks) GF mice, which is consistent with the previous study from our lab[45]. Overall, the behaviour profile suggests that GF mice displayed abnormal behaviour and that bacterial colonization normalizes this behaviour.

A lack of overt inflammation in the colon after colonization (ASF or *E. coli*) indicates that the mechanism for the behavioral change is different from those observed in chemically induced colitis (DSS or DNBS) or pathogenic models. Despite absence of overt inflammation, the MPO values in GF mice were slightly

higher compared to 4 weeks after colonization. This could be due to a lack of Treg activation and expansion in GF mice leading to a hyperactive immune response in the colon, which was normalized after bacterial colonization[187].

BDNF and cfos expressions in the brain have emerged as prominent features of the microbiota gut-brain axis communication. Many studies have shown changes in expression in both GF and antibiotic treated mice[204]. We demonstrated altered BDNF and c-fos expression in the hippocampus and amygdala regions of mono-colonized mice. The decrease in BDNF expression in colonized mice is consistent with previous literatures[23, 205]; however there are also contradicting results published previously[22, 42]. This discrepancy could be attributed to a number of different factors including bacterial diversity in the gut, the age and genetic background of the mice, and differential housing environments which has been reported to effect hippocampal BDNF expression levels[206]. Even though there are reports to support a decrease in BDNF levels in colonized mice, it seems that BDNF expression may also depend on other systems besides the microbiota. How these microbiota-induced changes in neurotrophin factor expression affect the structure and function of the GF brain warrants further investigation.

Continuous presence of bacteria is not required to maintain normal behaviour

Although it is evident that bacterial colonization of GF mice can restore behavioral effects similar to conventionally raised mice, it is yet to be determined

whether the continuous presence of bacteria is required to maintain these changes or whether the initial priming of the mucosal immunity following bacterial colonization is sufficient to maintain normal behaviour. One can speculate based on literature that a continuous microbial signalling, either directly or indirectly, may be required to maintain normal behaviour. Specifically, the production of a number of microbiota induced metabolites such as SCFAs, fermentation products (lactic acid and propionic acid), and neuroactive substances (GABA, tryptophan, serotonin, catecholamines) can all signal to the host brain[12]. Most metabolites identified in the circulation of the host are of gut microbial origin[116], providing the theoretical basis for the microbiota-gut-brain axis signaling system. On the other hand, bacteria in the gut may also interact with neural circuitry indirectly through non-neuronal intermediary cell types such as endocrine or immune cells of the gut. Therefore, we investigated whether the initial priming of the mucosal immunity following bacterial colonization is sufficient to maintain normal behaviour.

We showed that a continuous presence of gut bacteria is not required to maintain normal behaviour since behavioural changes persist even after the gut reverts to germ free status using a transiently colonizing bacterium, *E.coli* HA107. This suggests that the immune activation/maturation following mono-colonization of GF mice is sufficient to maintain normal behaviour. GF animal studies have shed light into the importance of bacterial colonization on proper development of the ENS[100]. During embryogenesis the ENS develops within a largely sterile

environment, but the postnatal stages of ENS development and maturation take place under strikingly different conditions due to the ingestion of food and the establishment of microbiota, which result in a multitude of changes to the maturation of the immune system[203]. It is plausible that maturation of the immune system following bacterial colonization could lead to the development of the ENS, which in turn would bring about permanent changes to brain function and normalization of behaviour.

In addition to direct sensing of bacteria by gut-innervating sensory neurons, metabolic communication in the gut–brain axis occurs when bacterial metabolites become absorbed into the portal vein and enter circulation. These bacterial metabolites may then cross the BBB to directly affect brain function[41]. It seems in our study that normalization of behaviour was not due to the direct effect of the microbial metabolites since GF mice developed normal behaviour after oral administration of *E.coli* LPS for 2 weeks. This suggests that the development of normal behaviour was through an immune-dependent pathway and not a direct bacterial metabolite pathway. That being said, microbial metabolites can regulate the function of the immune system directly through the aryl hydrocarbon receptor (AHR). Several reports have shown that AHR has the capacity to mediate the differentiation and function of microglia, macrophages, and dendritic cells[208]. Recently it was shown that microbial metabolites can modulate astrocyte activity and CNS inflammation through the AHR[209], thereby

linking the microbiota directly to neuroinflammatory mechanisms through the AHR.

Mice administered *E.coli* LPS displayed normal behaviour suggesting that the TLR-4 receptor is crucial for the development of normal behaviour. This is consistent with our gene expression profile that showed TLR-4 to be an important gene responsible for the change in behaviour. Poly I:C administration only partially affected the behaviour with one out of the three behavioural tests being positive, which suggests that other TLRs may also contribute for behavioural change and this requires further investigation.

Potential pathways for microbiota-gut-brain signalling

Understanding the mechanisms underpinning the extent of the influence exerted by the microbiota on host physiology, brain and behaviour is of utmost importance. A number of pathways and potential mechanisms which may regulate the bi-directional communication between the gut-brain interactions are under investigation. The messengers involved in this complex dialogue between the gut and the brain include neural, metabolic, endocrine and immune mediators responsive to diverse environmental cues, including nutrients and components of the intestinal microbiota. To investigate potential immune-mediated pathways in the gut-brain communication, we used knockout mice to determine the role of innate and adaptive immunity, transcriptome analysis of inflammatory genes in colon and brain tissues, and tryptophan metabolism through the kynurenine pathway (**Fig 18**).

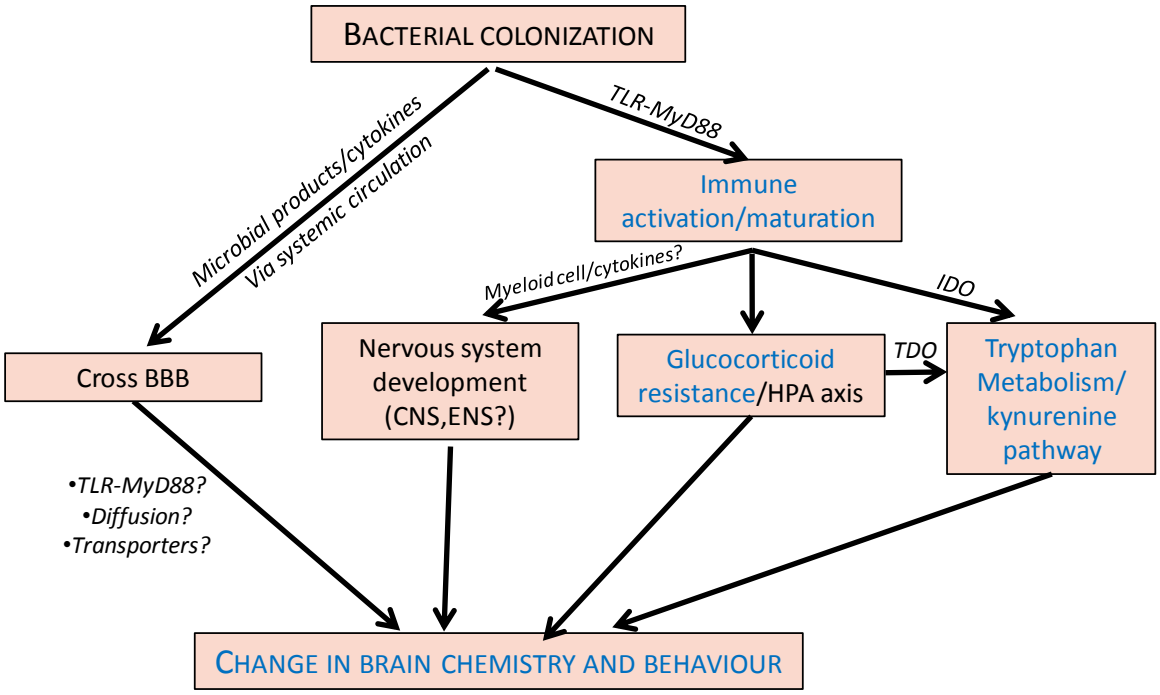


Figure 18: Potential pathways for microbial-induced behavioral change. It is evident that activation and maturation of the immune system through the TLR-MyD88 pathway is important for the change in behaviour. Based on the results we hypothesize that a number of different pathways is involved in the behavioural change (*illustrated above*). Maturation of the immune system post-bacterial colonization changes behaviour possibly through the development of the nervous system (ENS or CNS), glucocorticoid receptor and HPA axis signalling, or through the modulation of tryptophan metabolism. Direct pathway for the induction of behavioural change post-bacterial colonization may involve microbial products or cytokines crossing the blood-brain-barrier (BBB). **Abbreviations**: TDO, tryptophan 2,3-dioxygenase; IDO, Indoleamine-pyrrole 2,3-dioxygenase. *Blue highlights show results currently presented in this thesis*.

INNATE IMMUNITY: The immune system integrates and modulates bi-directional signals between the CNS and the gut, and mechanistically links alterations in function in both brain and gut. An altered immune system can affect brain function and behaviour as evidenced during sickness behaviour, and the brain has the potential to profoundly affect the immune function via glucocorticoids and

catecholamines[210]. In this study we focused on the immune-mediated pathway, but other pathways such as neuronal or humoral, including microbial metabolites are likely involved in the microbiota gut-brain communication. We used a reductionist approach with *E. coli* mono-colonization, which mimicked most of the behavioural changes induced by colonization with complex microbiota, and knockout mice to determine the role of the innate or adaptive immunity. We assessed mouse behaviour before and after colonization with *E. coli* JM83 in MyD88$^{-/-}$ Ticam$^{-/-}$ and SCID mice with an impaired innate and adaptive immunity, respectively. Both wild-type C57BL/6 (genetic background of the knockout mice) and SCID displayed a change in behaviour but this was not observed in MyD88$^{-/-}$ Ticam$^{-/-}$ mice. This suggests that the innate immunity, specifically through the MyD88-dependent TLRs is crucial for the development of normal behaviour. BDNF and c-fos expression in the hippocampus and amygdala regions of MyD88$^{-/-}$ Ticam$^{-/-}$ mice showed no changes in behaviour before and after mono-colonization, confirming the importance of the MyD88/Ticam pathway. Colonic gene expression data confirmed MyD88 to be one of the key genes involved in the development of normal behaviour. It is well established that gut microbiota is essential for the priming and maturation of the mucosal immunity via the TLR-MyD88 pathway[203] by affecting the development of lymphoid structures and epithelial cell proliferation. Moreover, the TLR-MyD88 pathway is crucial for the mucosal homeostasis following bacterial colonization early in life[211]. Our results suggest that the immune activation following bacterial colonization is transmitted

to the brain through a TLR-MyD88 dependent pathway. However, it is unknown whether this signal to the brain is initiated at the level of epithelial or myeloid cells. The adaptive immunity has been shown to be important for cognition and behaviour, especially the adaptive meningeal immunity was reported to be crucial for regulating neural activity and social behaviour through the IFN-γ signalling[170]. We found that the adaptive arm of the immune system does not play a role in the development of normal behaviour. We hypothesize that shaping the neuronal circuitry to develop everyday behaviour may be under the influence of the innate immunity since it evolved earlier compared to higher cognitive functions, such as learning and memory, which evolved later under the adaptive immune response[170].

We have shown multiple innate immune related genes that are responsible for the normalized behaviour, with some having dual functions in both innate immune response and neural system development. For instance, members of the Rho family of small GTPases, *RhoA* and *Cdc42* genes that were down-regulated after colonization, were previously reported to be important for the chemotaxis response of leukocytes to sources of chemoattractants[212] and nervous system development, including neuronal migration, axon guidance and synapse formation[213]. Another up-regulated gene, CC chemokine receptor 2 (*CCR2*) was shown to be important for monocyte trafficking and inflammation within the CNS, and was extensively studied in the context of multiple sclerosis and Alzheimer's disease[214]. We speculate that bacterial colonization primes and matures the

innate immune system, which influences the development of the nervous system leading to normal behaviour (**Fig 18**). It was previously shown using transcriptome analysis that conventionalized GF mice displayed strong induction of the innate immune response followed by the stepwise development of the adaptive immune response. This peaking of innate immunity occurred 4 days after *de novo* microbial colonization[197]. From our gene expression data, we see that many inflammatory genes (*Hmgb1, Tgfb, Tlr4, Myd88*) were down-regulated, suggesting that the strong innate immune response following bacterial colonization subsided by 2 and 4 weeks after colonization. Although TLR-4 expression is down-regulated in colon tissue, qPCR showed an up-regulation of TLR-4 gene in *E. coli* LPS treated mice. It is unclear as to the reason for this discrepancy, but we speculate this could be attributed to the absence of live bacteria in the LPS treated GF mice.

Gene expression profiles from the hippocampus and amygdala regions of the brain showed ten genes that were significantly altered between GF and colonized mice. These genes play an important role in neuronal development, inflammatory response, and blood-brain-barrier function. Glutamate NMDA receptor subunit, NR2B that was up-regulated in colonized mice was previously reported to be a critical receptor in amygdala synaptic plasticity and development, and in learning and memory[215]. Also NMDA receptor antagonist has been shown to block anxiety in mice[216]. The NR2B gene expression is consistent with the results previously reported by Neufeld et al[23] where they showed an increase in

NR2B expression in SPF mice compared to GF in the central amygdala region of the brain. It is plausible that the up-regulation of NR2B receptor in colonized mice compared to GF mice is contributing to the change in behaviour. Another up-regulated gene, neuropeptide S100B, was shown in *in-vitro* studies to have played a beneficial role in neuroprotective and neurotrophic functions[217, 218]. In an *in vivo* model of traumatic brain injury, intracerebroventricular infusion of S100B improved cognitive recovery and enhanced hippocampal neurogenesis[219]. P75 neurotrophin receptor was down-regulated in colonized mice and this receptor has been previously reported to be a strong inhibitor of neural growth and activity in the hippocampus[220]. P75 knockout mice showed improved spatial memory and enhanced long-term potentiation in the Schafer collateral fiber synapses of the hippocampus[221]. Glial fibrillary acidic protein (GFAP) is an intermediate filament that is highly expressed on astrocytes and is important for the maintenance and activation of astrocytes. It has been shown in animals that GFAP expression is decreased in the hippocampus of depression models induced by maternal deprivation[222] and chronic stress[223]. Our gene expression data showed a significant increase in the GFAP expression in colonized mice, which implies an increase in astrocyte activation and function in the brain. Another up-regulated gene was the tight junction, *occludin (Ocln)*, which is known to regulate blood-brain-barrier function in endothelial tissues. This is consistent with another study that showed GF mice have increased blood-brain barrier permeability and was associated with reduced *Ocln* gene expression compared to conventional mice[92].

Perhaps decreased blood-brain-barrier permeability in colonized mice may prevent bacterial metabolites and/or cytokines from entering the brain, leading to normal behaviour (**Fig 18**).

GLUCOCORTICOIDS AND HPA AXIS: Glucocorticoid receptor (GR) signaling was one of the significant canonical pathways detected in the colon tissue post-colonization. It is well known that inflammatory cytokines can trigger glucocorticoid resistance by inhibiting GR translocation to the nucleus or by diminishing GR transcriptional activity via the mitogen-activated protein kinase (MAPK) signaling pathway[224]. This is consistent with our gene expression data that showed down-regulation of a number of *MAPK* genes (Mef2d, Tgfb2, Map2k4, Mapkapk2) after colonization, suggesting an increase in the GR activity and a decrease in HPA axis hypersensitivity. Altered HPA axis activity due to immune-inflammatory signals is one of the most consistent biological findings in major depression patients[225]. It has been documented that cytokines, particularly IL-1 and IL-6, are potent activators of the HPA axis which influence the release of corticotrophin-releasing hormone (CRH) in hypothalamic neurons[226]. It is known that GF mice have a hyperactive HPA axis and this can be reversed after bacterial colonization[42]. We speculate that an immature immune system in GF mice results in a hypersensitive inflammatory response leading to a sequence of events, including glucocorticoid resistance, hyperactive HPA axis, and abnormal behaviour. However, bacterial colonization reverses all of these effects and normalizes behaviour (**Fig 18**). Emerging evidence indicates that microbiota may

govern behaviour indirectly through regulation of monocyte trafficking to the brain and subsequent microglia activation and HPA axis activity[227, 228].

KYNURENINE PATHWAY METABOLISM: Tryptophan metabolism has been hypothesized to be a focal point in understanding the influence exerted by the gut microbiota on brain function and behaviour. Changes in the supply and availability of the essential amino acid tryptophan may have many implications for brain-gut axis signalling. We accessed the plasma kynurenine, kynurinic acid, tryptophan, and kynurenine:tryptophan ratio (used as an index of IDO or TDO activity) in GF mice colonized with ASF and *E. coli*. Although the higher levels of plasma tryptophan in GF mice compared to colonized is consistent with previous literature[43], our results contradict previously published data that show an increase in both plasma kynurenine concentrations and kynurenine:tryptophan ratios following colonization[229]. This discrepancy could be attributed to the fact that bacteria can directly utilize tryptophan for growth, thereby limiting its availability to the host. It has been reported that *E.coli* harbours tryptophanase enzyme (TnaA) that produces indole from exogenous tryptophan, which is important for various microbial processes such as bacterial motility, biofilm formation, and antibiotic resistance[60]. Bacterial tryptophan utilization and metabolism play an important role in determining circulating tryptophan availability for the host, which could explain the decrease in kynurenine metabolites production following mono-colonization with *E. coli*. We showed that GF mice colonized with ASF microbiota also display a decrease in the production of kynurenine metabolites and this

could be due to the lactobacillus bacteria producing large amounts of hydrogen peroxide known to inhibit IDO/TDO enzyme activity[58].

The rate of tryptophan metabolism along the kynurenine pathway in the plasma is dependent on the expression of TDO which is localized mainly to the liver and modulated by glucocorticoids[230]. As such, TDO activity may at least partly be a link to the microbial-neuroendocrine with significant implications for brain function and behaviour. The normalization of behaviour that we observe could be due to a decrease in the hyperactivity of the HPA axis, glucocorticoids production and subsequent reduction in TDO expression and kynurenine metabolites. All of these changes could be initiated by the priming and maturation of the immune system following bacterial colonization (**Fig 18**). Interestingly, one of the major AHR ligand is kynurenine and kynurenic acid. AHR plays a crucial role in the CNS development and maintenance of the blood-brain-barrier integrity[231], and in mice it was shown to alter hippocampal neurogenesis and behaviour[232]. Despite experimental difficulties in definitively linking the microbiota, kynurenine metabolites and behaviour, it seems there is an overlap in microbiota-immune-induced behaviour and those modulated by neuroactives derived from kynurenine metabolism.

Limitations, strengths, and future directions

One of the biggest criticisms in microbiota-gut-brain research using a GF mouse model is the lack of clinical relevance, since humans are never truly germ free. Even though there is merit to this criticism for obvious reasons, it should be

clear that the use of GF animals is not to directly mimic human conditions but rather to understand the role of bacteria on host development and function. Its artificially controlled state allows us to study the dysfunction arising in the absence of any microbial input on behavior, anatomy, and physiology. One of its greatest strengths is that it provides a clean background onto which we can add single bacteria, such as mono-colonization, or a defined bacterial ecosystem to determine its impact on the host. Behavioural experiments with GF animals are technically challenging since repeated manipulations including import/export of behavioural equipment and animal cages can increase the risk of environmental contamination.

One potential limitation with this study is the use of only one type of bacteria (*E. coli)* for mono-colonization. As such, it is unknown whether mono-colonization with another bacterial strain will produce a similar behavioral profile as *E. coli*. Further experiments are needed to confirm whether the change in behaviour is dependent on the type of bacteria used for mono-colonization. Another limitation is the uncertainty as to which brain regions govern the different behavioural tests, since many of the behavioral tests do not complement each other. For example, MyD88$^{-/-}$Ticam$^{-/-}$ mice showed no changes in behavior except for the step-down test. One can speculate that MyD88-Ticam pathway may not be involved in the bacteria induced signal to the brain region measured by the step-down test. Further research is required to understand which brain regions are associated with the different behavioral tests. One of the strengths is that

behaviour was measured in each mouse before and after colonization rather than having GF and colonized groups measured separately. This allowed for better assessment of changes in behaviour and the accompanying changes in gene expression following colonization.

Although this study provides evidence that the intestinal microbiota influences brain function and behaviour, further research is required to understand the mechanisms underlying this link. The primary focus will be to address the connection as to how gut bacteria uses the neuroimmune pathway to communicate with the CNS. Although a number of potential pathways were highlighted in this study, one immediate pathway to address is whether microbial products are responsible for the normalization of behaviour. Adoptive transfer of blood serum from conventional or ex-GF into GF mice and subsequent behavioural assessment is one way to address whether particular bacterial metabolites or LPS directly is involved in the gut-brain communication. Metabolomic studies before and after colonization of GF mice can also be used to gain insight into the involvement of bacterial metabolites. Our results from this study suggest that the immune activation following bacterial colonization is signaled to the brain through a TLR-MyD88 dependent pathway. However, it is unknown whether this is initiated at the level of epithelial or myeloid cells. Adoptive transfer of primed mucosal myeloid cells, such as macrophages and dendritic cells, from transiently colonized mice into GF mice can be used to address whether the signal for normalization of behaviour is initiated by myeloid

cells. Further research is needed to investigate whether maturation of the immune system following bacterial colonization could lead to the development of the ENS resulting in normal behaviour.

TDO expression can be modulated by glucocorticoids and is responsible for the rate of tryptophan metabolism along the kynurenine pathway. Thus, it could potentially be a link to the microbial-neuroendocrine with significant implications for brain function and behaviour. We hypothesize that the normalization of behaviour could be due to a decrease in the hyperactivity of the HPA axis and glucocorticoid production with subsequent reduction in TDO expression and kynurenine metabolites. Future experiments will be needed to measure plasma ACTH and corticosterone levels before and after colonization and the expression of TDO enzyme in the liver. In addition, it will be beneficial to measure kynurenine metabolites in the brain to correlate with changes in brain chemistry and behaviour.

Concluding remarks

The work in this thesis provides insight into the role of gut microbiota in brain function and behaviour. Furthermore, these findings establish the CNS and peripheral immune system as important cellular mediators of communication across the microbiota gut-brain axis. Through the examination of GF animals both before and after introduction of bacteria (single strain or bacterial community), I showed that bacterial colonization is necessary for the development of normal behaviour. The specific molecular mechanisms for how

host–microbe interactions in the intestine remotely change brain chemistry and behaviour remains largely unknown, but we have established that the innate immune system through TLR signalling plays a crucial role. It is known that gut microbiota is important for the epithelial barrier function, gut homeostasis, and immune functions. In light of this study, we can begin to appreciate its influence on the development of normal behaviour as well. For this reason, we can consider humans (and most other organisms) as holistic functioning units working together with their bacterial counterparts for good health.

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