From the Department of Neuroscience
Karolinska Institutet, Stockholm, Sweden

GUT MICROBES AND THE DEVELOPING BRAIN

Tim Ben Frans Arentsen

Karolinska Institutet

Stockholm 2017

Cover Illustration by Laleh Maleki

Published by Karolinska Institutet.
Printed by E-Print AB, 2017

ISBN 978-91-7676-613-2

Gut Microbes and the Developing Brain

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

For the degree of PhD at Karolinska Institutet

This thesis will be defended in public at the Hillarp Lecture Hall, Retzius väg 8, Karolinska Institutet, Stockholm, Sweden

Friday the 7th of April, 2017, 09:30

By

Tim Ben Frans Arentsen

Principal Supervisor:
Assoc. Prof. Rochellys Diaz Heijtz
Karolinska Institutet
Department of Neuroscience

Co-supervisor:
Prof. Hans Forssberg
Karolinska Institutet
Department of Women's and Children's Health
Neuropediatric Unit

Opponent:
Prof. John Cryan
University College Cork
Department of Neuroscience/Anatomy

Examination Board:
Assoc. Prof. Elisabeth Norin
Karolinska Institutet
Department of Microbiology, Tumor, and Cell Biology

Prof. Jon Laman
University of Groningen
Department of Neuroscience

Prof. Klas Blomgren
Karolinska Institutet
Department of Women's and Children's Health

Dedicated to my beloved mother, father, and sister
and to my dearest wife Laleh.

ABSTRACT

The discovery that commensal gut microbiota can influence host development and physiology beyond the gastrointestinal (GI) tract has triggered a paradigm shift in our conceptualization of the origin of human diseases. A growing body of preclinical research has demonstrated that gut microbiota exert a modulatory role on the development and function of brain circuits involved in motor control, emotion and cognition. These findings have lent support to the hypothesis that gut bacteria may play a role in the etiology and/or pathophysiology of human brain disorders. The current challenge is to understand the precise mechanisms mediating the communication between the microbiota and the brain. In the present thesis, we used a combination of mouse models (e.g., germ-free; GF, antibiotic treated, and transgenic mice), molecular, biochemical, and behavioral approaches to gain a deeper insight into the role of gut microbiota on brain development and behavior. A major goal was to explore whether microbial products from the commensal gut microbiota can be translocated into the developing brain and be sensed by pattern recognition receptors (PRRs) of the innate immune system.

In **Paper I**, we took advantage of the GF mouse model (mice raised throughout development in an environment devoid of bacteria) to study the influence of gut microbiota on social behavior. Using the three-chamber social approach task, we demonstrated significant differences in social approach behavior between GF and conventionally raised mice (specific pathogen-free, SPF). Adult GF Swiss-Webster mice displayed higher levels of sociability than SPF mice, as indicated by a stronger preference for time spent close to the unfamiliar stimulus mouse versus the novel object. In addition, they showed reduced expression levels of total BDNF and BDNF exon-containing transcripts I-, IV-, VI-, and IX in the amygdala, a brain region involved in the processing of social stimuli. These findings suggest that alterations in the expression of specific BDNF exon transcripts within the amygdala may contribute to the abnormal development of social behavior in GF mice.

In **Paper II**, we investigated whether antibiotic-induced perturbation of the maternal gut microbiota during pregnancy influences brain development and behavior of the offspring. The juvenile offspring of antibiotic-treated dams showed hyperactivity and sex-specific changes in social behavior (similar to that observed in GF mice), without changes in body weight. In addition, the male juvenile offspring had reduced BDNF mRNA and protein expression in the amygdala. Interestingly, we found a negative correlation between time spent interacting with an unfamiliar stimulus mouse and levels of BDNF protein. These findings in mice indicate that antibiotic-induced perturbation of the maternal gut microbiota during pregnancy has profound effects on brain development leading to abnormal motor and social development of the offspring.

In **Paper III**, we examined the possibility that fragments of bacterial peptidoglycan (PGN), a major component of the bacterial cell wall, derived from commensal gut microbiota can cross the blood brain barrier under normal conditions and influence the developing brain via activation of PRRs. Using various expression-profiling techniques (i.e., qRT-PCR, Western Blot and immunohistochemistry), we showed that two families of PRRs that specifically detect PGN and its derivates (PGN recognition proteins and NOD-like receptors), and the PGN transporter PepT1 are highly expressed in the developing brain during critical windows of postnatal development. In addition, we demonstrated that the expression of several of these PGN-sensing molecules are sensitive to manipulation of the gut microbiota (i.e., GF conditions and antibiotic exposure in early life). Finally, we demonstrated that the absence of PGN

recognition protein 2 (Pglyrp2; which is an N-acetylmuramyl-L-alanine amidase that hydrolyzes bacterial PGN between the sugar backbone and the peptide chain) leads to sex-specific changes in social behavior in the prepubertal period. However, we did not observe changes in motor or anxiety-like behavior at this age. These novel findings support the notion that central activation of PRRs by bacterial PGN fragments could be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain.

In **Paper IV**, we tested the hypothesis that the modulatory role of PGN recognition proteins (PGRPs) on behavior changes with age, by using Pglyrp2 knockout (KO) mice. Using a battery of behavioral tests, we demonstrated sex-dependent alterations in motor and anxiety-like behavior in 15-month-old Pglyrp2 KO mice, as well as mild changes in the expression of synaptophysin (a presynaptic marker) and gephyrin (a protein associated with inhibitory synapses) in key brain regions implicated in the processing of emotional stimuli. These observations indicate that the mammalian Pglyrp2 plays an important role in the modulation of brain circuits involved in motor control and anxiety in later life.

In summary, this thesis provides conceptually novel evidence that the central activation of PRRs by bacterial PGN fragments could be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain. This new signaling pathway may be a new entry point for the exploration of the role of gut microbiota on brain development, function and behavior. Finally, we propose that alterations within different components of this signaling pathway could lead to deviations in brain developmental trajectories, thus increasing risk for neurodevelopmental and psychiatric disorders.

LIST OF SCIENTIFIC PAPERS

I. **Arentsen T**, Raith H, Qian Y, Forssberg H, Diaz Heijtz R. Host microbiota modulates development of social preference in mice. *Microbial Ecology in Health & Disease, 2015, volume 26: 29719*

II. **Arentsen T**, Mina D, St. Pierre J, Foster J, Forssberg H, Diaz Heijtz R. Perturbation of maternal gut microbiota during pregnancy influences offspring brain development and behavior. *Submitted manuscript.*

III. **Arentsen T**, Qian Y, Gkotzis S, Femenia T, Wang T, Udekwu K, Forssberg H, Diaz Heijtz R. The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Molecular Psychiatry (2017) 22, 257–266; doi:10.1038/mp.2016.182 published online 15 November 2016.*

IV. **Arentsen T**, Khalid R, Qian Y, Diaz Heijtz R. Sex-dependent alterations in motor and anxiety-like behavior of aged bacterial peptidoglycan sensing molecule 2 knockout mice. *Submitted manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
ACTH	Adrenocorticotropic hormone
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
BDNF	Brain-derived neurotropic factor
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
CNS	Central nerous system
CNV	Copy number variation
CREB	cAMP response element binding protein
DA	Dopamine
DAP	Diaminopimelic acid
DSS	Dextran sulfate sodium
ENS	Enteric nervous system
EPM	Elevated plus maze
GF	Germ-free
GI	Gastrointestinal
GlcNAc	B-(1→4)-linked *N*-acetylglucosamine
HPA-axis	Hypothalamic-pituitary-adrenal -axis
KO	Knockout
LD-test	Light-dark box test
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeats
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
meso-DAP	Meso-diaminopimelate
MIA	Maternal immune activation
MurNAC	*N*-acetylmuramic acid
NA	Noradrenaline
NDD	Neurodevelopmental disorder

NF-κB	Nuclear factor κB
NLR	Nod-like receptor
NMDA receptor	N-methyl-D-aspartate receptor
NOD	Nucleotide-binding oligomerization domain-containing protein
OF	Open field test
PCoA	Principal component analysis
PD	Postnatal day
PepT1	Peptide transporter 1
Pglyrp1	Peptidoglycan recognition protein 1
Pglyrp2	Peptidoglycan recognition protein 2
Pglyrp3	Peptidoglycan recognition protein 3
Pglyrp4	Peptidoglycan recognition protein 4
PGLYRP	Peptidoglycan recognition protein
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PRR	Pattern recognition receptor
PSD-95	Postsynaptic density protein-95
qRT-PCR	Quantitative real time polymerase chain reaction
RIPK2	Receptor-interacting serine/threonine-protein kinase 2
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
SLC15	Proton-coupled oligopeptide transporter family
SLP	Silkworm larvae plasma
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
TLR	Toll-like receptor
WT	Wild-type
5-HT	Serotonin
5-HIAA	5-hydroxyindoleacetic acid

1 INTRODUCTION

Human brain development is a protracted process that starts in the third gestational week and extends into adulthood. This process involves a complex, constantly changing interaction between genes and environmental factors (Stiles and Jernigan, 2010). Over the past decades, it has become clear that environmental influences during pre- and/or early postnatal life may profoundly affect brain developmental trajectories and function in later life. One such environmental factor is the commensal gut microbiota, the microorganisms that inhabit the gastrointestinal (GI) tract. The interaction between the host and the microbiota is an evolutionary driven process in which the gut microbiota adapted to coexist in a commensal or mutually beneficial relationship with the host (Hooper and Gordon, 2001). The gut microbiota play a crucial role in overall health and in aspects of host development and function such as in digestion, immune system maturation and gut homeostasis (Sommer and Backhed, 2013). Research has revealed that the gut microbiota can also impact developmental processes beyond the GI tract including brain development, function and behavior (Sekirov et al., 2010, Cryan and Dinan, 2012). The mechanisms mediating the crosstalk between the gut microbiota and the brain are still poorly understood (Collins et al., 2012). Novel insights into the pathways by which the gut microbiota can impact brain development and function may provide new treatment opportunities for neurodevelopmental and psychiatric disorders.

1.1 The gut microbiota

Although microorganisms reside on almost all mucosal surfaces, the majority can be found in the intestinal tract where they are in close contact with the mucosal tissues. It has been estimated that the GI tract contains 10 to 100 trillion microorganisms (Gill et al., 2006, Sender et al., 2016). These microorganisms represent a diverse community of predominantly bacteria, but also archaea, viruses, protozoa and fungi. Anaerobic bacteria dominate the typical gut flora and more than 1000 different bacteria species reside in the adult gut. Firmicutes and Bacteroidetes are the two most abundant phyla in the adult intestine, while other phyla such as Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria and Cyanobactera are present in lower numbers (HumanMicrobiomeProject, 2012). The combined genome of all gut microorganisms is 150 times larger than the human genome, however the number of bacterial cells has been estimated to be similar to the total number of human cells (Qin et al., 2010, Sender et al., 2016).

Neonatal colonization process. Bacterial colonization starts during birth and thereafter, when the newborn is rapidly and densely populated by microbes. The primary colonizers are facultative anaerobes that are thought to change the intestinal milieu in a way that allows anaerobic bacteria to colonize the GI tract (e.g., *Bacteroides* and *Clostridium*) (Rodriguez et al., 2015). The composition and diversity of the gut flora changes dramatically during the first two years of life. The microbial community stabilizes around the second year of life and resembles an “adult-like” composition (Sekirov et al., 2010, Sommer and Backhed, 2013). Interestingly, the bacteria colonization period coincides with key stages of brain development (Borre et al., 2014). The

human brain undergoes pronounced changes during the first years of postnatal life. This period represents the peak of synaptogenesis (the formation of new synapses) and myelination (the formation of myelin sheaths around axons). Moreover, during the first two years of life the human brain grows from approximately 36% to about 80% of its adult size (Tau and Peterson, 2009, Diaz Heijtz, 2016).

Factors influencing the infant microbiome. The microbial colonization of the infant intestinal tract is a crucial process. Several factors are known to affect the assembly of the neonate gut microbiota including the mode of delivery (vaginal birth versus caesarean (c) -section), diet (breastfeeding versus formula), early-life antibiotics, prenatal stress and life-style, among others (**Fig. 1**). The mode of delivery is a crucial factor that shapes the initial neonate microbiome. Vaginally delivered babies are colonized by the maternal vaginal and faecal bacteria (e.g., *Lactobacillus* and *Prevotella*) (Dominguez-Bello et al., 2011). C-section born babies, on the other hand, do not directly acquire maternal microbes, but are populated by bacteria related to the skin (not necessarily related to the mother) and the hospital environment. It has been shown that the mode of delivery impacts the gut microbiota composition and diversity up to months or even years after birth (Dominguez-Bello et al., 2011, Eggesbo et al., 2015, Jakobsson et al., 2014). Another relevant factor that shapes the neonate gut microbiota is the mode of feeding (breastfeeding or formula feeding). Mother's milk has a rich and diverse microbiota and also contains a large number of oligosaccharides that enhance the growth of favorable bacteria for instance *Bifidobacterium* (Zivkovic et al., 2011). This may facilitate beneficial health effects (e.g., improved cognitive development) (Quigley et al., 2012).

Early-life antibiotic use is another common perinatal intervention that disturbs the assembly of the neonate gut microbiota and has long lasting effects on microbial diversity (Mueller et al., 2015a). For example, higher proportions of Proteobacteria and lower proportions of *Bifidobacterium* (Fouhy et al., 2012). Moreover, several epidemiological studies have shown that prenatal or early-postnatal antibiotic treatments were associated with metabolic alterations and obesity (Ajslev et al., 2011, Trasande et al., 2013, Azad et al., 2014, Murphy et al., 2014, Cox and Blaser, 2015b, Mueller et al., 2015c) and increased risk for immune related disorders (e.g., asthma) (Stokholm et al., 2014, Metsala et al., 2015, Stensballe et al., 2013). These findings highlight that antibiotic treatment during early life, before the microbial colonization process is completed, may result in adverse effects later in life. Other factors that have been shown to affect the assembly of the infant microbiota include preterm birth, prenatal stress and life-style (**Fig. 1**).

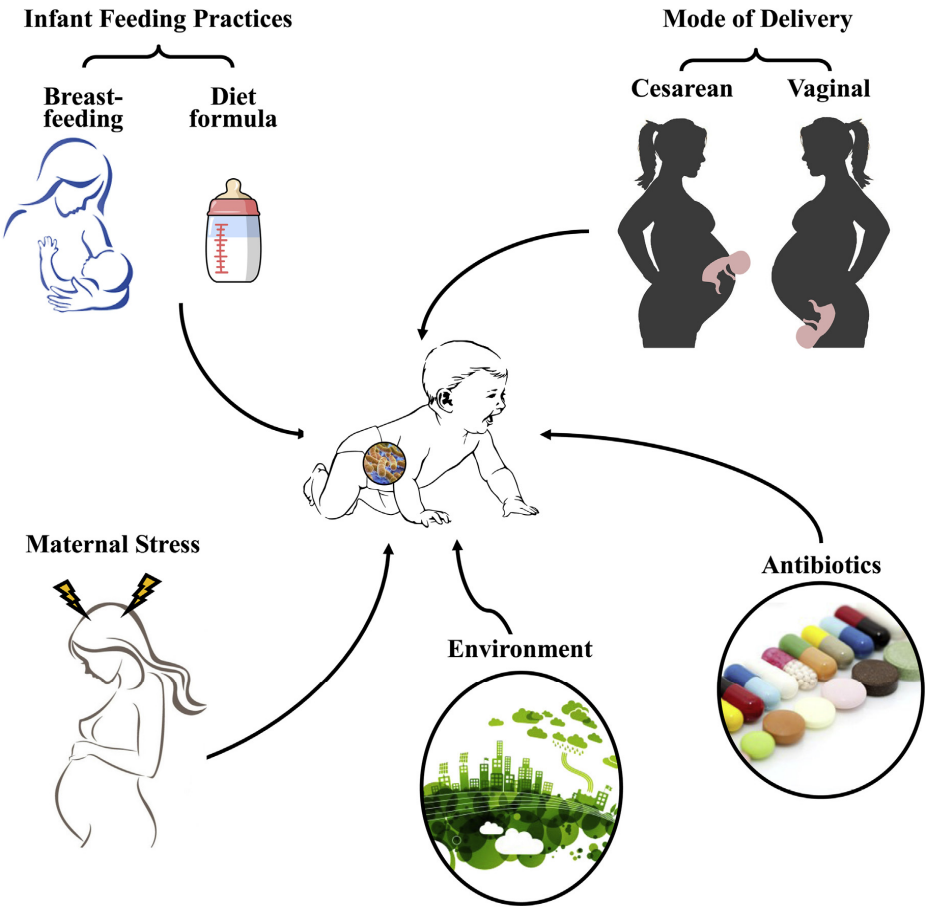

Figure 1. Factors that can affect the development of the neonate gut microbiota. A large number of factors impact the assembly of the neonate gut microbiota including the mode of feeding, mode of delivery, early-life antibiotic treatments, prenatal stress, the environment, pre- and/or early postnatal infections, gestational age and genetics. Cartoon from (Diaz Heijtz, 2016).

1.2 Models of gut microbiota-host interactions

Several experimental approaches have been used to investigate gut microbiota-host interactions, which include both invertebrate and vertebrate model systems including Hawaiian bobtail squid, fruit fly, zebrafish, and mice. The mouse model system is highly relevant because of its similarity to the human in terms of genetics and microbiota composition (Kostic et al., 2013). A wide range of mouse model systems are currently used to explore the potential modulatory effects of the gut microbiota on host development and physiology (Mayer et al., 2015). Since the late 1950s researchers have been able to raise germ-free (GF) mice (devoid of microorganisms) throughout development in sterile isolators (Reyniers, 1959, Midtvedt, 1997). These isolators are carefully monitored for contamination and incoming air, food and water are sterilized. GF animals can be colonized with one or more bacteria strains (i.e., gnotobiotic animals) enabling the study of taxa-specific effects on the host. The microorganism composition of gnotobiotic animals is known (Williams, 2014). Treatment with antibiotics (mainly broad-spectrum) is another approach to perturb the intestinal microbiota. The first two to three years in humans, and the first three to four weeks in mice are crucial to establish an “adult-like” gut microbiota. Antibiotic treatment during this critical time window can alter the bacterial composition permanently and result in profound

later life consequences. For example, Martin Blaser and colleagues showed that antibiotic-induced microbiota perturbations early in life can affect metabolic activity later in life, influencing body mass by either promoting weight gain or inhibiting growth (Cox and Blaser, 2015a). Over the last years, faecal microbiota transplantation gained interest to "repair" a perturbed microbial gut ecology (Khoruts and Weingarden, 2014), but also to investigate the impact of the microbiota composition on host physiology and behavior (Bercik et al., 2011a). Together these experimental approaches are very important tools to further clarify the interaction between the gut microbiota and the host.

1.3 The role of the gut microbiota on host development and physiology

1.3.1 Gut microbiota effects on the periphery

Gut microbes are involved in numerous developmental and physiological processes including the development and maturation of the immune system and GI tract, bone homeostasis, absorption of nutrients, metabolism and digestion (Sommer and Backhed, 2013). For instance, studies in GF mice have shown that the gut microbiota is required for the development of Peyer's patches and mesenteric lymph nodes (Round and Mazmanian, 2009). Moreover, the intestinal microbiota plays an important role in the immune cell differentiation of T-cells. Deshmukh and colleagues showed that antibiotic treatment decreased the number of interleukin-17 producing cells and reduced the production of granulocyte colony-stimulating factor, which was restored by the introduction of "normal" gut microbiota (Deshmukh et al., 2014). The gut microbes also play a crucial role in inflammation responses, by directly or indirectly affecting the production of cytokines, chemokines, and antimicrobial peptides (Sommer and Backhed, 2013, Hooper et al., 2012). The gut microbiome has also been strongly linked to the development, vascularization, and homeostasis of the GI tract. Sharma and collaborators showed that the development of the intestinal mucus layer, that covers the epithelial cells and forms a physical barrier to protect against bacteria, depends on the presence of gut microbiota (Sharma et al., 1995). Devoid of a gut microbiota, GF mice developed a larger caecum and have reduced intestinal surface area, as well as, increased epithelial permeability (Sommer and Backhed, 2013). The gut microbiota is also beneficial to the host by digesting otherwise indigestible polysaccharides and carbohydrates. Interestingly, GF mice showed reduced adiposity and displayed an altered metabolism (i.e., reduced energy extraction from food) therefore a higher calorie intake is necessary to achieve a similar weight as control animals (Backhed et al., 2004). In addition, to the modulatory role of gut microbiota in the periphery, an impact of the intestinal microbiota on the central nervous system (CNS) is increasingly being recognized.

1.3.2 Effect on brain development, function and behavior

Over the last few years, studies have shown that the actions of gut microbiota have much wider effects on host physiology than originally believed, including the modulation of brain development and function (Diaz Heijtz et al., 2011, Cryan and Dinan, 2012). In 2004, the pioneer

study of Sudo and collaborators showed that the gut microbiota can modulate the hypothalamic-pituitary-adrenal (HPA) axis stress response in mice. They exposed GF and control (with a "normal" gut microbiota; specific-pathogen free, SPF) mice to mild restraint stress and evaluated the HPA reaction to this stressor. GF animals displayed elevated adrenocorticotropic hormone (ACTH) and corticosterone levels in response to the stressor compared to controls. Interestingly, the exaggerated stress response of GF mice could be normalized by exposing GF mice to a specific bacteria strain (*Bifidobacterium infantis*) and was partly reversed with inoculation of SPF feces during early, but not later developmental stages (Sudo et al., 2004). Thus, providing the first experimental evidence for the existence of a developmental time window in which the intestinal microbiota may influence brain development. Surprisingly, it took almost seven years until neuroscientists started to explore the role of the microbiota on brain development, function and behavior.

In 2011, Diaz Heijtz and collaborators showed that adult male GF mice exhibited elevated motor activity and decreased anxiety-like behavior compared to SPF mice. Importantly, conventionalization (microbial colonization) in early life normalized their behavioral phenotype, whereas colonization of adult GF mice had no effect (Diaz Heijtz et al., 2011). These findings also supported the notion that a sensitive time window of development exists during which the intestinal microbiota may impact early brain development and consequently, brain function and behavior later in life. Around the same time, the laboratory of Jane Foster (Canada) demonstrated that adult GF females displayed decreased anxiety-like behavior. However, GF female mice did not display alterations in motor activity as observed in male GF mice, indicating sex-specific effects (Neufeld et al., 2011b). The same authors also observed that microbial colonization of adult GF females with SPF feces did not alter their anxiety-like behavior (Neufeld et al., 2011a). In addition, another independent study by the laboratory of John Cryan confirmed that adult GF male mice displayed decreased anxiety-like behavior, and they demonstrated that microbial colonization from weaning onwards normalized anxiety-like behavior to SPF levels (Clarke et al., 2013). Gareau and collaborators observed impaired object recognition and working memory in female GF mice. In contrast to previous mentioned studies, female GF animals did not show alterations in anxiety-like behavior (Gareau et al., 2011). Bercik and colleagues showed that fecal microbiota transplantation between two behaviorally different mouse strains successfully altered their behavioral phenotype; colonization of the highly anxious GF BALB/c mouse with NIH Swiss microbiota increased their exploratory behavior, while colonization of the less anxious GF NIH Swiss with BALB/c microbiota decreased their exploratory behavior (Bercik et al., 2011a). Recent studies also have shown an influence of the gut microbiota on social behavior in GF rodents (Crumeyrolle-Arias et al., 2014, Desbonnet et al., 2014, Buffington et al., 2016). Desbonnet and collaborators observed decreased social behavior and social cognition in adult GF male, but not female mice. GF male mice also exhibited increased levels of self-grooming, an indication for repetitive behaviors. Some of the behavioral impairments were rescued by microbial colonizing of GF mice, social behavior, but not social cognition was normalized after colonization (Desbonnet et al., 2014). Similar to these findings, Buffington and colleagues

reported reduced social behavior in GF mice (Buffington et al., 2016). Together, these studies highlight the importance of the gut microbiota in brain development and behavior. However, it is important to point out that some of the reported behavior phenotypes in GF mice may differ between mouse strains.

In addition to their distinct behavioral phenotype, GF mice also exhibit abnormalities in neurochemistry and in the expression of genes involved in synaptic plasticity and myelination. Several authors reported that GF male mice exhibit decreased brain-derived neurotrophic factor (BDNF) expression levels in the cortex, hippocampus and amygdala (Sudo et al., 2004, Diaz Heijtz et al., 2011, Clarke et al., 2013). BDNF plays many roles in neurodevelopmental processes including neurogenesis, synaptic growth, synaptic plasticity and transmission (Vicario-Abejon et al., 2002, Benarroch, 2015). Abnormalities in BDNF signaling have been linked to several neurodevelopmental and psychiatric disorders that often co-occur with GI problems (Adachi et al., 2014, Castren, 2014). Moreover, synaptic related proteins synaptophysin and postsynaptic density protein-95 (PSD-95) were found to be significantly increased in the striatum of GF mice (Diaz Heijtz et al., 2011). GF animals also showed increased neurogenesis in the hippocampus (Ogbonnaya et al., 2015), a process which has been linked to cognitive and emotional responses (Burokas et al., 2015). The same research group showed increased expression levels of myelination related genes specifically in the prefrontal cortex of GF male mice. Upregulation of myelin-related genes led to increased myelin sheath thickness, indicating that the intestinal microbes may play a role in myelination (Hoban et al., 2016b). Additionally, a study by Erny and colleagues demonstrated that the gut microbiota is required for microglia maturation and function. Microglia from GF mice display altered cell proportions and an immature phenotype. Following exposure to a bacterial or viral immune challenge, microglia of GF mice displayed inactive morphology and blunted immune response. Interestingly, conventionalization of GF mice restored microglia morphology and function, indicating that the microbiota plays an important homeostatic role in the CNS immune system (Erny et al., 2015). Braniste and colleagues showed another possible role of the gut microbiota. They reported that GF mice displayed a more permeable blood-brain barrier (BBB) from prenatal life until adulthood, which was associated with decreased expression levels of tight junction proteins. Conventionalization reduced permeability and restored protein expression levels (Braniste et al., 2014). These studies identified that commensal gut microbiota may play an important role in neurogenesis, synaptogenesis and myelination.

A number of studies also described differences in dopaminergic, serotoninergic and glutamatergic signaling in GF mice. In the striatum, GF male mice displayed elevated noradrenaline (NA), dopamine (DA) and serotonin (5-HT) turnover compared to SPF mice (Diaz Heijtz et al., 2011). N-methyl-D-aspartate (NMDA) receptor subunits NR1 and NR2A were also decreased in the hippocampus and cortex of male GF mice (Sudo et al., 2004). On the other hand, in female GF mice only NMDA receptor subunit NR2B was found to be reduced in the amygdala (Neufeld et al., 2011b), suggesting a potential sex-dependent effect in the expression of NMDA

receptor subunits. One key finding was the discovery that the microbiota regulates the hippocampal serotonergic system in a sex-dependent manner. In the hippocampus, increased 5-HT and its main metabolite (5-HIAA) were observed in male, but not female GF mice (Clarke et al., 2013). Interestingly, microbial colonization from weaning onwards normalized tryptophan levels in the periphery, but not 5-HT and 5-HIAA within the hippocampus (Clarke et al., 2013). These findings suggest that the time-window during which the gut microbiota can modulate the central serotonergic system may be during the perinatal period.

Antibiotic-treated animals. Another way to perturb the gut microbiota is by the administration of antibiotics. Bercik and colleagues showed that exposure to antibiotic for 7 days perturbed the intestinal microbiota in adult mice. These antibiotic-treated mice displayed increased exploratory behavior and exhibited alteration in the expression of BDNF in hippocampus and amygdala (Bercik et al., 2011a). It has been also demonstrated that antibiotic-induced perturbations of the intestinal microbiota in adult rodents impaired object recognition memory (Frohlich et al., 2016), decreased spatial memory (Hoban et al., 2016a), and reduced neurogenesis and cognitive function in the hippocampus (Mohle et al., 2016). Another study found that antibiotic treatment from weaning onwards decreased anxiety-like behavior, produced cognitive deficits, and reduced BDNF, oxytocin and vasopressin expression in adulthood (Desbonnet et al., 2015a), whereas antibiotic treatment during pregnancy affected anxiety-like behavior and spatial memory of the offspring during adulthood (Zhang et al., 2016).

Probiotics. Studies have also explored potential beneficial effects of probiotics on brain neurochemistry and behavior by bacteria. For example, treatment with *Lactobacillus* (Bravo et al., 2011) or two strains of Bifidobacteria (Savignac et al., 2014) reduced anxiety-like behavior in an intrinsic anxious mouse strain (BALB/c). Moreover, long-term administration of Bidfidobacteria improved learning and memory in BALB/c mice (Savignac et al., 2015). Hsiao and colleagues showed beneficial behavioral effects with *Bacteroides fragilis* treatment. They used the maternal immune activation (MIA) mouse model that show some autism spectrum disorder (ASD) relevant features (e.g., gut permeability, stereotypic and anxiety-like behavior). Oral treatment of MIA-offspring with *Bacteroides fragilis* rescued most behavioral abnormalities. Moreover, a bacteria-derived metabolite (4-ethylphenyl sulfate) was found to exert the same effects as treatment with the bacterium (Hsiao et al., 2013). Other authors showed that maternal high fat diet during pregnancy decreased social behavior and increased anxiety and repetitive behavior in the offspring. Social, but not anxiety-like behavior and repetitive behavior were normalized by co-housing offspring of high fat diet dams with dams on a normal diet. The authors identified that a probiotic, *L. reuteri*, was also able to normalize social behavior, but not anxiety and repetitive behaviors in offspring of high fat diet dams (Buffington et al., 2016). These studies suggest that some probiotic bacteria may have a beneficial effect on brain function and behavior.

Taken together, the above results provide critical evidence supporting the notion that the gut microbiota can influence brain development, function and behavior. However, the interactions between the gut microbiota and developing brain are not straightforward. Numerous other factors play a role including time-window of development, diet, species, strain, age, sex, and genetic factors. The next challenge is to bring this large body of experimental information to the clinics and provide new treatment opportunities for human brain disorders in which an altered gut microbiota has been implicated.

1.4 Potential mechanisms mediating the gut microbiota-brain interactions

The brain can influence processes in the gut such as GI tract functioning (e.g., motility, secretion, epithelial functioning and mucin production (Mayer, 2011)) and gut immune function (e.g., the modulation of cytokine production in mucosal cells (Tracey, 2009)). In turn, the gut microbiota can affect brain development, function and behavior including stress responsiveness, anxiety-like behavior, depression-like behavior, nociceptive responses, mood, social behaviors, feeding behavior, and taste preference, among others (Mayer et al., 2015, Sampson and Mazmanian, 2015, Sharon et al., 2016, Luczynski et al., 2016). The precise mechanisms mediating interactions between the gut microbes and the developing brain remain largely unknown, but likely involve multiple direct and indirect signaling pathways. Multiple mechanisms have been proposed to mediate the influence of the gut microbiota on brain development and behavior including neural, hormonal, immunological signaling, and bacterial metabolites and products (**Fig. 2**).

Neuronal pathways. The neuronal signaling pathways mediating communication between the gut and the CNS include the central, autonomic (the sympathetic and parasympathetic nervous system) and enteric nervous system (ENS) (Collins et al., 2012). The sympathetic nervous system is primarily involved in the regulation of motility, secretion and bowel transit time, barrier function, and immune system activation (Cervi et al., 2014). Neural circuits facilitate the ENS-CNS communication in which the ENS receives input from the brain and vice versa. Interestingly, the ENS can function autonomously from the CNS, and therefore it is often referred to as the 'second brain' (Collins et al., 2012). The *vagus nerve* is the main component of the parasympathetic nervous system and sends information from numerous peripheral organs such as heart, intestines, pancreas and stomach to the brainstem via sensory fibers (Zhou and Foster, 2015, Forsythe et al., 2014). Several studies indicate that some of the beneficial probiotic effects depend upon an intact vagus nerve. For example, Bravo and colleagues showed that the treatment with probiotic *Lactobacillus rhamnosus* affected neurochemistry (altered GABA receptor expression in the brain) and emotional behavior (e.g., anxiety- and depression-like behavior) in healthy animals. Using vagotomized mice the authors showed that the behavioral and neurochemical effects of this probiotic required an intact vagus nerve (Bravo et al., 2011). Another study demonstrated that vagal integrity was required for the anxiety reducing effects of probiotic *Bifidobacterium longum* (Bercik et al., 2011b). On the other hand, the same authors found that antibiotic-induced

perturbations of the gut microbiota led to vagus-independent alterations in brain chemistry and behavior (Bercik et al., 2011a), indicating that non-neuronal signaling mechanisms may also play a role in the gut microbiota-brain crosstalk.

Endocrine system. The endocrine signaling pathways involve the release of gut peptides, such as galanin, orexin, ghrelin, leptin and gastrin by enteroendocrine cells, which can directly affect the brain (Forsythe and Kunze, 2013, Wren and Bloom, 2007, Cameron and Doucet, 2007). Gut bacteria can initiate peptide release of enteroendocrine cells. Intriguingly, a recent study showed that proteins released by commensal gut microbiota can directly communicate with the brain, thereby affecting appetite. The authors showed that after food intake a growing population of *E. coli* bacteria can produce proteins that directly act on anorexigenic signaling pathways in the brain (Breton et al., 2016). The gut microbiota has the capacity to produce neurotransmitters such as GABA, noradrenaline and 5-HT. Certain bacteria can play a regulatory role in the availability

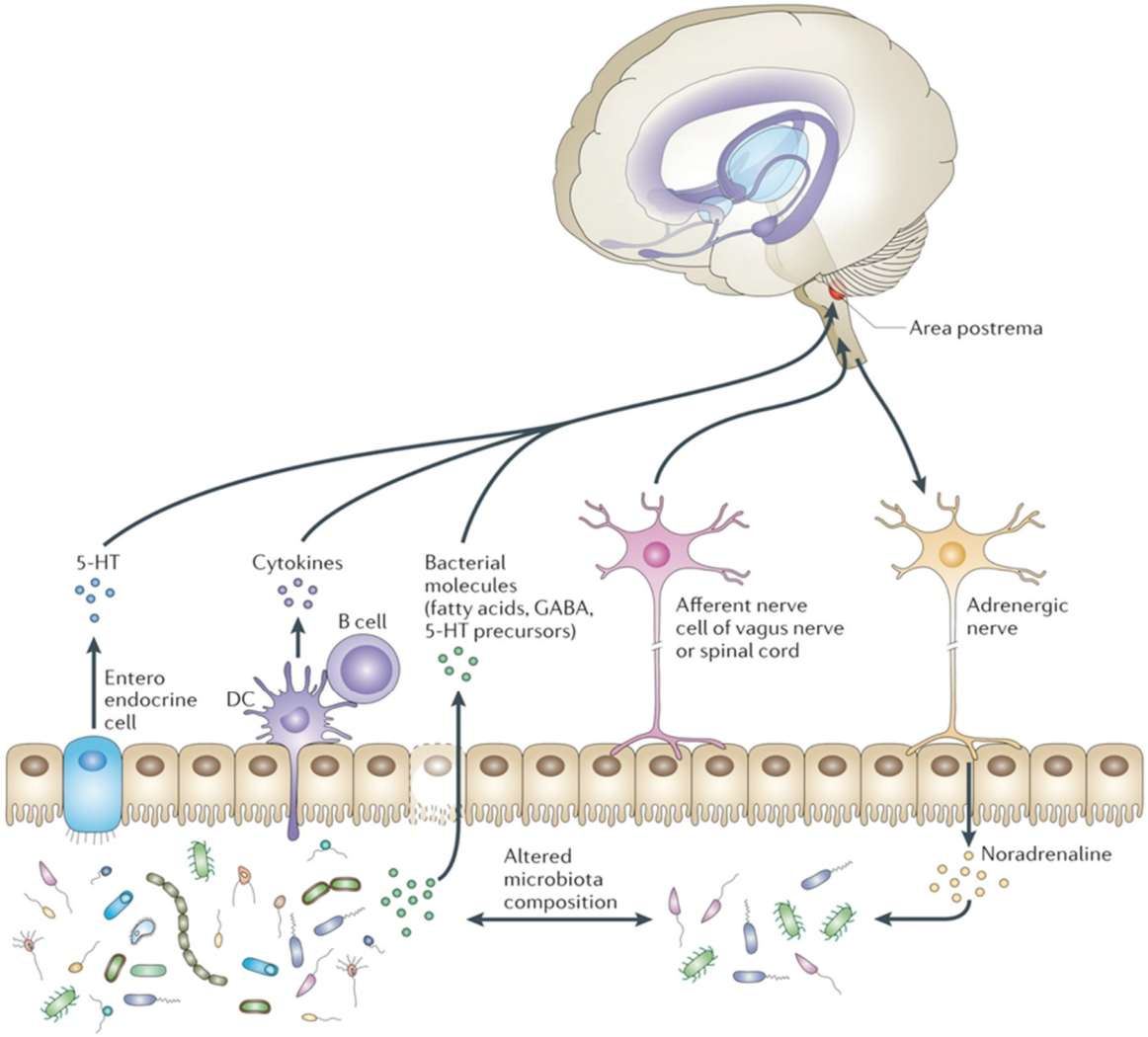

Figure 2. Pathways involved in the bi-directional communication between the gut microbiota and brain. Multiple pathways have been proposed to be involved in the gut-microbiota-brain axis including neuronal, hormonal and immunological pathways, and more recently bacteria-derived molecules that may modulate brain and behavior. Abbreviations: dendritic cell (DC); Serotonin (5-HT). Modified from (Collins et al., 2012).

of neurotransmitters in the periphery. For example, Yano and collaborators demonstrated that the gut microbiota is an important regulator of 5-HT homeostasis in colon and blood. They showed that certain spore-forming bacteria can produce metabolites that increase 5-HT production, which in turn affected host physiology (i.e., gut motility and homeostasis). Moreover, exposing GF mice to these microbial metabolite was sufficient to increase 5-HT levels in their colon and blood (Yano et al., 2015). Given that GF mice have impaired BBB integrity it will be interesting to investigate whether these microbial metabolites can impact 5-HT levels in the GF brain.

The immune system. In a recent publication, a role for Ly6C^{hi} monocytes in the communication between the gut microbiota and the brain was proposed. It was found that antibiotic treatment decreased neurogenesis and spatial learning and memory in adult mice. Treatment with probiotics or voluntary exercise restored neurogenesis and cognitive functioning. They also observed that antibiotic treatment reduced the number of Ly6C^{hi} monocytes in the periphery and brain. When bone marrow derived Ly6C^{hi} monocytes were transplanted into antibiotic treated animals, neurogenesis was restored to control levels and brain monocyte levels increased, thus suggesting a potential role of Ly6C^{hi} monocytes in the gut microbiota-brain crosstalk (Mohle et al., 2016). Also, it has been shown that perturbations of the gut microbiota alter the homeostasis between the microbiota and the mucosal immune system which can result in the activation of immune responses such as the production of cytokines. These cytokines may in turn affect brain function and behavior (Honda and Takeda, 2009).

***Microbial metabolites*.** Short chain fatty acids (SCFAs) are produced when gut bacteria ferment dietary fibers in the colon. SCFAs such as acetate, propionate and butyrate have multiple effects on the host physiology including energy metabolism in the colon and periphery, food intake, modulation of immune cells functioning, and activation of epithelial cell signaling pathways (Pomare et al., 1985, Mayer et al., 2015). It has been suggested that these bacterial metabolites can exert a direct effect on brain development and behavior (MacFabe et al., 2007, MacFabe et al., 2011). Over the last years several studies reported beneficial effects of SCFA in the context of brain development and function. For instance, GF mice have a more permeable BBB compared to SPF mice. These BBB abnormalities are mostly normalized after microbial colonization of GF mice. Interestingly, the introduction of a single bacteria strain that produces SCFAs, such as *Clostridium tyrobutyricum* which produces butyrate or *Bacteroides thetaiotaomicron* that produces acetate and propionate, into GF mice partly restored BBB integrity. Moreover, treatment with sodium butyrate alone was enough to improve BBB integrity in GF mice (Braniste et al., 2014). SCFAs also seem to play an important role in microglia homeostasis. Erny and collaborators showed that treatment of GF mice with SCFA restored microglia maturation and function. In addition, SCFA receptor knockout mice (i.e., FFAR2) showed similar microglia deficits as observed in untreated GF mice (Erny et al., 2015), suggesting that SCFA play a role in microglia maturation. In a transgenic mouse model of Parkinson's disease, it was demonstrated that the presence of a normal gut microbiota produced Parkinson's-like deficits (motor problems and Parkinson's related brain pathology) by activating disease-competent microglia. GF

conditions or treatment with antibiotics significantly reduced Parkinson's related deficits which was associated with the absence of disease-competent microglia. Treatment with SCFAs produced a similar disease phenotype in GF mice as observed in mice with a normal gut microbiota, indicating that SCFAs were required for the maturation of disease-related microglia (Sampson et al., 2016). These findings indicate a potential pathway by which the gut microbiota may contribute to the pathophysiology of neurodegenerative disorders.

Bacterial-derived products. One possibility that has not been fully explored is the direct actions of bacteria-derived products that are recognized by pattern recognition receptors (PRRs) of the innate immune system. PRRs are typically expressed in immune cells and can detect conserved microbe-associated molecular patterns (MAMPs). MAMPs such as lipopolysaccharide (LPS), bacterial lipoprotein, flagellin, CpG DNA, and peptidoglycan are common among microbes. MAMPs are universally conserved, generally invariant, and essential in all microorganisms. Importantly, these "motifs" are not restricted to pathogens and are abundantly produced by the commensal gut microbiota (Chu and Mazmanian, 2013). Moreover, emerging evidence suggest that MAMPs from commensal gut microbiota signal through PRRs to promote host development, homeostasis, and protection against pathogens (Clarke et al., 2010). PRRs of the innate immune system include different receptor families, including the Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs), and peptidoglycan recognition proteins (PGRPs or PGLYRPSs).

1.5 Toll-like receptors of the innate immune system

TLRs were first discovered in Drosophila melanogaster where they play immune and developmental roles. In mammals, TLRs mediate immune responses such as the immediate immune response via nuclear factor-κB (NF-κB) activation resulting in cytokine and chemokine production, and facilitating the adaptive immunity by activation of antigen-presenting cells. These receptors (TLR1-11 in mice) are typically expressed in immune system-related cells including B-cells, natural killer cells, mast cells and macrophages. Interestingly, TLRs were also found to be expressed by astrocytes, microglia and neurons (Okun et al., 2011), suggesting that mammalian TLRs may play developmental roles beyond the classical immune functions. In the CNS, TLRs are important mediators for the initiation of immune responses. However, some TLRs are highly expressed during mouse brain development and are able to modulate key neurodevelopmental processes including neurogenesis, synaptogenesis, and synaptic plasticity. Studies have shown neurodevelopmental roles for Tlr2 in neuronal differentiation, Tlr3 in neurite outgrowth, Tlr4 in cell proliferation and Tlr8 in neuronal death (Okun et al., 2010b, Lathia et al., 2008, Shechter et al., 2008, Ma et al., 2006). Other studies suggest that TLRs may impact motor, anxiety and cognitive functions. Okun and collaborators showed that Tlr3 has a developmental role in memory retention, whereas Tlr4 plays a role in learning and memory (Okun et al., 2010a, Okun et al., 2012). Another study reported a developmental role for Tlr2 in anxiety and cognitive functioning (Park et al., 2015). These studies indicate that TLRs play multiple roles in the

immune system, neurodevelopment and the programming of brain circuits linked to motor control, memory and cognition.

1.6 Bacterial peptidoglycan and its sensing molecules

Clarke and colleagues demonstrated that the microbiota is a source of peptidoglycan (PGN), which is translocated from the gut into the circulation under normal conditions without any indications of infection. This microbial product was found to influence the function of bone marrow-derived neutrophils, which are cells residing outside the gut. The authors showed that low levels of PGN were required for optimal neutrophil immune function. Moreover, neutrophils obtained from mice with a perturbed gut microbiota (induced by broad-spectrum antibiotics or raised under GF conditions) showed reduced immune function compared to controls. The PGN sensor, nucleotide-binding oligomerization domain-containing protein-1 (Nod1) was identified as the homeostatic regulator mediating the systemic effects of PGN (Clarke et al., 2010). These novel findings raise the possibility that a similar mechanism may be operating within the brain.

PGN is a polymer consisting of amino acids and sugars, which is a fundamental component of the bacterial cell wall. The PGN structure is built from β-(1→4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). A small peptide chain of three to five amino acids is attached to the MurNAc. These short chains can cross-link (via the L- and D- amino acids) with other peptide chains to form a 3D mesh-like layer. Some of the amino acids in the peptide stem differ between Gram-positive and Gram-negative bacteria; the first two amino acids are normally L-alanine and D-glutamine or isoglutamine, the third residue is Lysine in Gram-positive bacteria and in Gram-negative bacteria meso-diaminopimelate (*meso*-DAP), the last two residues are generally D-alanine (Schleifer and Kandler, 1972). The PGN layer is much thicker in Gram-positive bacteria compared to Gram-negative bacteria. Approximately 90% of the dry weight of Gram-positive bacteria is PGN, in contrast, in Gram-negative bacteria PGN only makes up for around 10% of its dry weight. When bacteria divide, PGN is partly degraded and released into the surrounding milieu. These small molecules are perfect markers for the immune system to detect bacterial activity. Several PRRs are able to detect PGN, including Tlr2 a member of the TLR-family, cytosolic Nod1 and Nod2 of the NLR-family, as well as all four mammalian PGN recognition proteins (PGRP1–4) (Dziarski, 2003). These PGN sensing molecules can detect different "PGN motifs", for instance, Nod1 specifically detects tripeptides containing *meso*-DAP, which are predominantly found in Gram-negative bacteria and some Gram-positive bacteria. Nod2 detects muramyl dipeptides present in both Gram-negative and Gram-positive bacteria and some muramyl tripeptides found in Gram-positive bacteria (Wheeler et al., 2014). PGRPs typically detect muramyl tripeptides (Dziarski and Gupta, 2010). A few decades ago, systemic PGN molecules were observed in urine and cerebral spinal fluid of patients with sleep disorders and in murine brain, liver, and kidney (Sen and Karnovsky, 1984, Martin et al., 1984, Krueger et al., 1984). However, the detection methods have been criticized and it has been controversial whether bacterial-derived PGN molecules can reside in host tissue in the absence of infections or inflammation. (Wheeler et al., 2014). Using a different experimental approach, PGN fragments

have also been observed in antigen presenting cells in brain tissue of multiple sclerosis patients (Schrijver et al., 2001). Moreover, the presence of PGN was correlated with demyelination in multiple sclerosis patients (Branton et al., 2016). In 2010, Clarke and colleagues showed that bioactive *meso*-DAP PGN can cross the intestinal epithelium and was detectable in sera and bone marrow (Clarke et al., 2010), indicating that PGN fragments can affect immune system development and host physiology.

1.6.1 NOD-like receptors.

The NLR-family are cytosolic innate immune proteins. Nod1 and Nod2 are NLR members that recognize specific PGN molecules were Nod1 detects meso-DAP containing PGN and Nod2 detects muramyl di- and tri-peptides. Receptor activation initiates NF-κB-dependent and mitogen-activated protein kinase (MAPK)-dependent pro-inflammatory gene transcription. NOD1 and NOD2 proteins can sense PGN motifs via Leucine-rich-domains (LRRs). Upon ligand binding, the caspase recruitment domain (CARD) recruits downstream adaptor proteins such as receptor-interacting serine/threonine-protein kinase 2 (RIPK2) to initiate downstream signaling (Philpott et al., 2014). Activation of Nod1 and Nod2 depends on binding with PGN in the cytosol, the mechanisms by which PGN molecules are transported into the cell are not well established. Several uptake mechanisms have been reported, including phagocytosis, endocytosis and uptake of outer membrane vesicles via neighboring cells through tight junctions and GAP junctions. Active uptake via proton-coupled oligopeptide transporters (SLC15), specialized in the cellular uptake of di/tripeptides has also been proposed (Caruso et al., 2014, Philpott et al., 2014, Irving et al., 2014). Mainly Slc15a1 (PepT1) and Slc15a4 have been shown to transport PGN into the cytosol, thereby facilitating Nod receptor activation (Smith et al., 2013). Overall, several uptake mechanisms seem to be involved in delivering extracellular ligands to intracellular Nod1 and Nod2 receptors.

Nod signaling plays key roles in maintaining the integrity of the epithelial barrier and regulating the immune homeostasis in the gut. Nod1 is widely expressed by both immune and non-immune cells, for example Nod1 is abundantly expressed by epithelial cells in the intestinal tract. Nod2 expression is mostly limited to immune cells including T-cells, neutrophils, macrophages and dendritic cells. In the intestine, Nod2 is expressed in Paneth cells (Caruso et al., 2014, Kaparakis-Liaskos, 2015). Both Nod1 and Nod2 receptors have been detected in the adult brain. For instance, functional Nod2 receptors have been found in both microglia and astrocytes (Chauhan et al., 2009, Sterka and Marriott, 2006). Another study showed that Nod1 and Nod2 were expressed in the adult rat brain and Nod2 expression increased in response to a Gram-positive bacterial infection (Liu et al., 2014). Consistent with their roles in immunity, Nod1 and Nod2 knockout mice showed increased susceptibility for pathogen infections. Studies with Nod2-deficient mice have shown that Nod2 has a protective role in the development of intestinal inflammation (Petnicki-Ocwieja et al., 2009). Moreover, Nod2 models uncovered that Nod2 regulates the expression and secretion of antimicrobial peptides, important to sustain the delicate microbiota-host balance (Philpott et al., 2014). Disrupted Nod2 signaling has been associated

with an increased risk for Crohn's disease (Inohara et al., 2003). A zebrafish study suggested that NLRs may be required for normal microglial development (Shiau et al., 2013). Thus far, direct neurodevelopmental roles for Nod1 and Nod2 receptors have not been discovered.

1.6.2 Peptidoglycan recognition proteins

The peptidoglycan recognition proteins (PGRPs) are highly conserved in vertebrates and invertebrates. They were first discovered in the silkworm (*Bombyx mori*) as immune proteins that detect bacteria-derived PGN. Insect PGRPs initiate an antibacterial defense mechanism, called melanization, when they bind PGN. Insects have many different PGRPs with a wide range of functions. For example, the fruit fly has 13 PGRP genes that are transcribed into 19 different PGRPs, mammals on the other hand, have only four specialized PGRPs: PGLYRP1 (S), PGLYRP2 (L), PGLYRP3 (Iα), and PGLYRP4 (Iβ) (Liu et al., 2001, Royet et al., 2011). All mammalian PGRPs have at least one PGRP domain and the relatively low number of PGRPs in mammals is explainable by the large number of other PRRs active in the mammalian innate immune system. The general structure of the four mammalian PGRPs is similar; a specialized PGN-binding grove typically binds muramyl peptide fragments, and can distinguish between different amino acids in the peptide (Dziarski and Gupta, 2010, Royet et al., 2011). However, PGLYRP2 is the only mammalian PGRP with amidase activity that hydrolyzes the lactyl bonds in bacterial PGN (see **Fig. 3**) (Dziarski and Gupta, 2006). PGRPs are capable of binding to both Gram-positive and Gram-negative bacteria. Crystallographic analysis of the PGN binding groves revealed some of the ligand-PGRP binding characteristics, for example, PGLYRP1 and PGLYRP3 detect specifically muramyl-tripeptides (Guan et al., 2004a, Guan et al., 2004b, Guan et al., 2005).

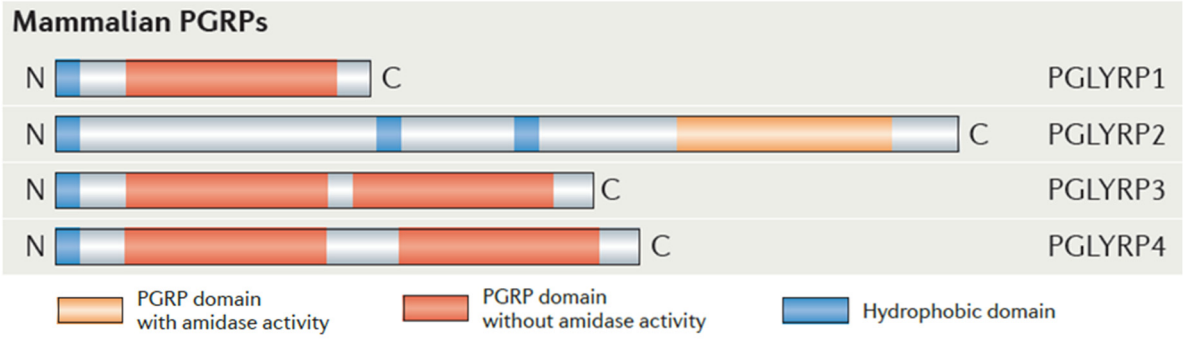

Figure 3. The structure of mammalian PGRPs. Modified from (Royet et al., 2011).

The mechanisms by which the PGRPs exert their antimicrobial function are still poorly understood. Recent studies suggested that the bacteria killing may depend on a synergistic effect of reactive oxygen species (ROS), thiol, and metal stress (Kashyap et al., 2014). Another potential mechanism involves that PGRPs bind to the bacterial cell wall and over-activate the bacteria stress response system to kill the bacteria for example via the CssR–CssS system in Gram-positive bacteria and CpxA–CpxR system in Gram-negative bacteria) (Dziarski et al., 2012, Royet et al., 2011).

PGRPs are widely expressed throughout the body, typically in areas that come into close contact with microbes (Royet et al., 2011). High expression of PGLYRP1 was observed in the bone marrow in leukocytes, in the lactating mammary glands (Kappeler et al., 2004), in epithelial cells, and fibroblasts. PGLYRP2 has been detected in the liver, blood, spleen, epithelial cells and fibroblasts. PGLYRP3 and PGLYRP4 were detected in skins epidermis, hair follicles, sweat glands, corneal epithelial, oral epithelial cells and in specific cells in the stomach and intestinal tract (Dziarski and Gupta, 2010). Rehman and colleagues have previously reported the expression of PGLYRP1 in various brain regions such as the hippocampus, hypothalamus and brain stem in adult rats (Rehman et al., 2001). The mechanisms by which the expression of PGRPs in mammals is regulated are poorly understood, however, a mouse study showed that PGLYRP1 expression may depend on NF-κB signaling (Lang et al., 2008).

A genetic association study showed that genetic variants in PGRP genes were associated with risk for inflammatory bowel disease (Zulfiqar et al., 2013), suggesting that PGRPs are important in the protection and maintenance of a beneficial homeostasis in the colon. Saha and colleagues showed that PGRP1–4 knockout mice had increased sensitivity to an inflammatory agent, dextran sulfate sodium (DSS) (Saha et al., 2010). Another study showed that double knockout PGRP3-Nod2 mice were more sensitive to DSS induced colitis compared to single gene knockout mice (Jing et al., 2014). Recently, a novel non-immune related role for PGRPs was described. Harris and colleagues showed that in the Drosophila brain, PGRP-LC was required for presynaptic homeostatic plasticity (Harris et al., 2015). In humans, a genetic association study found several single-nucleotide polymorphisms (SNPs) in PGRP genes to be associated with increased risk for Parkinson's disease (Goldman et al., 2014). Interestingly, the Mazmanian group recently reported that the gut microbiota play a crucial role in motor deficits and brain pathology related Parkinson's diseases (Sampson et al., 2016). Mounting evidence revealed that the gut microbiota exert an effect on the development of brain circuits involved in motor control, emotion and cognition. One of the current challenges is to understand the precise mechanisms mediating the interactions between the gut microbiota and the developing brain. This knowledge will provide new insights into how behavior and personality traits are shaped, and perhaps offer novel biological mechanisms involved in neurodevelopmental and psychiatric disorders.

1.7 The gut microbiota in neurodevelopmental disorders

Neurodevelopmental disorders (NDDs) are a group of conditions characterized by an early onset, typically in childhood, and include ASD, attention deficit hyperactivity disorder (ADHD), dyslexia, language disorders, intellectual disability, cerebral palsy, developmental coordination disorder, and Tourette's syndrome among others. The range of developmental deficits varies between different NDDs and may affect learning ability, self-control, emotions, social skills, intelligence and memory, which in turn can impair personal, social, academic, or occupational functioning (5th edition of the Diagnostic and Statistical Manual of Mental Disorders). ASD is one NDD that has been particularly linked to disturbances of the commensal gut microbiota is ASD. ASD is characterized by two key symptoms, impaired social communication/interaction

and restrictive/repetitive behavior or interest. In Europe, ASD is estimated to affect 1,3% of children and adolescents (www.autismeurope.org) and is more prevalent in boys than girls. Although ASD has a high heritability, a causal relationship between genomic alterations and ASD has been difficult to explain in many cases. This suggests a contribution of environmental factors as well, which has in recent years been increasingly recognized (Hallmayer et al., 2011). Genetic factors that contribute to ASD include SNPs, copy number variants (CNVs) and syndromic forms of ASD (Rett-syndrome, fragile-X and Tuberous sclerosis complex). Environmental risk factors that have been associated with ASD are, for instance, microbial pathogen infections (Atladottir et al., 2012, Zerbo et al., 2013, Bilbo et al., 2005) and prenatal stress (Beydoun and Saftlas, 2008, Boersma et al., 2014). Also, the gut microbiota has gained interest as a potential environmental factor that may play a role in ASD, since many affected individuals with ASD suffer from GI problems such as constipation, increased intestinal permeability (Coury et al., 2012). Intriguingly, studies have indeed reported a positive association of ASD severity with GI dysfunction (Adams et al., 2011, Wang et al., 2011). ASD patients often exhibit an atypical bacterial composition (Finegold et al., 2010, Williams et al., 2011) and excessive use of antibiotics during early-life has been implicated as a contributor to the unbalanced gut microbiota in ASD individuals. Probiotics, on the other hand, may have a beneficial effect on ASD outcome by restoring the atypical microbiota (Critchfield et al., 2011).

In trying to link both genetic and environmental factors, the proto-oncogene *C-MET*, a receptor tyrosine kinase, is of particular interest given its strong link to ASD individuals with co-occurring GI problems (Campbell et al., 2009). The ligand for the MET receptor is Hepatocyte growth factor (HGF). MET is important in various biological processes such as cell proliferation, intestinal epithelial development, immune function, angiogenesis and brain development which involves neuronal growth, morphology, and synapse maturation (Peng et al., 2013, Qiu et al., 2014, Hsiao, 2014). Disrupted MET signaling has been linked to morphological and functional alterations in neurons in brain regions linked to ASD (Peng et al., 2013). In line with this finding, HGF levels were decreased in serum of autistic children suffering from severe GI problems (Russo et al., 2009). A postmortem study showed decreased MET protein levels in the temporal cortex of ASD individuals (Campbell et al., 2007). A common SNP in the MET promoter region (rs1858830) known to increase the risk for ASD is specifically associated with ASD in patients with comorbid GI problems (Campbell et al., 2009). Functional magnetic resonance imaging (fMRI) studies showed that individuals with this risk allele show reduced connectivity in response to social stimuli (Rudie et al., 2012), and showed reduced gray matter growth in typically developing children with the risk allele (Hedrick et al., 2012). White matter tract and functional connectivity abnormalities were also observed in ASD subjects (Berg and Geschwind, 2012). These studies led to a growing appreciation that the gut microbiota may contribute to the etiology and pathophysiology of ASD.

2 AIMS OF THE THESIS

The overarching aim of this thesis is to improve our understanding of the mechanisms by which the gut microbiota modulates brain development, function and behavior. The specific aims of the thesis are:

- To assess the impact of the host microbiota on social behavior by using the germ-free mouse model.

- To study whether antibiotic-induced perturbations of the maternal gut microbiota during pregnancny impact the neurobehavioral outcomes of the offspring.

- To determine whether bacterial PGN fragments can be translocated into the developing brain and detected by PGN sensing molecules. Additionally, to explore the possible roles of PGN sensing molecules on brain function and behavior in early and later life.

3 METHODS

3.1 Animals

The following animals were used throughout the four studies included in this thesis:

- In paper I, we used adult Swiss-Webster germ-free (GF) and specific pathogen-free (SPF) male mice (Taconic Farms Inc., Germantown, NY, USA).
- In paper II, we obtained adult C57BL/6N male and female mice from Charles River Laboratories (Sulzfeld, Germany).
- In paper III, we obtained C57BL/6N male and female mice from Charles River Laboratories (Sulzfeld, Germany), C57BL/6 GF and SPF male and female mice from the Core Facility for Germ Free Research (Karolinska, Institutet, Sweden) and the hetrozygous *Pglyrp2*-breeding pairs from the Jackson Laboratory (Bar Harbor, ME, USA).
- In paper IV, heterozygous *Pglyrp2*-breeding pairs were obtained from the Jackson laboratory (Bar Harbor, ME, USA).

Upon arrival, all animals were housed in same-sex groups in standard plastic cages (Makrolon® Type III, Tecniplast, Buguggiate, Italy) under controlled temperature, humidity, and light (12:12 h light–dark cycle) conditions. Food and water were available *ad libitum*. Pregnant C57BL/6N female mice were housed individually under the same conditions as above. We defined the day of birth as postnatal day (P) 0. All offspring was weaned from their mothers on P21. All experiments were conducted according to a protocol approved by the Ethics Committee on Animal Research, Stockholm North and in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.1.1 Prenatal and perinatal antibiotic treatment

Prenatal antibiotic treatment. One week after arrival in the animal facility, mice were mated (one male with two females). Females with a vaginal plug were individually caged and exposed to ampicillin (cat no. A8351, 0.6 mg/ml; Sigma-Aldrich, St. Louis, MO, USA; freshly made every day) in their drinking water throughout the entire pregnancy. Antibiotic treatment was discontinued on the day of delivery. The control group received fresh water on daily basis. Offspring of antibiotic-treated dams and controls were housed in same-sex and -treatment groups.

Perinatal antibiotic treatment. Pregnant females were exposed to ampicillin (0.6 mg/ml; cat no. A8351, Sigma-Aldrich, St. Louis, MO, USA; freshly made every day) starting 5 days before delivery until P3. Importantly, both prenatal and perinatal antibiotic treatment did not affect the body weight of the dams or their offspring.

3.1.2 Germ-Free animals

In paper I, we used three-month old adult Swiss-Webster GF and SPF male mice. Both GF and SPF animals arrived at the animal facility in guaranteed Taconic GF shippers with free access to food and water. GF and SPF male mice were transferred to sterile isolators and allowed to acclimatize for one week before testing took place. In the isolators, animals had free access to autoclaved water and food and were housed under similar temperature, humidity and light conditions as previously described in section (**3.1**).

C57BL/6 GF and SPF male and female mice (paper III) were obtained from our own Core Facility for Germ Free Research at Karolinska Institutet. Animals were transported to our animal facility in special GF shippers. Immediately after arrival, brain tissues of GF and SPF mice were collected. **Figure 4** shows GF isolators used for breeding GF colonies and long-term GF housing (GF facility, Karolinska Institutet).

Figure 4. Germ-Free isolators in the GF-core facility at Karolinska Institutet. GF and SPF animals are bred and raised in GF isolators.

3.1.3 Pglyrp2 knockout mouse model

Pglyrp2 knockout and wildtype mice were obtained from multiple heterozygous *Pglyrp2*-breeding pairs. Heterozygous *Pglyrp2* male and female mice were bred and housed in our animal facility in standard plastic cages and under controlled conditions (see section **3.1**).

3.2 Behavioral Tests

3.2.1 Behavioral studies

All behavioral testing took place between 0900 and 1600 hours under low illumination to reduce stress. Prior to any behavioral procedure, animals were brought in their home cages to the experimental testing room and allowed to habituate for at least 1 h before testing sessions were started in order to reduce stress caused by environmental changes. All animals were naïve to the behavioral experiment. The experimenter was blind to the genotype of Pglyrp2 animals during testing. Test chambers were cleaned first with disinfectant and then with 70 % ethanol and water after each animal.

3.2.2 Open-Field test

The open-field box is normally used to measure the general locomotor activity, exploration and anxiety-like behaviors in rodents (**Fig. 5**). Mice exposed to a novel open-field box typically

show high levels of locomotion and exploratory behavior during the novelty period (i.e., the first 15-min of exposure to the novel open-field box). Prolonged exposure to the open-field results in a progressive reduction in exploratory behaviors as the novel environment becomes more familiar (i.e., the habituation phase). All animals were exposed to the open-field test for 90 minutes. The open-field test was performed by placing animals individually in the center of the Acti-Mot detection system (48 cm × 48 cm; TSE, Bad Homburg, Germany). This system is able to automatically track and record movements of the animal using infrared photobeams. For spontaneous locomotor activity two parameters were analyzed: the distance traveled (in meters) and rearing activity, measured by the number of rears (the animal standing on its hind legs). The following parameters were analyzed: time spent and distance traveled in the center and periphery of the open-field box during the first 5-min of testing, as an index of anxiety-like behavior.

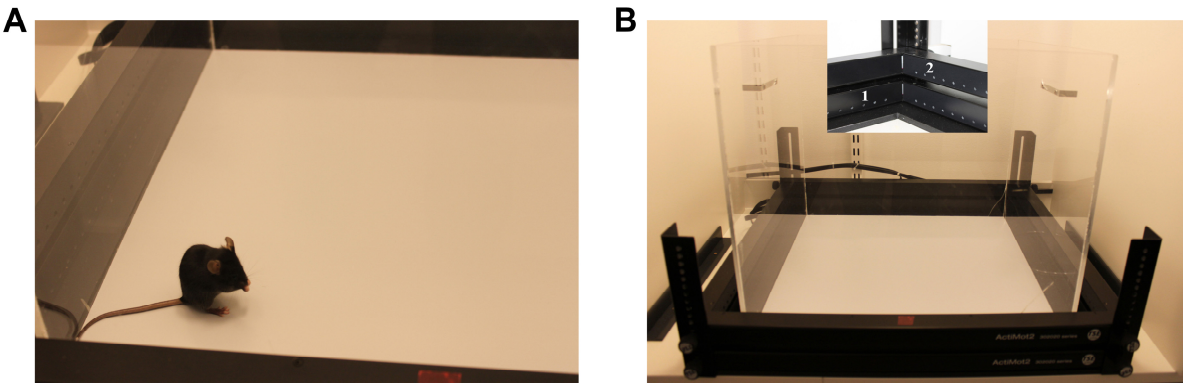

Figure 5. The Open-Field box system. (A) A mouse in the open-field box. (B) The Acti-Mot detection setup. The first pair of photobeam bars (1) is able to detect the animal's movement in a horizontal way, whereas the second pair of beams (2) detect the vertical activity (rearing activity). The open-field box is 48 x 48 cm.

3.2.3 Elevated Plus Maze test

The Elevated Plus Maze (EPM; **Fig. 6A**) test is a well-established behavioral paradigm commonly used to investigate anxiety-like behaviors in rodents. This test takes advantage from the fact that mice are afraid of light and open areas, and prefer dark and closed (well protected) areas. Typically, mice subjected to the EPM test will spent more time in the closed arms than in the open arms. The total time spent in the open and closed arms of the EPM are an indication for anxiety-like behavior. Typically, an anxious mouse will spent significantly more time in the closed arms than in the open arms compared to a less anxious animal. The EPM test was performed by individually placing the test animal in the center (intersection) of the EPM apparatus (Kinder Scientific, California, USA), facing an open arm. Mice were allowed to explore the different areas of the EPM for 5 minutes. The following parameters were analyzed: the time (in seconds) spent in, and entries into, the open and closed arms, and total distance traveled (in meters) in the different compartments of the apparatus. The EPM system uses infrared photobeams to automatically track movements of the animal. Motor Monitor™ software was used to analyze the data (Kinder Scientific, California, USA).

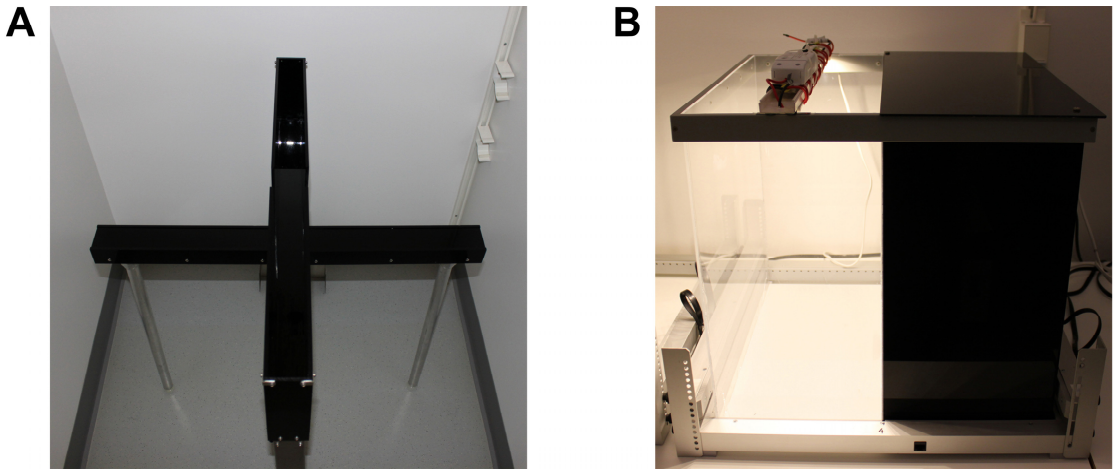

Figure 6. Two behavioral paradigms used to examine anxiety-like behaviors in mice (A) The elevated plus maze test, consisting of two open and two closed arms. (B) The light-dark box test, with a light and dark compartment of equal size.

3.2.4 Light-Dark box test

The Light-Dark box (LD; **Fig. 6B**) is another test to evaluate anxiety-like behaviors in rodents. When investigating anxiety-like behavior in mice, we always subjected test animals to both the EPM and LD-test since these two closely related tasks are complementary. The size of the LD test box is similar to that of the Open-Field box (48 x 48 cm). The apparatus is divided into a dark and light zone of equal seize with a rectangular door in the middle wall connecting the light and dark compartments. The light zone was lit by a light-bar consisting of 8 LED lights. The LD-test was performed by placing the test animal into the dark compartment of the LD-box and allowing it to freely explore the apparatus for 5 minutes. The following parameters were analyzed: the time (in seconds) spent, distance traveled (in meters) and numbers of rears in the dark and light compartments. These parameters were automatically recorded by the Acti-Mot detection system (TSE, Bad Homburg, Germany) using photocells.

3.2.5 Three-chambered sociability and social cognition test

Sociability and social cognition were evaluated in a three-chambered apparatus as described in **paper II**, **III** and **IV**. The apparatus consists of three equal sized chambers that were accessible via doors in the dividers. The sociability test consisted of two 10-min sessions in the three-chambered apparatus, whereas the social cognition test required an additional 10-min session. To test for sociability, the time the test mouse spent interacting with the novel stimulus mouse and novel object was analyzed. To assess social cognition, the time the test mouse spent with the familiar and the unfamiliar stimulus mice was analyzed. Briefly, during the first 10-min session (i.e., exploration/habituation phase), the test mouse was allowed to freely explore all chambers. The test mouse was then briefly confined in the center chamber during which the experimenter placed the stimuli for the sociability session. For the 10-min sociability session, a novel stimulus mouse (i.e., same sex, age and strain) in a grid enclosure was positioned in one of the side-chambers and an identical grid enclosure without stimulus mouse was placed in the opposite chamber (see **Fig. 7A**). After a 10-min testing period, the test mouse was again briefly confined in the center chamber to allow the placement of the stimuli for the social cognition session. To test for social cognition, a novel stimulus mouse was place in the

previously empty grid enclosure (**Fig. 7B**) after which the test animal was again allowed to freely explore the three-chambered apparatus for 10-min. Time spent in each chamber and time spent around the enclosures was recorded and analyzed using an automated tracking system (EthoVision XT version 7 and 11, Noldus, Wageningen, The Netherlands). Detailed analysis of the time the test mouse spent interacting with the stimulus mouse was manually scored from recorded videos.

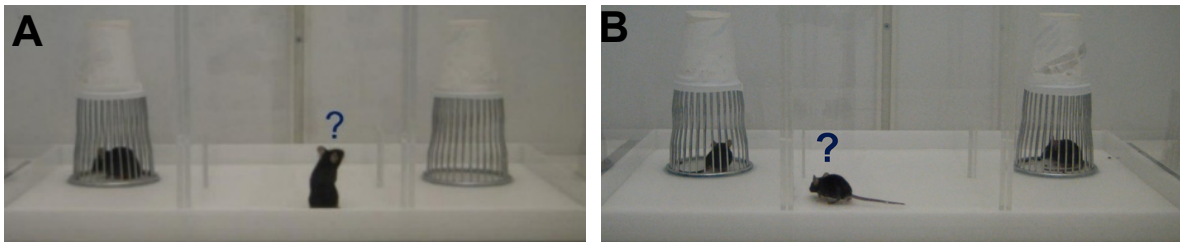

Figure 7. The three-chambered social approach task. (A) During the 10-min sociability phase, the test mouse can freely explore all three chambers and choose to interact with the unfamiliar stimulus mouse in the grid enclosure or with the empty grid enclosure (i.e., object). (B) In 10-min social cognition phase, the test mouse can freely explore the apparatus and choose to interact with the familiar stimulus mouse or the novel stimulus mouse.

3.2.6 Social interaction test

Social interaction and approach were evaluated in the social interaction test (**Fig. 8)** as previously described (Sato et al., 2013). Stimulus mice of the same age, weight and sex as the test mouse were tail-marked 24h before testing. Test animals were transferred to the testing room and individually caged in new standard plastic cages (Makrolon® Type III, Tecniplast, Buguggiate, Italy) for at least 2 hours prior to testing. During the 2h habitation period, animals had access to water and food, which was removed just before the start of the experiment. The behavioral task started with the introduction of an unfamiliar stimulus mouse into the cage of the test animal. Social interactions were video recorded for 10 min. The time the test mouse spent interacting with the stimulus mouse was manually scored. Active interaction was defined as sniffing, close following and allo-grooming. All sessions were recorded from the front of the cage using a Samsung (Seoul, South Korea) HMX-H100P high-definition camcorder.

Figure 8. The social interaction test. Test mice were habituated for 2h prior testing in standard plastic cages. An unfamiliar stimulus mouse (same sex, age and strain) was introduced and social interactions were video recorded for a 10-min time period. Interaction time (i.e., sniffing, close following and allo-grooming) was manually scored.

3.2.7 Rotarod

Motor coordination and balance were evaluated in the accelerating rotarod apparatus (Ugo Basile, Varese, Italy). The apparatus consisted of a rotating rod (3 cm in diameter) divided into 5 running lanes allowing up to 5 mice to be tested simultaneously (**Fig. 9**). One day before testing, animals were habituated to the rotarod apparatus by placing them on the rotating rod at a fixed speed of 4 rotations per minute (r.p.m.) for two 90-second periods, 2 hours apart. On the day of testing, animals were placed on the rotating cylinder, and the time each animal was able to maintain its balance was recorded. The rotarod was set to accelerate in a linear manner from 4 to 40 r.p.m. over a 5-min time period. Latency to fall was measured in seconds after a mouse fell off the rod, clung to the rod for two consecutive rotations or rotated three times within 10 seconds. The maximum latency time of 300 s was assigned when a mouse did not fall from the rod or did not perform the above rotation mistakes. Mice performed 5 trials over 1 day, with a 5 min inter-trial interval.

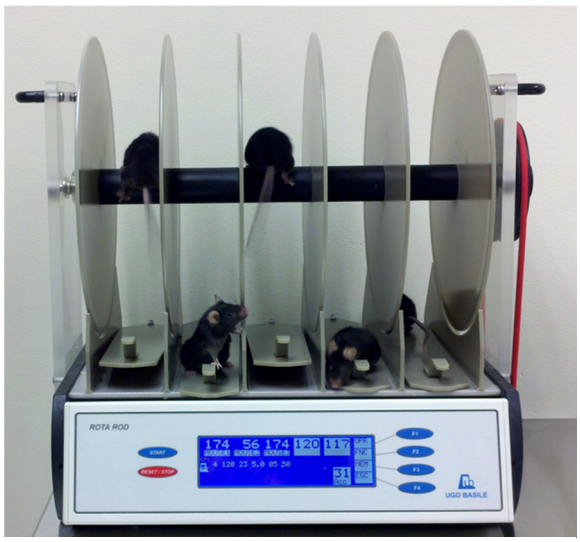

Figure 9. The rotarod apparatus. Up to 5 mice can simultaneously perform the 5-min motor coordination task.

3.2.8 Treadmill

Motor coordination of mice was examined in great detail by using the automated treadmill apparatus (Exer Gait XL, Columbus Instruments, USA; **Fig. 10A**) as previously described (Bonito-Oliva et al., 2014). In short, the test animal was place on the motorized transparent treadmill belt and the ventral view of its motions was recorded using a high-speed digital video camera (100 frames per second; **Fig. 10B**). Prior to testing, each mouse was allowed to habituate on a still treadmill belt for 1 min, followed by a phase of 1 min during which the speed of the treadmill belt was increased from 0 to 17 cm/s (i.e., 10 meter/min). Once the belt reached the testing speed (i.e., 17cm/s), gait was recorded during 3 trials of 20s each, with a 1

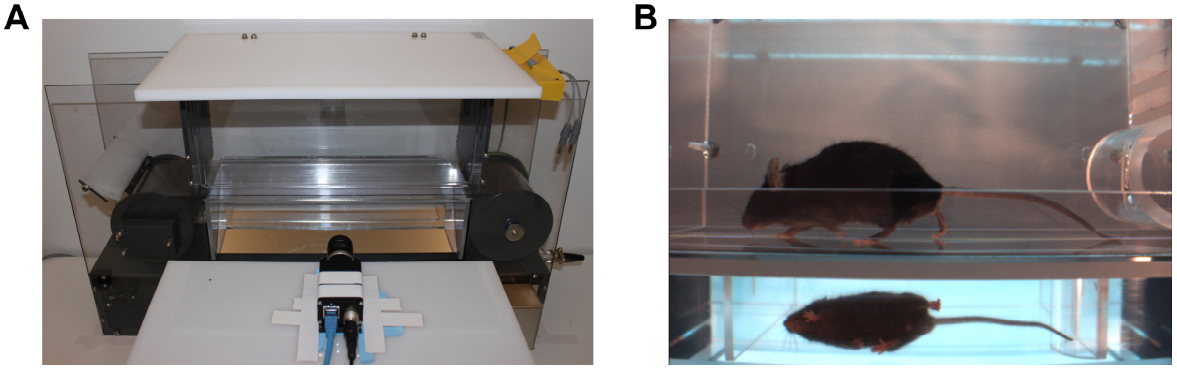

Figure 10. The automated treadmill apparatus. (A) The automated treadmill with the high-speed camera mounted to record the ventral view of the transparent treadmill belt as reflected by an angled mirror below. (B) A test mouse walking on the transparent automated treadmill belt from the camera's point of view.

min inter-trial interval. Motor coordination and movement on the treadmill was examined by measurement of individual paws and the whole body. Recordings were analyzed using the TreadScan software (Treadscan 4.0, Clever Sys, Inc., Reston, VA, USA) and all measurements were averaged across the 3 trails. Numerous parameters were measured including stance time, swing time, stride time, body length, and body width.

3.3 Molecular Techniques

3.3.1 Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) is a widely used molecular biology technique based on the PCR principal to quantify the expression of a target gene. In contrast to conventional PCR, qRT-PCR can detect and quantify amplification products in real-time as they are formed in the reaction, and not only at the end of the reaction as in PCR. Detection of the PCR product can be accomplished using either non-specific DNA binding dyes (e.g., SYBR® Green) or sequence-specific DNA probes (e.g., TaqMan, PrimePCR). In this thesis, we mostly used SYBR® Green, a fluorescent dye that binds to double-stranded DNA. Upon binding, a high fluorescent emission can be detected, allowing measurement of the total amount of double-stranded DNA products in the reaction after each cycle (see **Fig. 11A**). The main advantages of the SYBR® Green methods are the relatively low costs, ease of use and a simplified primer design compared to probe-based detection methods. Probe-base detection is more sensitive than non-specific DNA binding dyes because the probe is sequence specific. Therefore, SYBR® Green detection requires the use of melt curves after amplification to ensure specificity of the amplified products (**Fig. 11B** and **C**). To confirm gene expression data (for the primers used in paper III) we used Bio-Rad PrimePCR Probe assays (FAM- and HEX- labeled, Biorad, Sweden), which are fluorescent-labeled sequence-specific probes. The target-specific primers used in this thesis were designed using the Primer-BLAST web-based software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were designed using

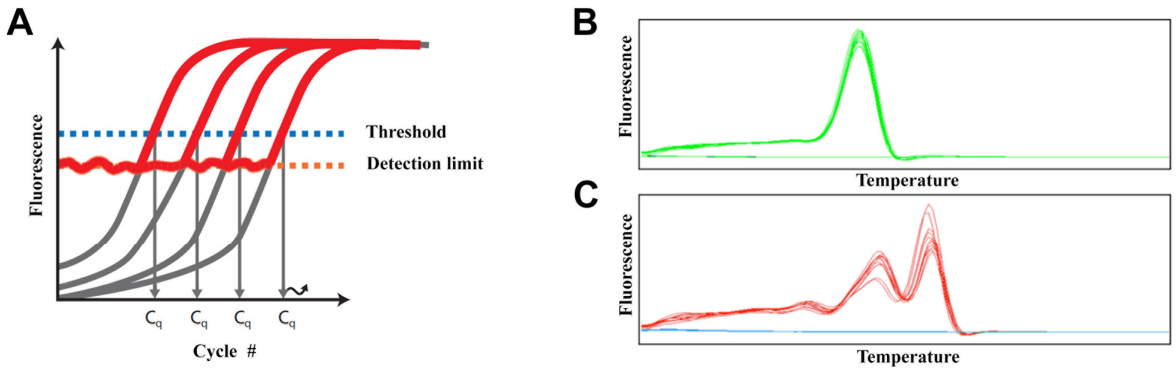

Figure 11. qRT-PCR principal and melt curve analysis. (A) During the qPCR cycles, the fluorescent signal emitted by SYBR® Green bound to the double-stranded DNA can be measured in real-time after each amplification cycle. The number of cycles required to reach the threshold level of detection is defined as the quantification cycle (Cq). A low Cq value represents a high concentration of the target gene, and a high Cq value a low concentration of the target. (B–C) Melt curve analysis. (B) shows a single melt peak, which is an indication for a specific amplification. In contrast, panel (C), shows two clearly distinct melt peaks suggesting a non-specific amplification. Thus, the primer pair of panel (C) cannot be used for qPCR. Figure (A) is modified from Sigma Aldrich "Primers and Fluorescent Probes" and Figure (B and C) are modified from Caister "PCR-troubleshooting".

the following parameters: PCR product seize: 90–150 nucleotides, Primer melting temperature: min 58 opt 60 max 62, Primer size: min 20 opt 22 max 25, Primer GC content (%): min 40 max 60. At least 2 primer pairs per target gene were ordered (25 nMole, desalted, and in dry format) from Invitrogen/Thermo Fisher Scientific. Upon arrival, the dry oligoes were reconstituted in nuclease-free water. The stock concentration of all primers was 125 pmol/ul and the working solution a ten-fold dilution of the stock solution (i.e., 12.5 pmol/ul). First, a gradient PCR was run to determine the optimal primer annealing temperature (usually between 55–60 °C), followed by an efficiency PCR with melt curve to determine primer efficiency (we aimed to have an efficiency between 95–105%). Finally, to confirm primer specificity, a conventional PCR was performed followed by the sequencing of the amplification product (KIGene, Stockholm, Sweden), and a BLAST search in the NCBI-BLAST website to confirm that the sequence of the amplified product is target gene specific. All primer sequences, annealing temperatures, and gene accession numbers of primers used in this thesis are listed in tables in **paper I–IV**.

Briefly, total RNA from various brain tissue samples was extracted using the RNeasy® Mini Kit (Qiagen AB, Sollentuna, Sweden) according to the manufacturer's instructions. RNA quality and quantity were determined spectrophotometrically using a NanoDrop® ND-2000 Spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA). We only included RNA samples with a 260/280 nm ratio of 2.0 and higher in our studies, since this is generally accepted as "pure" RNA. The 260/230 nm ratio was used as a secondary measurement of nucleic acid purity, 260/230 ratio between 2.0 and 2.2 were considered "pure". If ratios are appreciably lower than 2 it indicates the presence of protein, phenol or other contaminants that strongly absorb near 280 or 230 nm (NanoDrop Technical Bulletin 260/280 and 260/230 ratios). If a RNA sample did not pass quality check a clean-up step was performed to improve its purity (according to the RNeasy® Mini Kit instructions). Next, 1µg of each RNA sample was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Sundbyberg, Sweden) and stored in –20°C until used for qRT-PCR. Expression levels of genes of interest were quantified using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Sundbyberg, Sweden) as described in paper I. Bio-Rad CFX manager 3.1 software was used to analyze qPCR data and the fold changes were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.3.2 Western blotting

Western immunoblotting is a commonly used laboratory technique for protein detection and analysis. The first step in this technique is to separate the macromolecules by size using gel electrophoresis. After separation, proteins are transferred onto a polyvinylidene difluoride (PVDF) membrane. Next, the membrane is blocked to prevent non-specific binding of the antibody to the membrane. After blocking, the membrane is first incubated in a solution containing the target protein specific antibody (i.e., primary antibody) and then in a solution with an enzyme-labeled secondary antibody. Finally, the membrane is incubated with a chemiluminescent substrate that will emit light which can be detected using a CCD camera.

Briefly, various brain regions were dissected out on an ice-cold surface, immediately frozen on dry ice, and stored at –80°C until used. Sample were homogenized in 600 µl of 1% SDS, and boiled for 10 min as previously described (Qian et al., 2015). Protein concentrations were determined using the Bradford Protein Assay kit (Bio-Rad, Sundbyberg, Sweden). Next, protein samples (5 µg) were loaded into polyacrylamide gels (10–15% depending on the molecular weight of the target protein). Proteins were separated by SDS-PAGE and transferred to Immobilon® PVDF membranes (Millipore, Solna, Sweden). The membranes were then immunoblotted using polyclonal antibodies against Pglyrp2 (cat no. sc-50471, 1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and Pglyrp3 (cat no. MBS852923, 1:5000; MyBioSource, San Diego, CA, USA). Rabbit polyclonal antibody against Heat Shock Protein 90 (Hsp90; cat no. 4874, 1:10 000; Cell Signaling Technology, Beverly, MA, USA) was used as loading control. After incubation with primary antibodies, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Sundbyberg, Sweden) in combination with the chemiluminescent substrate (Clarity™ western ECL substrate, Bio-Rad, Sundbyberg, Sweden). Protein bands were detected using the ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad, Sundbyberg, Sweden) and quantitated using NIH Image J version 1.29 (National Institutes of Health). To verify equal loading of proteins, nitrocellulose membranes were stained with Coomassie Blue (Bio-Rad, Sundbyberg, Sweden).

3.3.3 BDNF ELISA assay

To quantify Brain-derived neurotrophic factor (BDNF) protein levels in the brain (see paper II), we used the ChemiKine™ BDNF sandwich enzyme immunoassay. BDNF ELISA principal: The microplate of this assay is coated with a rabbit polyclonal capture antibody. Samples are incubated in the plate and BDNF antigens are bound by the capture antibody. Next, a biotin labeled mouse BDNF monoclonal antibody (detection antibody) is added that can bind to the BDNF antigens. Then, a streptavidin-HRP conjugate solution is added which binds to the biotin labeled detection antibody. Finally, a substrate is applied resulting into a colorimetric reaction, which can be read in a spectrophotometer (**Fig. 12**). By generating a standard curve BDNF concentrations can be calculated. Briefly, brain tissues were homogenized in ice cold homogenization buffer, centrifuged and supernatants together with standards incubated in the ELISA plate. Next, the detection antibody was added, then the streptavidin-HRP conjugate, followed by the tetramethylbenzidine substrate and the Stop Solution, after which the plate was read immediately at 450 nm using a Bio-Rad xMark Microplate Absorbance Reader (**Fig. 12B**). A standard curve was generated and BDNF protein concentrations were calculated using Microplate Manager® 6.

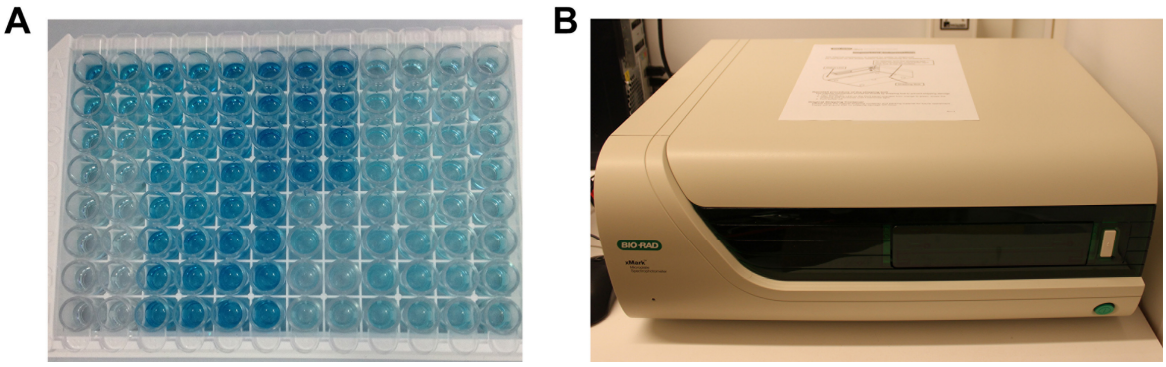

Figure 12. ELISA plate and spectrophotometer. (A) The colorimetric reaction in the ELISA plate after addition of the conjugate, just before the plate is read in the Bio-Rad xMark Microplate Absorbance Reader (B).

3.3.4 Peptidoglycan detection assay

To detect peptidoglycan (PGN) fragments (in **paper III**), we used the commercially available silkworm larvae plasma (SLP) detection kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's instructions. In short, the SLP detection kit is based on the following principal: SLP from the *Bombyx mori* contains an important innate self-defense mechanism, melanization. This process is controlled by the enzyme phenoloxidase (PO). The cleavage of the inactive proPO to the activate PO form is triggered by the recognition of bacterial-derived molecules such as PGN, by PGN-recognition proteins. Active PO, in turn, results in the production of melanin which serves as a measurement for PGN concentration. In short, we assayed 50µl of brain homogenate or serum in the detection plate together with a serial dilution of the PGN standard. After incubation, the plate was immediately read at 650 nm using a Bio-Rad xMark Microplate Absorbance Reader (**Fig. 12B**). A standard curve was generated and PGN concentrations were calculated using Microplate Manager® 6.3. Brain samples were wet weight normalized.

3.4 Statistical Analysis

Statistical analyses were performed using STATVIEW version 5.1 software. Data from behavioral studies were analyzed using either repeated-measures analysis of variance (ANOVA) or factorial ANOVA when appropriate. Post hoc comparisons were made using a Bonferroni/Dunn test when significant ANOVA effects were found. Data from gene and protein expression studies, as well as PGN studies were analyzed using one-way ANOVA. Post hoc comparisons were made using Fisher's Least Significant Difference (LSD) test when significant ANOVA effects were found. The threshold for statistical significance was set as $P \leq 0.05$. All data are presented as the mean ± SEM.

4 RESULTS & DISCUSSION

4.1 Host microbiota modulates development of social preference in mice

In **paper I**, the influence of the gut microbiota on motor and social behavior was assessed. For this purpose, three-month old Swiss-Webster GF and specific pathogen-free (SPF) male mice were subjected to an open-field test and the three-chambered sociability test. In the 90-min open field test GF mice traveled significantly greater distances during both the novelty period (i.e., the first 15-min of exposure) and habituation period compared to SPF mice (**Fig. 13A**). They also spent more time in the center of the open-field arena during the novelty period (**Fig. 13A inset**) and exhibited increased rearing activity throughout the 90-min task (**Fig. 13B**). These findings are in line with observations in adult NMRI GF mice that also displayed increased spontaneous locomotor activity (Diaz Heijtz et al., 2011). Another study reported increased home-cage activity counts in C57BL/6J GF male mice (Backhed et al., 2007). Neufeld and colleagues reported that female Swiss-Webster GF mice did not show alterations in locomotor activity during a 30-min open-field test (Neufeld et al., 2011b). This contrast with male GF mice can either be explained by the experimental setup (30- vs 90-min testing period) or sex-dependent differences. Next, social preference of Swiss-Webster GF male mice in the three-chambered sociability test was examined. GF animals spent significantly more time in the chamber containing the unfamiliar stimulus mouse, and less time in the chamber containing the object, compared to SPF mice (**Fig. 14A**). Further analysis revealed that GF mice displayed higher levels of social interaction with the stimulus mouse compared to controls (**Fig. 14B**), this observation was confirmed by calculating the sociability index (GF vs SPF: 81% vs 60%). To date, several studies in GF mice have shown an influence of the gut microbiota on social behavior (Crumeyrolle-Arias et al., 2014, Desbonnet et al., 2014, Buffington et al., 2016). In contrast to our findings, Desbonnet and colleagues found decreased social behavior in young adult Swiss-Webster GF male, but not female mice (Desbonnet et al., 2014).

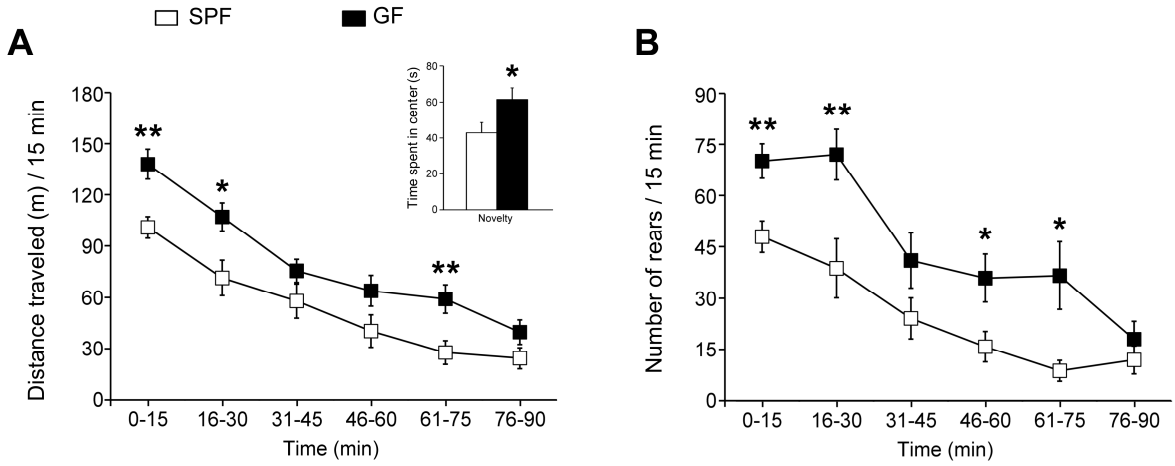

Figure 13. GF mice display increased locomotor activity. (A) Average distance traveled (meters) by GF male mice and controls in a 90-min open-field test. (Inset) The time the test animal spent in the center of the open-field arena during the novelty period (i.e., first 15-min of testing). (B) Rearing activity during a 90-min open-field box session. All data (A−B) are presented as means (± S.E.M; n = 10 per group). *P < 0.05, **P < 0.01 compared with SPF mice. Modified from (Arentsen et al., 2015).

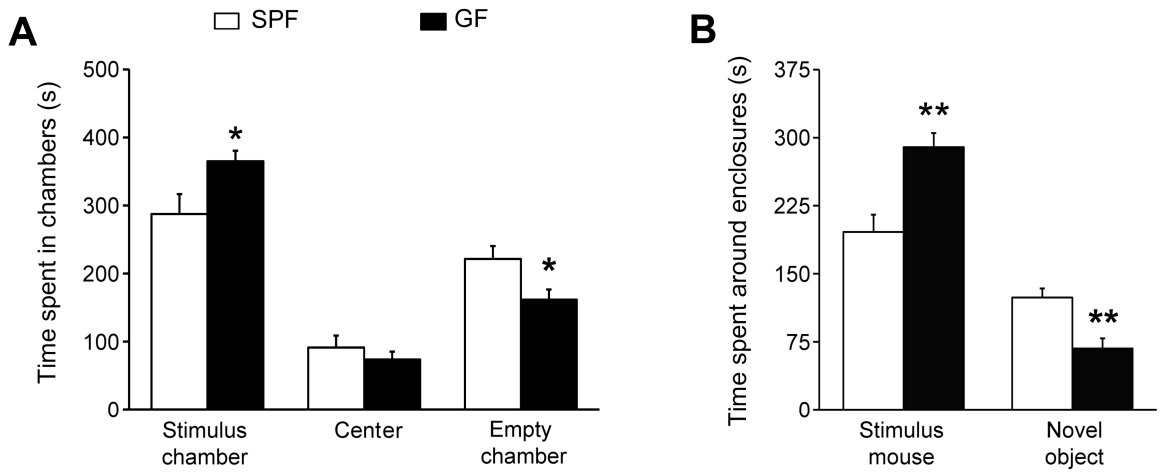

Figure 14. GF mice show increased social behavior. (A) Bars represent time spent (seconds) in the three chambers during a 10-min sociability session by GF and SPF male mice. (B) The time the test animals spent interacting with the unfamiliar stimulus mouse or the novel object. All data (A−B) are presented as means (± S.E.M; n = 10 per group). *P < 0.05, **P < 0.01 compared with SPF mice. Modified from (Arentsen et al., 2015).

The authors also reported increased self-grooming in GF male mice, in contrast no alterations in grooming behavior were observed in our study. The discrepancy between the two studies could be in part due to a different experimental design. An addition experiment with three-month old C57BL/6 GF and SPF male mice, obtained from the GF-core facility at Karolinska Institutet, was conducted with the inclusion of C57BL/6 controls from Charles River to ensure in-house SPF controls are similar to normal C57BL/6 inbred mice. It showed that GF mice displayed increased social behavior compared to SPF and C57BL6 controls (**Fig. 15**). In contrast to these findings, Buffington reported a decrease in social behavior and interaction in 7−12-week-old C57BL/6 GF male mice (Buffington et al., 2016). Both studies used C57BL/6 SPF stimulus mice and the experimental setup seems similar. Although inconsistencies in the direction of changes on social behavior observed in GF animals are evident in these studies, together, they all highlight that the absence of a gut microbiota can impact development of social behavior in rodents.

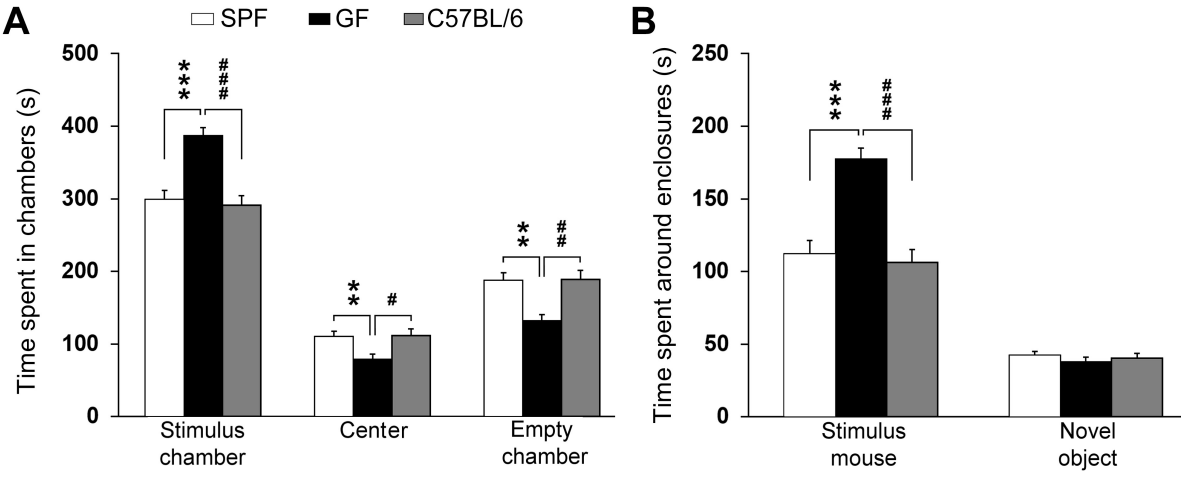

Figure 15. C57BL/6 GF mice show increased social behavior. (A) Bars represent time spent (seconds) in the different chambers during the sociability session by C57BL/6 GF and SPF mice from the KI GF-facility and for comparisons, C57BL/6 male mice obtained from Charles River Laboratories (Sulzfeld, Germany). (B) Bars show time spent interacting with the unfamiliar stimulus mouse or the novel object. All data (A−B) are presented as means (± S.E.M; n = 20 per group). **P < 0.01, ***P < 0.001 compared with SPF mice and $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001 compared with C57BL/6 mice.

To better understand potential molecular mechanisms underlying the observed behavioral alterations, the expression of brain-derived neurotropic factor (BDNF) in amygdala was investigated. The amygdala is important in the processing of social stimuli and BDNF is an important regulator of brain development, and involved in synaptic transmission and plasticity (Vicario-Abejon et al., 2002, Benarroch, 2015). In the amygdala of GF mice, mRNA expression levels of total BDNF were significantly reduced compared to SPF mice (**Fig. 16**). Moreover, four of the nine BDNF exon transcript variants (i.e., I, IV, VI and IX) were found to be significantly less expressed in GF mice compared to controls (**Fig. 16**). This data is in agreement with several studies in GF animals demonstrating that the BDNF expression is extremely sensitive to perturbation of the gut microbiota in the hippocampus, prefrontal cortex and amygdala (Sudo et al., 2004, Diaz Heijtz et al., 2011, Gareau et al., 2011, Neufeld et al., 2011b, Clarke et al., 2013). Similar to previous findings (Diaz Heijtz et al., 2011), expression levels of immediate-early gene NGFI-A were significantly reduced in GF mice (**Fig. 16**). In addition, several BDNF exon transcript variants were found to be significantly reduced in the amygdala of GF mice. The roles of these specific BDNF transcript variants are still poorly understood. Thus far, one study described an alteration in the expression of BDNF transcript variant (IV) in GF mice (Stilling et al., 2015). BDNF exon transcript IV is a direct target of transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). CREB binding to cAMP/Ca^{2+}-response element will result in the upregulation of BDNF exon transcript IV in the brain. Stilling and colleagues observed increased CREB signaling and upregulated levels of BDNF exon transcript IV (Stilling et al., 2015). Taken together, these data indicate that alterations in the expression of specific BDNF exon transcripts within the amygdala may contribute to the abnormal development of social behavior in GF mice.

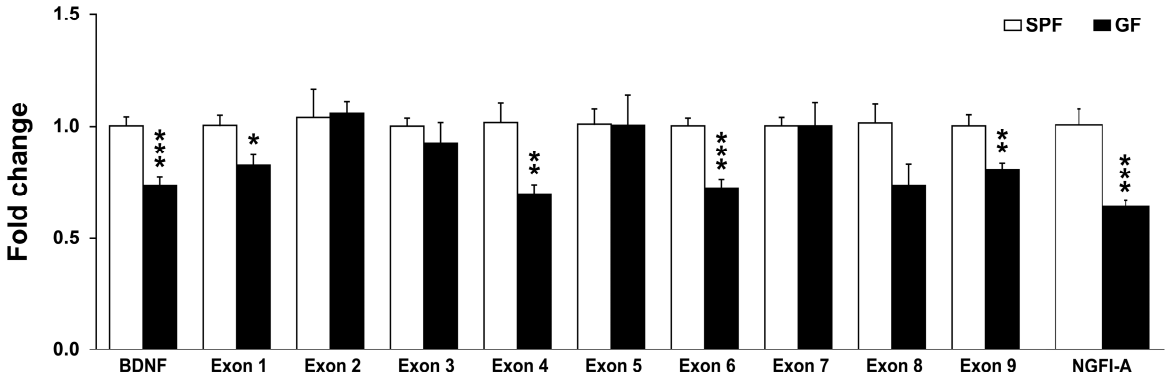

Figure 16. GF mice show decreased BDNF and NGFI-A expression levels. qRT-PCR was used to examine expression levels of the total BDNF, its exon transcript variants, and NGFI-A in the amygdala of GF and SPF mice. Expression levels were normalized to heat shock protein 90 (Hsp90) levels and expressed relative to the SPF group. Data are presented as means (± SEM; n = 5−6 per group). *P < 0.05 and ***P < 0.001 compared with SPF mice. Modified from (Arentsen et al., 2015).

4.2 Perturbation of maternal gut microbiota during pregnancy influences offspring brain development and behavior

The GF model has several limitations and lacks the clinical relevance since GF conditions are not translatable to humans. An alternative approach to study perturbations of the gut microbiota on health outcomes is through the use of antibiotics. Epidemiological studies have shown that antibiotic-induced perturbations of the gut microbiota during pregnancy and/or early postnatal life can have adverse outcomes in later life (Cox and Blaser, 2015b, Mueller et al., 2015b, Slykerman et al., 2017). Therefore, in **paper II**, we investigated whether perturbation of the maternal gut microbiota during pregnancy, induced by exposure to a broad-spectrum antibiotic, influences brain development and behavior in the offspring. For this purpose, C57BL/6 pregnant females were exposed to a clinically relevant dose of ampicillin in their drinking water throughout the entire pregnancy. To ensure the antibiotics were not having a major detrimental effect on overall health of the dams, their body weight and the body weight of their offspring was measured, and neither was different from controls.

Next, the impact of antibiotic treatment during pregnancy on the maternal gut microbiota was determined. Analysis of fecal samples of antibiotic-treated dams and controls revealed specific differences in the composition of the bacterial community within each group (alpha-diversity, see **paper II**). We also assessed the effect of antibiotic treatment on the relative abundances between groups (beta-diversity) and it was found that antibiotic treatment during pregnancy affected microbial diversity in the dams and their offspring, as shown in the unweighted UniFrac PCoA plots (**Fig. 17A** and **B**, respectively). Analysis of the relative abundances at the phylum level of fecal samples from antibiotic-treated dams and controls revealed a significant decrease in abundance of Bacteriodetes (antibiotic treatment vs control: 0.8% vs 46.8%; **Fig. 17C**), consistent with other report on the effect of antibiotic treatment on bacterial composition (Bercik et al., 2011a, Desbonnet et al., 2015b, Hoban et al., 2016a). Analysis of fecal samples from juvenile male and female offspring at the level of the phylum (**Fig. 17D)** revealed that the abundance of Deferribacteres was significantly altered compared to controls (**paper II**). Close examination at the family level revealed that several families belonging to the Bacteroidetes phylum (e.g., Bacteroidaceae, Prevotellaceae and Porphyromonadaceae) were in particularly affected by antibiotic treatment during pregnancy (**paper II**). These findings are consistent with previous reports using different antibiotics, time-windows of exposure and treatment duration (Bercik et al., 2011a, Desbonnet et al., 2015b, Hoban et al., 2016a, Frohlich et al., 2016, Tochitani et al., 2016), thus suggesting that specific microbes (e.g., members of the Bacteroidetes phylum) are highly sensitive to antibiotic exposure.

The impact of antibiotic treatment during pregnancy on the development of motor, emotional and cognitive functions in the offspring was examined next. For this purpose, juvenile offspring were subjected to a battery of tests for locomotor activity, anxiety-like and social behaviors.

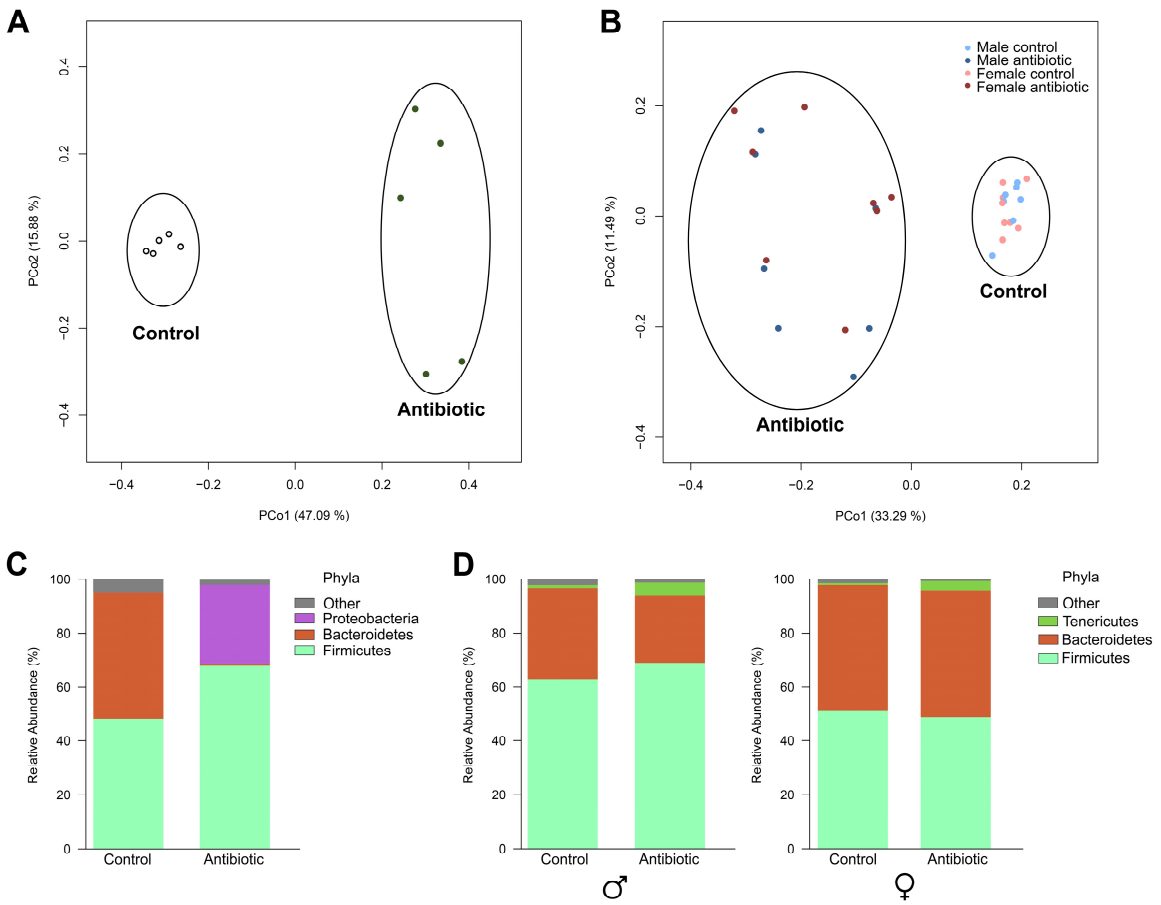

Figure 17. Antibiotic treatment during pregnancy altered fecal microbiota composition in antibiotic-treated dams and their offspring. (A) Unweighted UniFrac Principal Component Analysis (PCoA) of gut microbiota of antibiotic-treated dams and controls. (B) PCoA of gut microbiota of offspring from antibiotic-treated dams and controls. (C) Taxonomic distribution at the phylum level of fecal samples from the antibiotic-treated dams and controls. (C) Taxonomic distribution at the phylum level of fecal samples from male (left) and female (right) offspring of antibiotic-treated dams and controls. (A and C) n = 5 per group, (B and D) n = 7–8 per group. Modified from paper II.

In the first set of experiments, juvenile male and female offspring from antibiotic-treated dams and their respective controls, were placed in a novel open-field activity arena for analysis of their exploratory and habituation profiles. Both male and female offspring of antibiotic-treated dams displayed increased locomotor activity. Juvenile males displayed increased locomotor and rearing activity during the initial and early habituation phase (**Fig. 18A** and **C**), whereas females traveled a greater distance only during the habituation phase (**Fig. 18B** and **D**). These results are in line with previous literature (Diaz Heijtz et al., 2011, Bercik et al., 2011a) and our findings in paper I. Opposite to our results, a recent study using a high dose antibiotic cocktail during E9–E16 reported decreased locomotor activity in juvenile, but not adult offspring (Tochitani et al., 2016). Importantly, the antibiotic cocktail used in this study produced significant changes in the body weight of dams and their offspring, suggesting potential growth restriction in the offspring and subsequent detrimental effects. In contrast, we used a clinically relevant dose of ampicillin, which did not affect the body weight of dams, or their offspring.

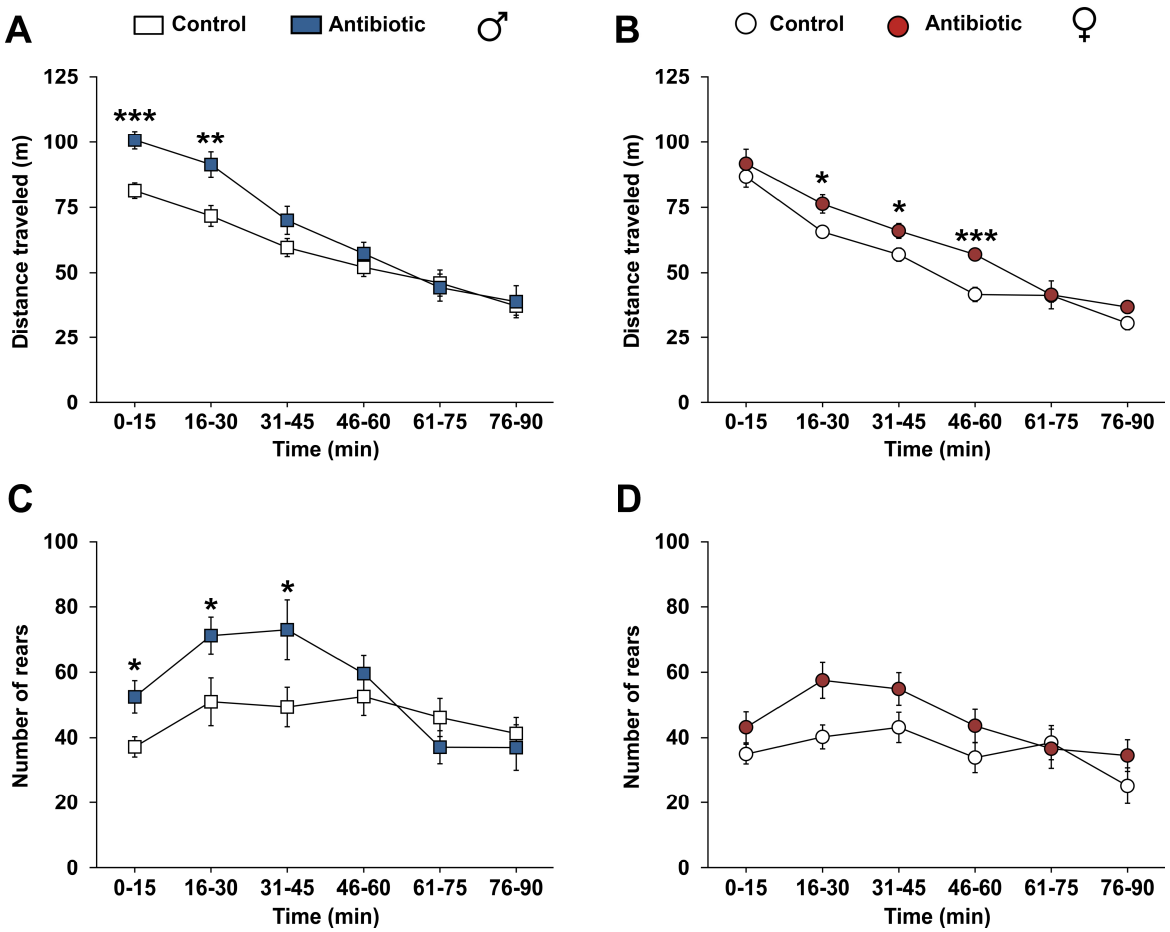

Figure 18. Juvenile offspring from antibiotic-treated dams show increased spontaneous motor activity. (A and B) Average distance traveled (meters) by juvenile male and female offspring of antibiotic-treated dams and controls in across a 90-min session in an open- field box. (C, D) Rearing activity of male and female offspring from antibiotic-treated dams and controls measured across a 90-min session in an open- field box. All data (A−D) are presented as means (± S.E.M; n = 10−12 per group). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control mice. Modified from paper II.

Previous studies have shown that mice devoid of a gut microbiota show decreased anxiety-like behavior (Diaz Heijtz et al., 2011, Neufeld et al., 2011b, Clarke et al., 2013). In the present study, no differences in anxiety-like behavior were observed between the offspring of antibiotic-treated dams and controls. Interestingly, studies in mice investigating the impact of antibiotic exposure during adulthood (Bercik et al., 2011a) or from weaning onwards (Desbonnet et al., 2015b) observed reduced anxiety-like behavior, similar to GF mice. Thus suggesting that the time-window of antibiotic exposure is particularly important for potential neurobehavioral outcomes.

Given that recent studies in GF mice have shown an influence of the gut microbiota on social behavior (**paper I**, (Desbonnet et al., 2014, Buffington et al., 2016)), an additional cohort was used to assess whether antibiotic treatment throughout pregnancy could affect social preference and social cognition of the offspring. For this purpose, juvenile male and female offspring of antibiotic-treated dams and controls were exposed to the three-chambered social approach task (see **material and methods**).

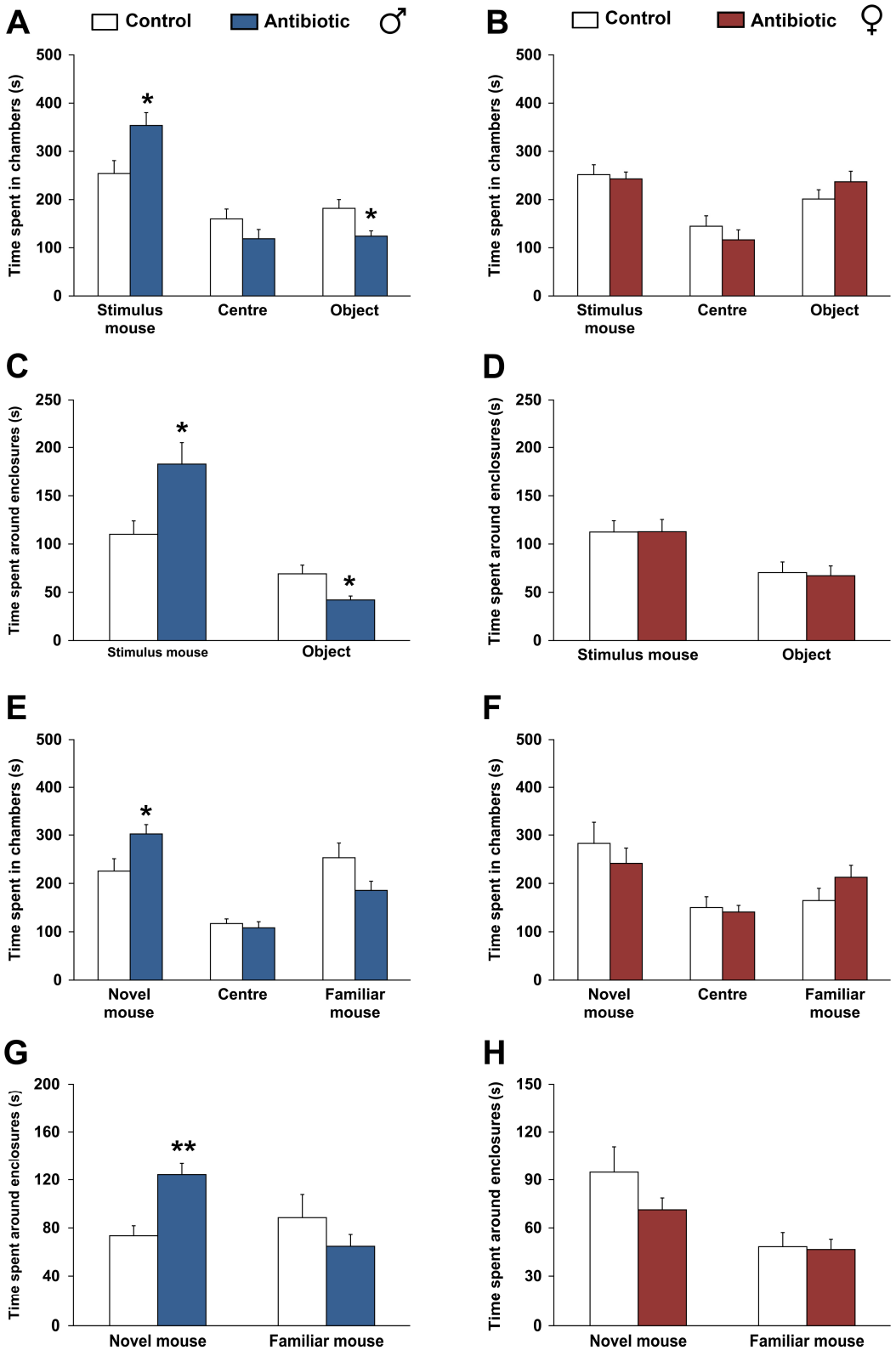

Figure 19. Perturbation of the maternal gut microbiota during pregnancy affect social behavior and cognition in a sex-dependent manner. (A, B) Bars show time (seconds) spent in the different chambers during the sociability session by juvenile male and female offspring of antibiotic-treated dams and controls. (C, D) Bars represent time spent interacting with the unfamiliar stimulus mouse or the novel object by juvenile offspring. (E, F) Bars show time spent in the different chambers during the social cognition session by juvenile male and female offspring of antibiotic-treated dams and controls. (G, H) Bars represent time spent interacting with the novel or familiar stimulus mouse by juvenile offspring. All data (A−H) are presented as means (± S.E.M; n = 8 per group). *P < 0.05, **P < 0.01 compared with controls. Modified from paper II.

We found that male, but not female offspring of antibiotic-treated dams displayed increased social behavior (**Fig. 19A–D**). These results were supported by the finding in the social interaction test, in which only male offspring of antibiotic-treated dams showed higher levels of social interaction. In the social cognition test, only male offspring of antibiotic-treated dams showed enhanced social memory (**Fig. 19E–H**), these results are in line with previous sex-specific findings in social behavior (Desbonnet et al., 2014). In contrast to our observations, Tochitani and colleagues did not observe an effect on social behavior in the offspring of dams treated with an antibiotic cocktail during pregnancy (E9−E16) (Tochitani et al., 2016). Using the GF-mouse model, Desbonnet and colleagues reported that adult males, but not females displayed altered social behavior and social cognition (Desbonnet et al., 2015b). This sex-dependent effect suggests that development of social brain circuits of males are more sensitive to perturbations of the gut microbiota. A recent study in humans found a moderate association between antibiotic treatment in the first year of life and poorer cognitive, behavioral and emotional outcomes during childhood (Slykerman et al., 2017). Although these findings require further validation, this study supports the notion that a sensitive period exists, early in life, in which antibiotic-induced perturbations of the gut bacteria may influence human brain development and cognitive function later in life.

Given the fact that BDNF plays a critical role in brain development and is also highly sensitive to perturbations of the gut microbiota (as shown in **paper I** and by others), BDNF expression levels were investigated in brain regions involved in the processing of social stimuli (e.g., prefrontal cortex and amygdala). We found that BDNF mRNA and protein levels were significantly reduced in the amygdala of juvenile male offspring of antibiotic-treated dams (**Fig. 20A** and **B**). These results are supported by the findings in **paper I** using the GF model which showed reduced BDNF mRNA expression in the amygdala of male GF mice. Interestingly, we found a negative correlation between BDNF protein expression levels in the amygdala and time spent interacting with the unfamiliar stimulus mouse (**Fig. 20C**). Several studies have suggested a relationship between BDNF and the functioning of brain circuits involved in social behavior (Branchi et al., 2006, Berry et al., 2015, Yeom et al., 2016). In addition, BDNF has been linked to several neurodevelopmental disorders including ASD (Adachi et al., 2014, Castren, 2014). A recent meta-analysis study found that children with ASD have increased serum BDNF levels compared with typically developing children (Qin et al., 2016), thus, supporting the notion that the BDNF signaling pathway is of importance in the development of social brain networks. Taken together, our results indicate that antibiotic-induced perturbations of the maternal gut microbiota during pregnancy may have long lasting effects on brain development and behavior in the offspring.

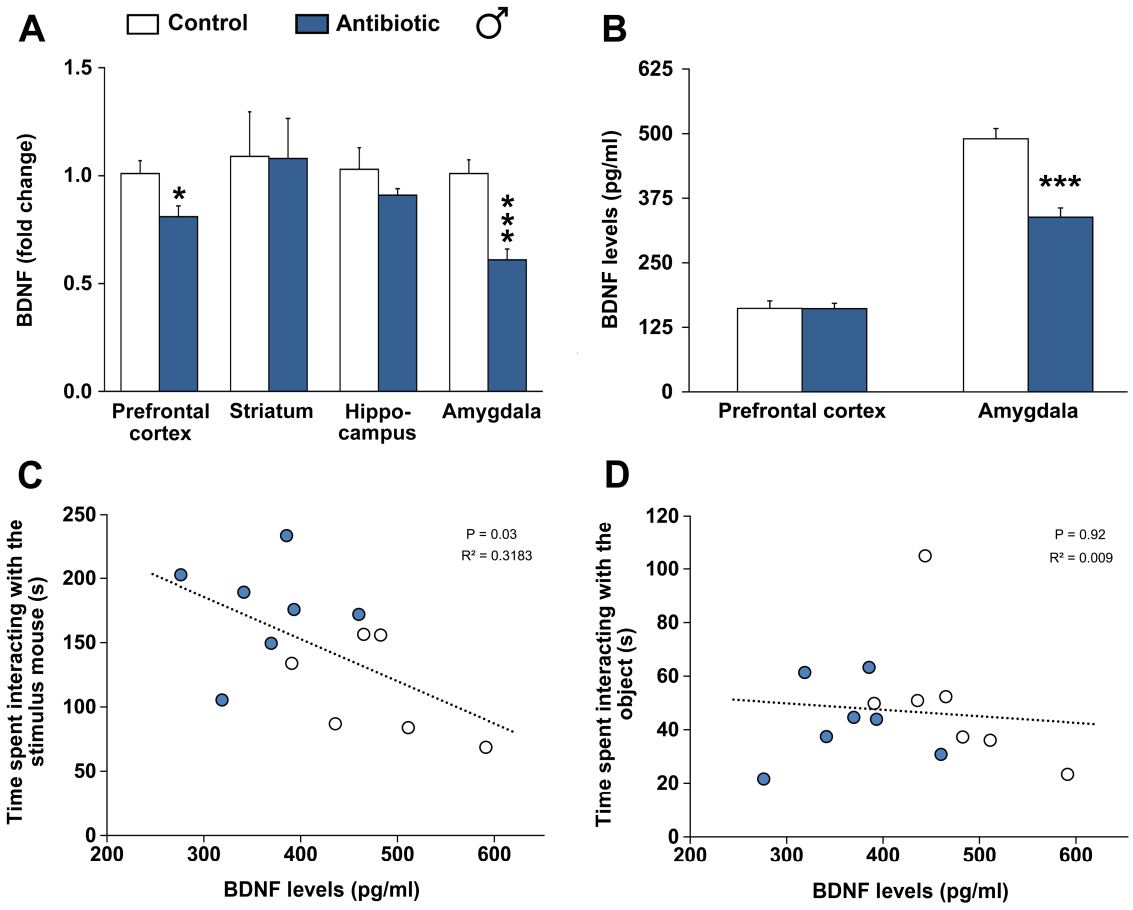

Figure 20. BDNF expression in the amygdala and the correlation between BDNF protein levels and social behavior. (A) Bars show mRNA expression levels in the prefrontal cortex, striatum, hippocampus and amygdala of naïve male offspring from antibiotic-treated dams and controls by means of qRT-PCR. Expression levels were normalized to heat shock protein 90 (Hsp90) levels and expressed relative to the control group (n = 5–6 per group). (B) An ELISA assay was used to examine BDNF protein levels in the prefrontal cortex and amygdala of behaviorally assessed juvenile male offspring (n = 7 per group). *P < 0.05, ***P < 0.001 when compared with control mice. (C) Simple regression analysis indicating a significant negative correlation between time spent interacting with an unfamiliar stimulus mouse and BDNF protein levels in the amygdala. (D) Correlation analysis of time spent interacting with a novel object and BDNF protein levels in the amygdala. (C and D) Filled circles represent values of individual mice. Modified from paper II.

4.3 The bacterial peptidoglycan-sensing molecule pglyrp2 modulates brain development and behavior

In **paper III** we first explored whether bacterial derived products were able to cross the BBB into the brain under normal conditions and sensed by specific pattern recognition receptors (PRRs). Using the silkworm larvae plasma (SLP) assay, we were able to determine that PGN can cross the BBB under normal conditions (**Fig. 21A**). It was also found that PGN levels increase in an age dependent manner, paralleling the microbial colonization process of the gut (**Fig. 21B**). Previous studies have shown that PGN can be detected in the brain and cerebral spinal fluid under compromised conditions (Schrijver et al., 2001, Krueger et al., 1984, Martin et al., 1984). The observation that PGN can cross the BBB under normal physiological conditions supports the notion that PGN might be of importance in the communication between the gut microbiota and the brain.

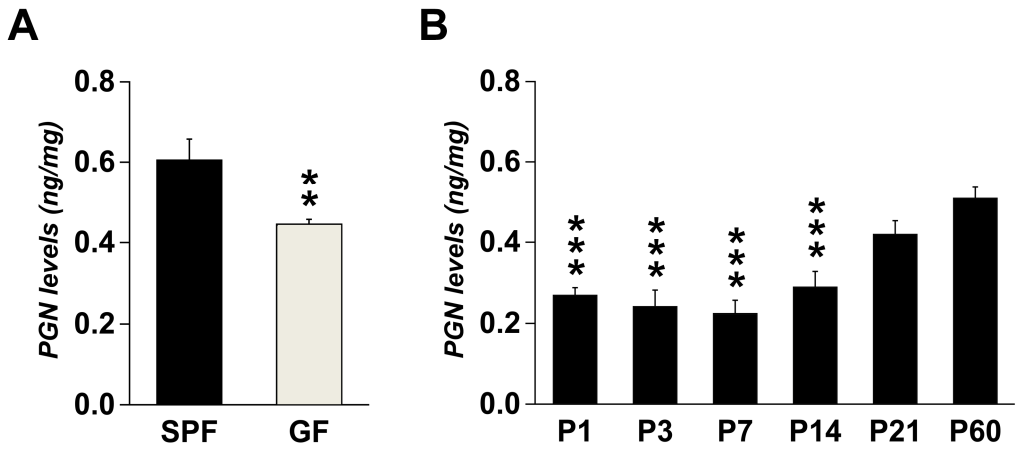

Figure 21. Bacterial peptidoglycan is present in the developing brain. (A) The silkworm larvae plasma assay was used to determine peptidoglycan (PGN) levels in the cerebellum of juvenile specific pathogen free (SPF) and germ-free (GF) male mice (n = 8 per group; **P < 0.01 when compared to SPF group). (B) PGN levels in developing cerebellum at postnatal (P) day 1, 3, 7, 14, 21 and 60 (n = 6 per group; ***P < 0.001 when compared with the P60 group). Modified from (Arentsen et al., 2017).

Next, it was assessed whether PGN sensing molecules were present in the developing brain using qRT-PCR. We found that members of three innate immune molecule families, the PGN recognition proteins (PGLYRPs), NOD-like receptors (NLRs) and Toll-like receptors (TLRs), were expressed in the developing brain during specific temporal windows (**Fig. 22A–C**). Pglyrp2–4, PGN transporter (PepT1) and Tlr2 were expressed at signficanlty higher levels during the first days of postnatal life, whereas, Pglyrp1, Nod1 and Nod2 were higher expressed later in life in the striatum, prefrontal cortex and cerebellum, (**Fig. 22A, B, C,** respectively). In addition, we also observed brain region- and sex-dependent differeces in the mRNA expression levels of PGN sensing molecules such as Pglyrp2 (**Fig. 22D–F**). These results suggest that the temporal expression of PGN sensing molecules during key neurodevelopmental stages, that co-occur with the bacterial colonization process of the gut, may play a role in brain development.

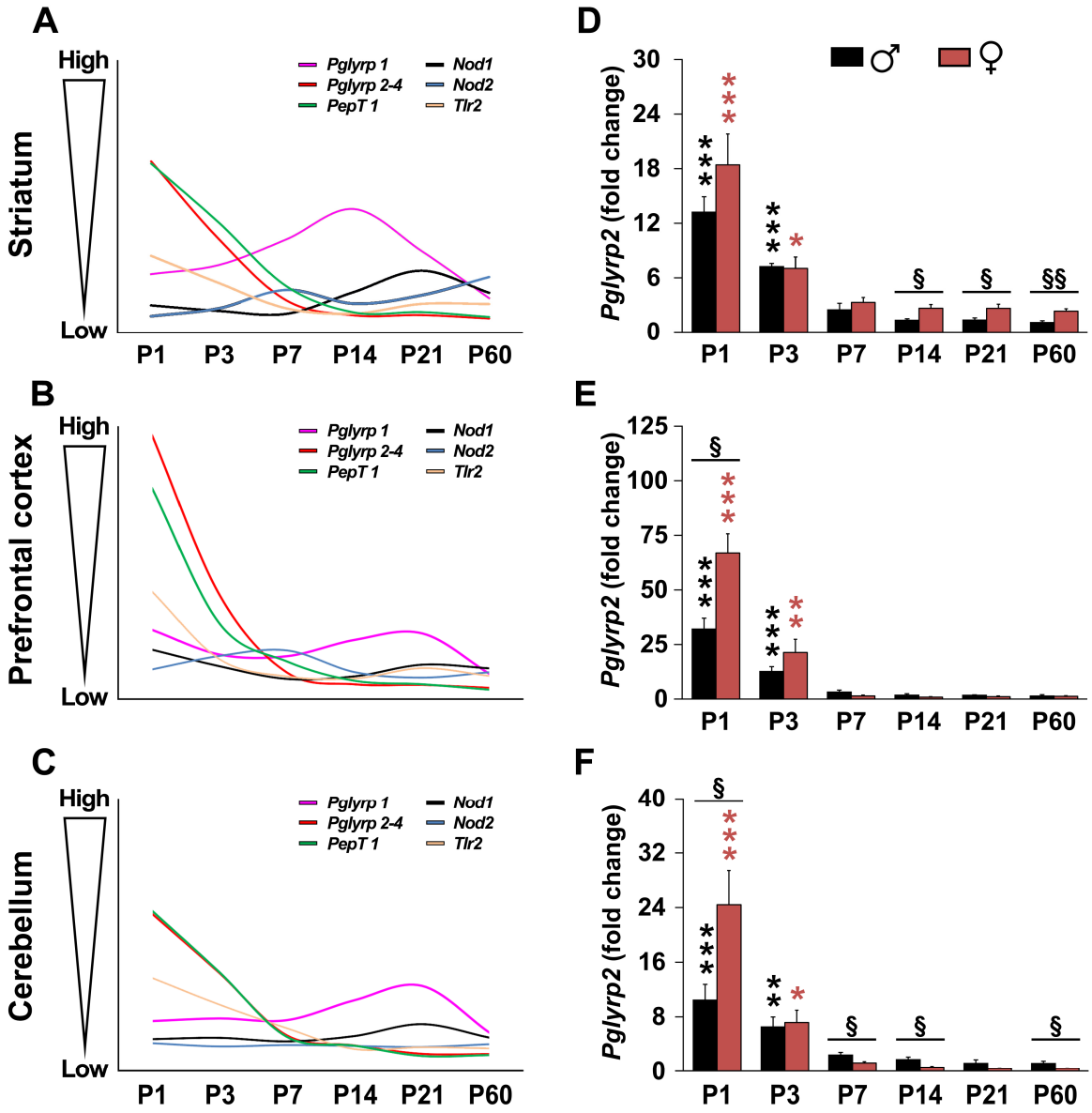

Figure 22. Expression of peptidoglycan-sensing molecules in the developing mouse brain. (A–C) Graphical visualization of mRNA expression levels of *Pglyrp1–4*, *Nod1, Nod2, Tlr2*, and *PepT1* in the striatum (A), prefrontal cortex (B), and cerebellum (C) during postnatal brain development. (D–F) mRNA expression levels of Pglyrp2 in the striatum (D), prefrontal cortex (E) and cerebellum (F) of male and female C57BL/6 mice during postnatal development. The expression level of each gene examined was normalized to *Hsp90ab1* levels and expressed relative to postnatal (P) day 60 male levels. Data of (D–F) are presented as means (± S.E.M; n = 4–6 per group). *P < 0.05, **P < 0.01, and ***P < 0.001 when compared with the P60 male group. Differences between male and female mice are indicated as follows: §P < 0.05, §§P < 0.01. Modified from (Arentsen et al., 2017).

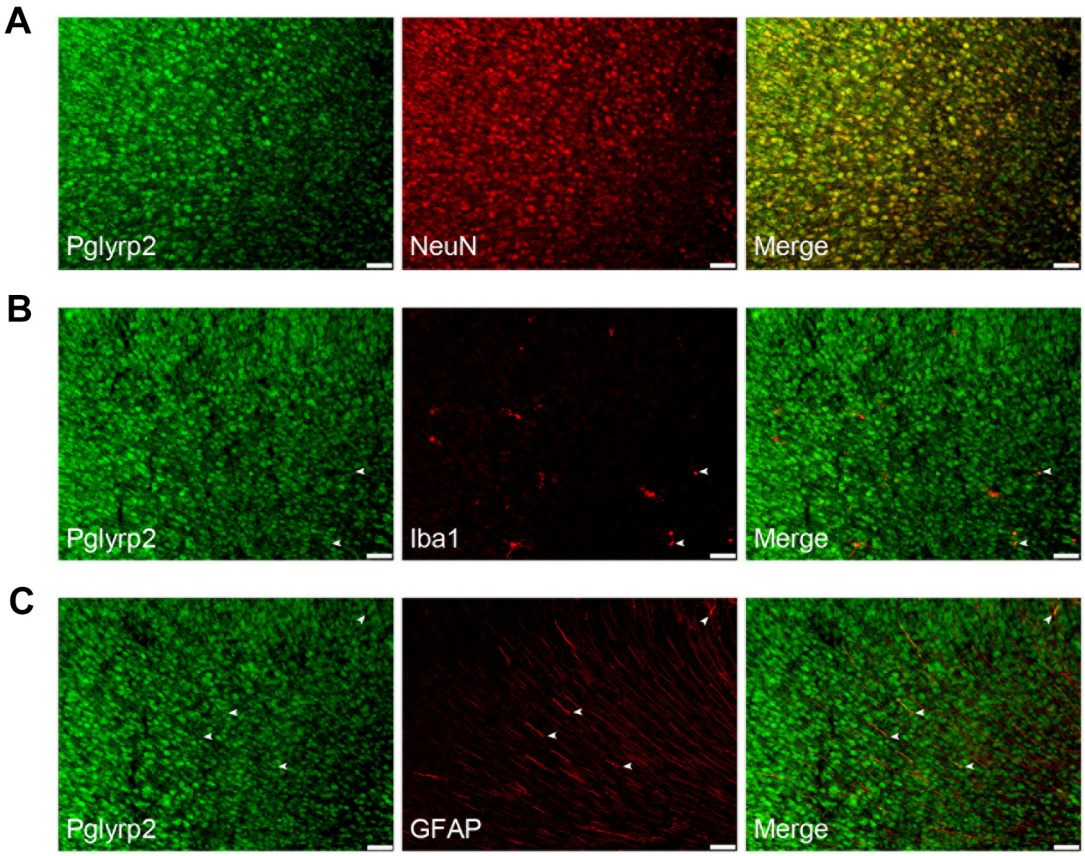

Figure 23. Representative images illustrating the cellular distribution of Pglyrp2 in the developing prefrontal cortex. (A–C) Double immunofluorescence staining shows Pglyrp2 (green) combined with NeuN (a neuronal marker; red), Iba1 (a microglial marker; red) or GFAP (an astrocyte marker; red) in prefrontal cortex sections of three-day-old C57BL/6 male mice. Merged images display the localization of Pglyrp2 with neurons (A), and to a lesser extent with microglia (B) and astrocytes (C). Arrows indicate Pglyrp2-positive microglia (B) and Pglyrp2-positive astrocytes (C). The white scale bars represent 50µm. Modified from (Arentsen et al., 2017).

To begin to address the potential role of PGN sensing molecules on brain development, the cellular distribution of some of these molecules was investigated, using immunohistochemical techniques. Similar to the mRNA expression data, the Pglyrp2 and Nod1 (Arentsen et al., 2017) proteins were detected in various brain regions including the prefrontal cortex, hippocampus and cerebellum (**Fig. 23**). Importantly, Pglyrp2 was highly expressed in neurons, indicating that PGN may affect neuronal development and function. In a recent study, Harris and colleagues described a novel role for PGRPs in the brain. Using the Drosophila model (Royet et al., 2011), the authors demonstrated that a member of the PGRP family (i.e., PGRP-LC) known to be the primary receptor that initiates an immune response against pathogens, is required for presynaptic homeostatic plasticity (Harris et al., 2015). In addition, other innate immune molecules have been linked to neuronal development including the C1q component of the complement cascade and several members of the TLR family. For example, it has been shown that C1q is required for synapse elimination (Stevens et al., 2007), Tlr3 and Tlr8 have been implicated in neurite outgrowth and neuronal cell death (Lathia et al., 2008, Ma et al., 2006), whereas neuronal differentiation and cell proliferation can be regulated by Tlr2 and Tlr4 (Okun et al., 2010b, Shechter et al., 2008). These findings suggest that PGRPs in the brain have roles beyond the “classical” innate immune system functions.

We then explored whether the expression of PGN sensing molecules in the brain would be sensitive to perturbations of the gut microbiota using two experimental models, i.e., the C57BL/6 GF mouse model and the perinatal antibiotic treatment model (see **material and methods**). A signficant decrease was found in the expression of Pglyrp2–4, Tlr2, and PepT1 in the striatum of three-day-old male and female mice (Pglyrp2 and Tlr2 are shown in **Fig 24A** and **B**). These results suggest that PGN sensing molecules are senstive to disruptions of the gut microbiota. Next, the effect of gut microbiota perturbation on the expression of BDNF and ASD risk gene c-Met was examined in GF and perinatal antibiotic-treated male and female mice. Consistent with observations in **paper I** and **II**, perturbations of the indigenous microbiota led to decreased BDNF expression levels in the striatum of three-day-old mice (**Fig. 24C**). This suggests that a disbalance in the gut microbiota early in life may affect BDNF levels during early-postnatal time windows when key neurodevelopmental processes occur. In addition to BDNF, proto-oncogene c-MET is a gene of particular interest given its strong link to ASD individuals with co-occurring GI problems, and the significant role it plays in brain development through involvement in the formation of synapses (Peng et al., 2013, Qiu et al., 2014). A decrease in c-Met expression levels in the striatum of male and female GF and perinatal antibiotic-treated mice was observed (**Fig. 24D**). Indicating that perturbations of the gut microbiota early in life can impact brain development and may subsequently increase the risk for neurodevelopmental disorders.

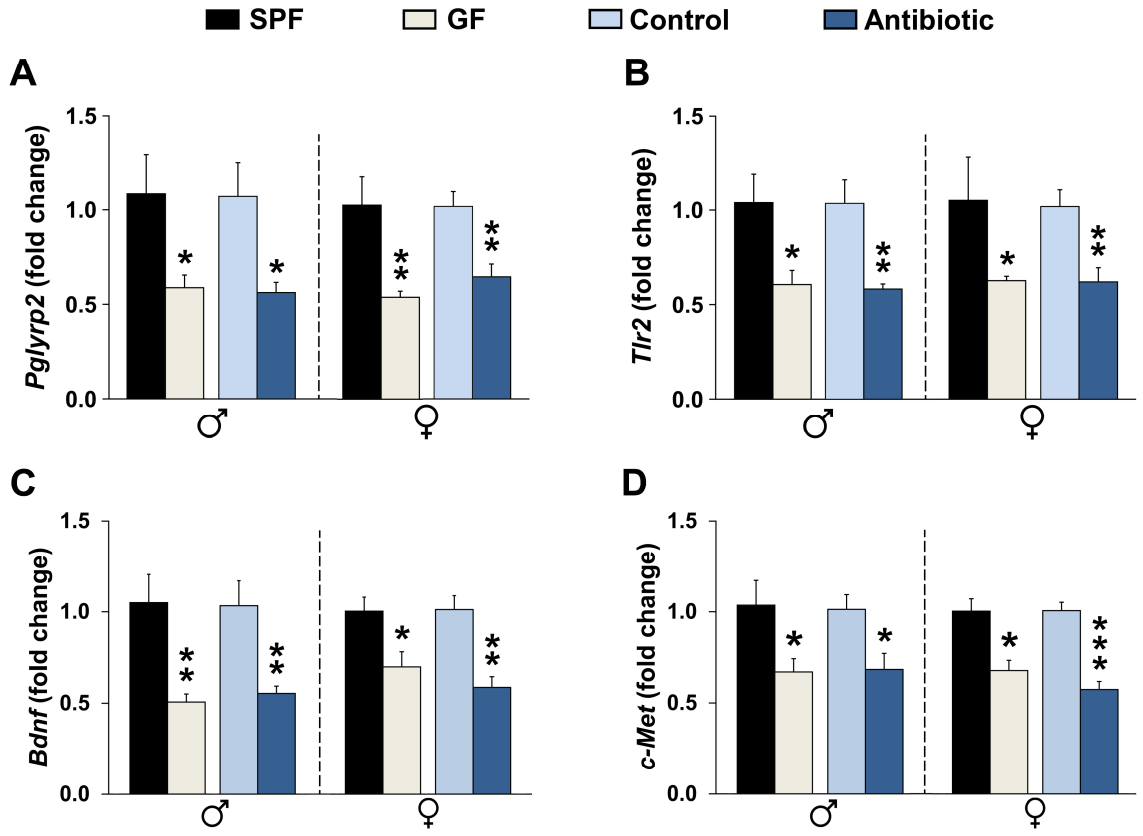

Figure 24. Perturbations of the gut microbiota alters the expression of PGN sensing molecules, BDNF and c-MET in neonatal striatum. Gene expression levels of *Pglyrp2* (A), *Tlr2* (B), *Bdnf* (C) and *c-MET* (D) in the striatum of three-day-old GF and perinatal antibiotic-treated male and female mice. All data (A–D) are presented as means (± S.E.M; n = 4–6 per group). *P < 0.05, **P < 0.01, and ***P < 0.001 their respective control groups. The labels are as follows: germ-free (GF); specific-pathogen free (SPF). Modified from (Arentsen et al., 2017).

In a new set of experiments, the potential role of PGN sensing molecules on brain development and behavior was further investigated. For this purpose, we took advantage of a transgenic mouse model lacking Pglyrp2. Pglyrp2 is the only mammalian PGRP with amidase activity (i.e., it can hydrolyze PGN) and it is one of the PGN sensing molecules that are highly expressed early in development. Juvenile (i.e., P22–24) Pglyrp2 knockout (KO) and wildtype (WT) mice were subjected to a battery of tests similar to those used in **paper II** (i.e., open-field, elevated plus maze, light-dark box and sociability tests). Juvenile male and female Pglyrp2 KO did not display alterations in locomotor activity and anxiety-like behavior compared to their respective WT controls. In the three-chambered sociability test, however, male Pglyrp2 KO mice exhibited increased social behavior (**Fig. 25A** and **C**), whereas, Pglyrp2 KO females spent similar amounts of time interacting with the stimulus mouse compared to WT controls (**Fig. 25D**). Interestingly, the observed sex-specific increase in social behavior is in line with our findings in the prenatal antibiotic treatment mouse model, as presesented in **paper II** (a schematic overview comparing the different animal models used in this thesis is shown in **Fig. 26**). Next, the expression of BDNF and c-MET in several brain regions of three-day-old Pglyrp2 KO and WT mice was examined. BDNF was found to be signficantly reduced in the striatum of neonate male mice (Arentsen et al., 2017). c-MET was less expressed in the striatum of neonatal female Pglyrp2 KO mice (**Fig. 25E**), in contrast, higher c-MET expression levels were observed in the prefrontal cortex of three-day-old male and female Pglyrp2 KO mice (**Fig. 25E**), and in the striatum of the juvenile male Pglyrp2 KO mice (**Fig. 25F**). Thus, the absence of Pglyrp2 led to a sex-specific increase in social behavior and to alterations in the expression of genes (i.e., BDNF and c-Met) involved in the formation and modulation of brain circuits, indicating that Pglyrp2 might play a role in neurodevelopment and behavior. This notion is supported by a recent study demonstrating that PGRP-LC is required for presynaptic homeostatic plasticity (Harris et al., 2015). Furthermore, studies investigating the role of TLRs on brain development and behavior have shown that TLRs may impact motor, anxiety and cognitive behaviors. Recently, Humann and colleagues showed that PGN was able to cross the placenta, activate Tlr2, induce transcription factor FoxG1, which led to increased neurproliferation in the fetal brain. *In utero* PGN exposure impaired memory and cognitive function in adulthood. Interestinly, the fetal brain was only senstive to incoming PGN-signals during a specific time window of brain development (i.e., E10) (Humann et al., 2016). Okun and collaborators showed that Tlr3 has a developmental role in memory retention, while Tlr4 is involved in shaping spatial reference memory and fear learning (Okun et al., 2010a, Okun et al., 2012). Other studies have also shown developmental roles for Tlr2 in anxiety and cognitive functioning (Park et al., 2015), Tlr7 in depressive-like behaviors (Kubo et al., 2013) and Tlr9 in sensory and motor behavior (Khariv et al., 2013). Given that PRRs seem to play multiple roles in immunity, in neuronal development, and in brain function, we propose that specific PRRs activitated by bacterial PGN, may be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain.

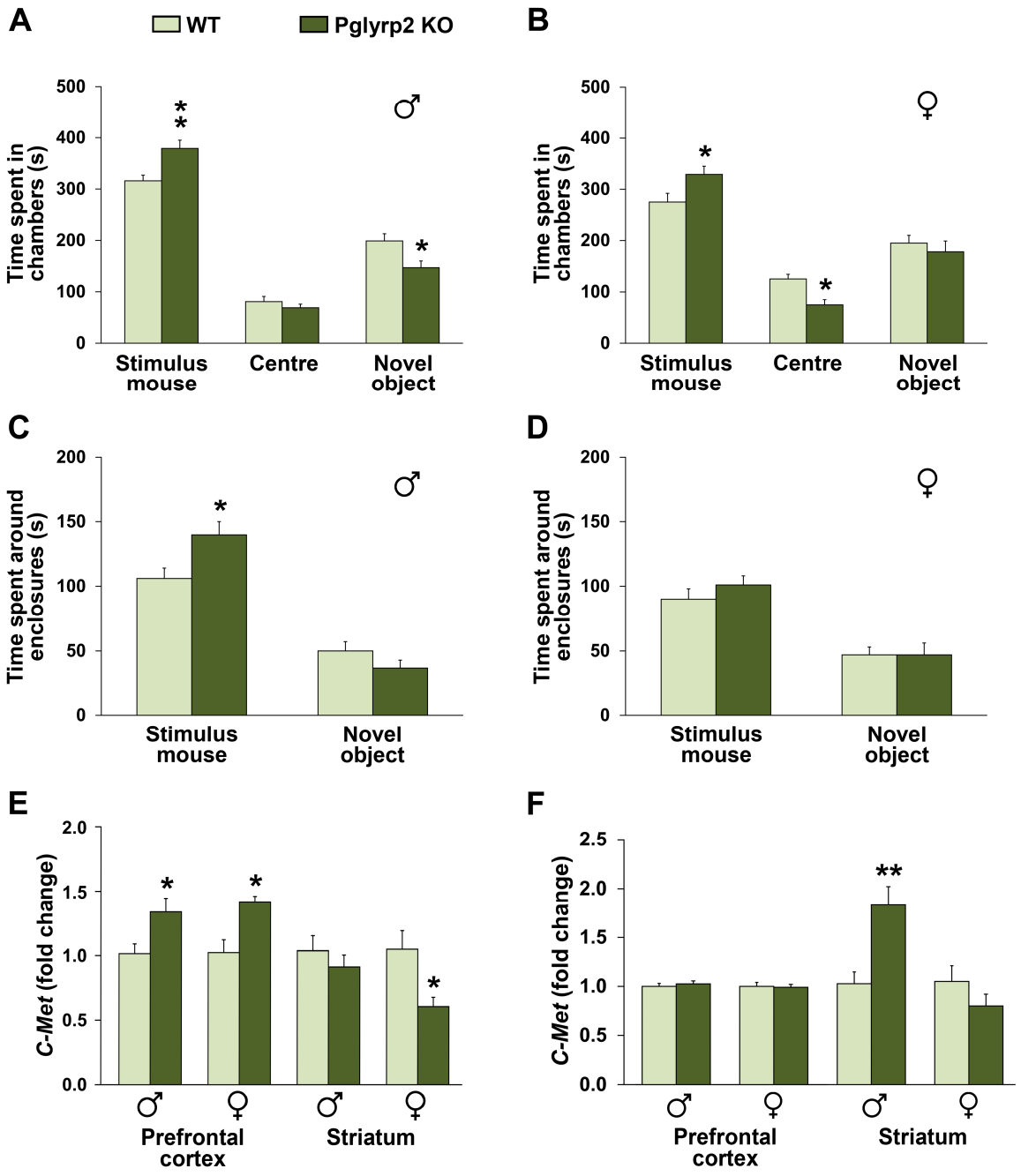

Figure 25. Mice lacking Pglyrp2 display increased social behavior in a sex-dependent manner and altered expression of c-Met. (A, B) Bars show time (seconds) spent in the different chambers during the sociability session by juvenile male and female Pglyrp2 KO and WT mice. (C, D) Bars represent time spent interacting with the unfamiliar stimulus mouse or the novel object by juvenile Pglyrp2 KO and WT mice (n = 8 per group). (E) c-Met mRNA expression levels in the prefrontal cortex and striatum of 3-day-old Pglyrp2 KO and WT mice (n = 5–6 per group). (F) mRNA expression levels of c-Met in the prefrontal cortex and striatum of behaviorally assessed juvenile Pglyrp2 KO and WT male and female mice (n = 6–8 per group). Data are presented as means (± S.E.M.). *P < 0.05 and ** P < 0.01 when compared with their respective WT group. The labels are as follows: knockout (KO) and wildtype (WT). Modified from (Arentsen et al., 2017).

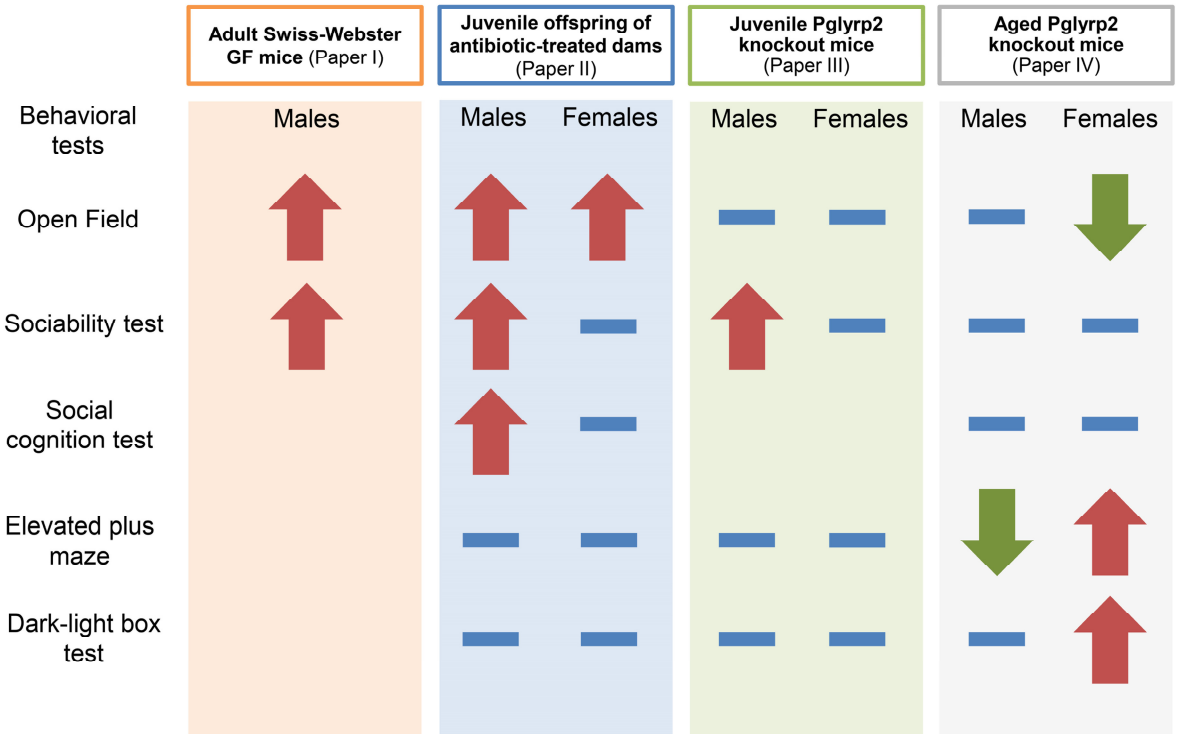

Figure 26. Schematic overview of the different animal models and their performance in various behavioral tests.

4.4 Sex-dependent alterations in motor and anxiety-like behavior of aged bacterial peptidoglycan sensing molecule 2 knockout mice

Recent genetic association studies found that several SNPs in PGRP genes were associated with increased risk for inflammatory bowel disease (Zulfiqar et al., 2013) and Parkinson's disease (Goldman et al., 2014). In a new study it was suggested that the gut microbiota may play a role in the progression of Parkinson's disease (Sampson et al., 2016). Parkinson's disease is characterized by motor deficits such as tremors, muscle rigidity and impaired gait, and frequently with co-occurring depression and anxiety as well as GI problems. Hence, in **paper IV** we investigated whether Pglyrp2 may play a role in motor control, anxiety-like behaviors and brain function later in life. For this purpose, 15-month-old Pglyrp2 KO and WT male and female mice were subjected to a battery of tests for spontaneous locomotor activity, motor coordination, anxiety-like behavior and social behavior. In the first set of experiments, Pglyrp2 male and female mice were exposed to the open-field test. The distance traveled by Pglyrp2 animals during the 90-min open-field test did not differ between groups. Analysis of the number of rears revealed that Pglyrp2 KO females, but not males exhibited reduced levels of rearing activity compared to WT controls (**Fig. 27A** and **B**).

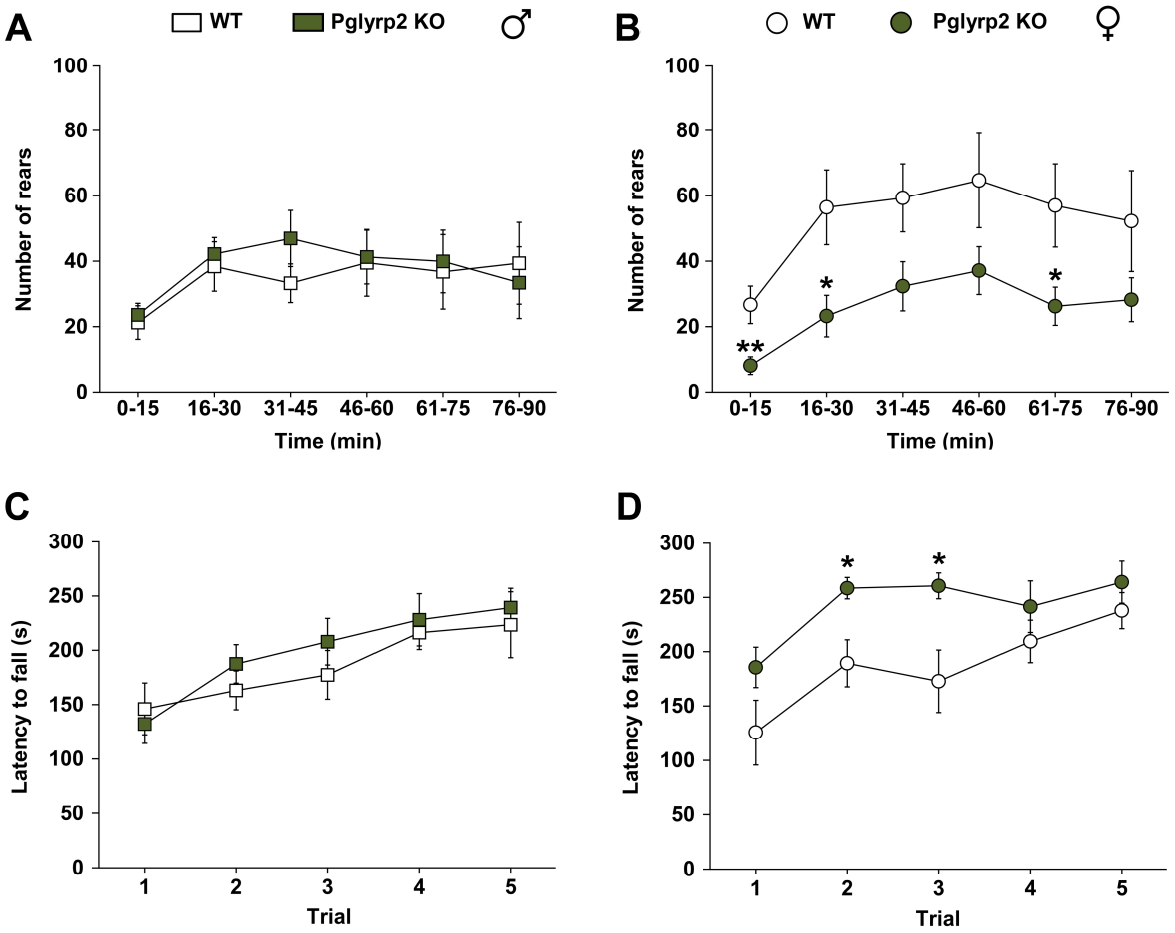

Figure 27. Pglyrp2 knockout females display decreased rearing activity and increased performance in the rotarod. (A, B) Rearing activity of Pglyrp2 KO and WT male and female mice measured in 15-min time bins across a 90-min open-field test. (C, D) Performance of Pglyrp2 KO and WT male and female mice on the rotarod apparatus, measured by the latency (in seconds) to fall during a 300s task. All data (A−D) are presented as means (± S.E.M; n = 8−12 per group). *P < 0.05, **P < 0.01 compared with their respective WT group. Modified from paper IV.

It has been previously proposed that rearing activity may reflect non-selective attention to novel environmental stimuli (Aspide et al., 1998), thus indicating that attention may be negatively affected in Pglyrp2 deficient female mice. Next, the motor coordination and balance of Pglyrp2 KO and WT mice was assessed in an accelerating rotarod apparatus. Pglyrp2 KO male mice performed similar to WT controls (**Fig. 27C**) and pglyrp2 KO females showed a better performance in the rotarod-test compared to controls (**Fig. 27D**), indicating better balance and coordination. Together, these data suggest a sex-dependent role for Pglyrp2 in the motor system.

Animals were next subjected to three different tests for anxiety-like behaviors; the elevated plus maze (EPM), light-dark (LD)-box test and open-field test. Pglyrp2 KO males displayed reduced anxiety-like behavior in the EPM and open-field test compared to controls (**Fig. 28A** and **E**). Pglyrp2 KO females showed the opposite pattern, increased anxiety-like behavior in the EPM, LD-box test, and open-field test (**Fig. 28B**, **D** and **F**, respectively). Interestingly, in **paper III** we did not observe any alterations in locomotor activity or anxiety-like behavior in juvenile (P22–P24) Pglyrp2 KO male and female mice compared to WT controls (for comparisons see **Fig. 26**). This suggests that the modulatory effects of PGN and its sensing

molecule (i.e., Pglyrp2) on brain circuits implicated in motor control and anxiety-like behaviors may change throughout life. The fronto-amygdala circuitry implicated in emotional regulation undergoes substantial developmental refinement from childhood through adulthood; highlighting that the developmental age of the animal can be an important factor in behavior outcomes.

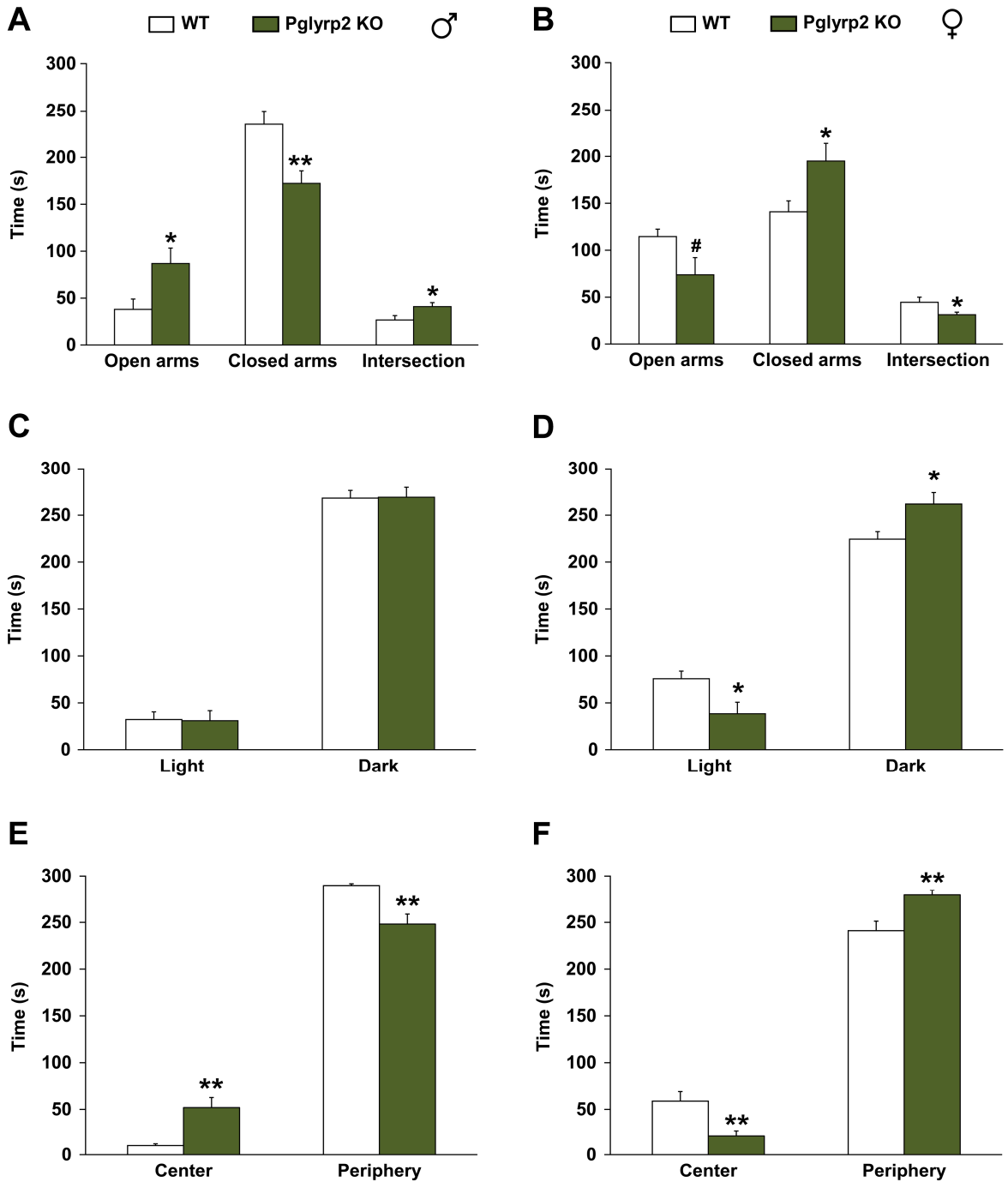

Figure 28. Pglyrp2 KO male and female mice display altered anxiety-like behaviors. (A, B) Bars show time (seconds) in each area of the elevated plus maze by Pglyrp2 KO and WT male and female mice during a 5-min test session. (C, D) Time spent in the light and dark compartment of the LD-box test by Pglyrp2 KO and WT mice. (E, F) Bars show time (seconds) spent in the center and periphery by Pglyrp2 KO and WT male and female mice during the first 5-min of an open-field test. All data (A−F) are presented as means (± S.E.M; n = 8−12 per group). #P < 0.058, *P < 0.05, **P < 0.01 compared with WT mice. Modified from paper IV.

In **paper III**, we observed that juvenile Pglyrp2 KO male mice displayed increased social behavior. In the present study, aged Pglyrp2 KO male mice tended to spend more time interacting with the stimulus mouse compared to WT controls, suggesting that social behavior may also change with age. This notion is supported by a study investigating the effects of strain and age (prepubertal to adulthood) on the development of social behavior (Fairless et al., 2012). To begin addressing potential effect of Pglyrp2 deficiency on brain function, the expression levels of synaptic-plasticity related genes in key brain areas involved in motor function and associated with anxiety were examined (i.e., prefrontal cortex and amygdala). A significant decrease in the expression levels of Synaptophysin (*Syp*), a presynaptic vesicle protein abundantly expressed in neurons, was observed in the prefrontal cortex of male Pglyrp2 KO mice compared to controls (**Fig. 29A**). Gephyrin (*Gphn*), a key postsynaptic scaffold protein of inhibitory synapses – involved in the maintenance of the excitatory-inhibitory balance (Sheng and Kim, 2011, Tyagarajan and Fritschy, 2014) – showed a similar expression pattern as *Syp* (**Fig. 29B**). This indicates that male, but not female Pglyrp2 KO mice have a potential excitatory-inhibitory (E-I) imbalance in the prefrontal cortex. *Syp* and *Gphn* mRNA expression levels were decreased in the amygdala of female, but not male Pglyrp2 KO mice (**Fig. 29A** and **B**). The same tendency was observed in the expression of postsynaptic density protein-95 (*Psd-95*), a postsynaptic scaffold protein of excitatory synapses (**Fig. 29C**), which may indicate a

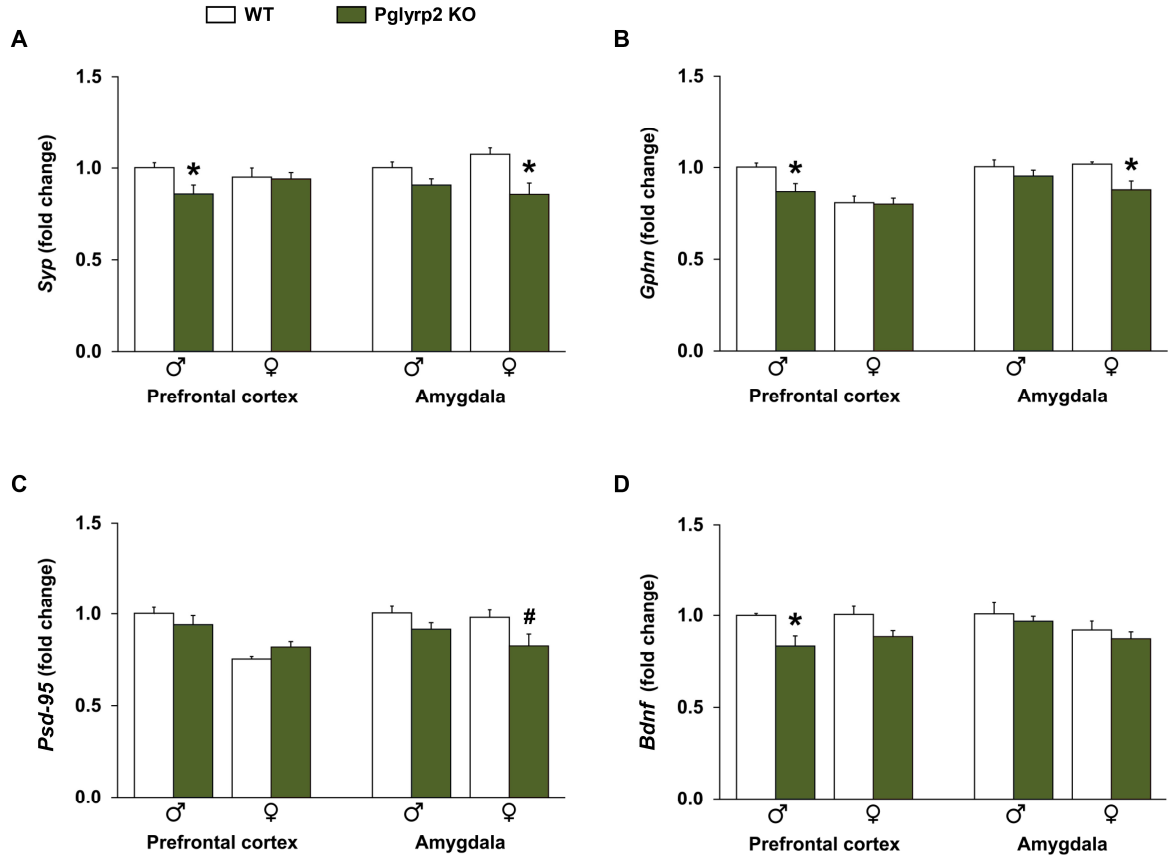

Figure 29. Pglyrp2 KO mice show alterations in the expression of synaptic-plasticity related genes. mRNA expression levels of *Syp* (A), *Gphn* (B), *Psd-95* (C), and *Bdnf* (D) in the prefrontal cortex and amygdala of aged Pglyrp2 KO and WT male and female mice. Expression levels were normalized to Peptidylprolyl isomerase A (*Ppia*) levels. Data are presented as means (± SEM; n = 4−6 per group. $^{\#}P < 0.01$, $^{*}P < 0.05$ when compared with their respective WT control group.

general reduction of synapses in the amygdala of female, but not male, Pglyrp2 KO mice. Further studies are required to investigate this possibility. Together, these results suggest a novel role for Pglyrp2 as a regulator of anxiety-like behavior and transcriptional control of synaptic-related genes.

5 CONCLUSIONS

There is a growing appreciation for the evidence showing that the gut-microbiota can modulate brain development, function and behavior. Sudo and collaborators provided evidence for the existence of an interaction between indigenous microbes and the central nervous system (Sudo et al., 2004). Since this hallmark study, the field of the gut microbiota-brain axis has moved forward and significantly expanded. This thesis focused on the impact of gut microbiota perturbations on brain development and behavior using the well-established GF mouse model in **paper I,** and a more clinically-relevant approach (i.e., prenatal antibiotic treatment model) in **paper II**. In **papers III** and **IV**, some light was shed on a potential novel signaling pathway involved in the communication between the gut microbiota and the brain.

A key finding in **paper I** was that male mice devoid of gut microbiota displayed altered social behavior and showed decreased BDNF expression levels in the amygdala, an important component of the social brain network. These results provide further evidence that the gut microbiota is of importance for normal brain development and social behavior. In **paper II**, it was shown that antibiotic-induced perturbations of the maternal gut microbiota during pregnancy altered the offsprings' motor and social behavior in a sex-specific manner. In parallel, reduced BDNF expression levels were measured in the amygdala which were negatively correlated with social interaction time. These observations indicate that a clinically-relevant dose of antibiotics during key stages of neurodevelopment can have adverse outcomes for offspring brain development and might involve alterations in the BDNF signaling pathway.

In **paper III**, we showed that peptidoglycan (PGN) – a crucial component of the bacterial cell wall – was able to translocate into the healthy brain and sensed by specific pattern recognition receptors (PRRs), such as PGN recognition protein 2 (Pglyrp2). PRRs were found to be highly expressed in the developing brain at specific stages, and their expression was sensitive to perturbations of the gut microbiota. Importantly, Pglyrp2 was expressed by neurons. Juvenile Pglyrp2 knockout (KO) mice were used to investigate the potential influence of Pglyrp2 on brain development and behavior; specifically there was a sex-specific increase in social behavior, without changes in anxiety-like behavior and motor control. These results suggest that activation of PRRs by bacterial PGN, may be one of the signaling pathways mediating the communication between the gut microbiota and the developing brain. **Paper IV** demonstrated that aged Pglyrp2 KO mice displayed sex-dependent alterations in anxiety-like behavior, motor coordination and balance, without changes in social behavior. In addition, aged Pglyrp2 KO male and female mice showed reduced mRNA expression of synaptophysin and gephyrin. These findings reveal a potential role for Pglyrp2 in the regulation of anxiety-like behavior and the expression of synaptic-related genes.

Taken together, the results presented in this thesis further support and expand our understanding of how the gut microbiota may influence brain development, function and behavior.

6 FUTURE PERSPECTIVES

In this thesis, the impact of the gut microbiota was studied mainly within the context of brain development and behavior. Here, a novel signaling pathway was proposed for a bacterial-derived cell wall product, peptidoglycan (PGN) that can cross the blood-brain-barrier into the brain, activate PRRs of the innate immune system, and impact brain function. Different PGN-sensing molecules can be activated by specific PGN fragments, and the assay used to detect PGN molecules in the current work cannot discriminate between different types of PGN molecules, such as muramyl dipeptides or mesoDAP PGN. Therefore, future studies investigating the precise types of PGN molecules that can translocate into the brain are warranted. Recently, several studies have challenged the idea of a sterile intrauterine environment by detecting bacterial DNA in placenta, umbilical cord blood and amniotic fluid from healthy pregnancies in the absence of infections or inflammation (Prince et al., 2015, Aagaard et al., 2014, Jimenez et al., 2005, Bearfield et al., 2002). Hence, it will be interesting to investigate whether PGN from maternal commensal microbiota crosses the placental barrier. Several sophisticated techniques such as liquid chromatography-mass spectrometry, ligand specific assays and PGN specific antibodies can be used for these purposes. This is a step towards elucidating the precise molecular mechanisms that mediate the bi-directional communication between the gut-microbiota and the developing brain. As the work moves forward and translates to the clinic, future work should address levels of PGN in cerebral spinal fluid across different developmental stages in humans.

Further, the effects of PGN molecules on the development, maturation, and homeostasis of brain cells including neurons, microglia, and astrocytes, are poorly understood. *In vitro* cell culture studies may clarify potential effects of different PGN motifs on cell development, morphology, and the CNS immune response via microglia. Moreover, cell culture experiments using siRNAs to knockdown or overexpress PRRs may provide a useful tool to further study potential roles of PRR signaling pathways in the context of brain development and function.

In vivo, it has been shown that PGN fragments derived from the commensal gut microbiota can prime peripheral immune cells (Clarke et al., 2010). Similarly, injections or treatment with specific PGN fragments during key stages of development can provide new insights on the effects of PGN on brain development and function. In this thesis, a total Pglyrp2 knockout model was used. The cre-loxP system can be used to generate tissue specific or inducible knockouts to further investigate developmental roles of PRRs in a brain region or cell specific manner.

It has been shown that the gut microbiota can affect the expression of genes involved in brain development and function such as BDNF and c-MET. Both genes have been implicated in neurodevelopmental disorders and c-MET is specifically associated with co-occurring GI problems in ASD individuals. These observations warrant further studies investigating the influence of the gut microbiota on the expression of crucial genes during key stages of development. Furthermore, the role of the gut microbiota in the etiology and/or

pathophysiology of clinical conditions that include neurodevelopmental and psychiatric disorders, warrants further investigation. Herein, clinical studies are extremely important to fill in the gaps in our knowledge, while different experimental models such as GF mice, antibiotic treatment, prenatal stress models, dietary models, and transgenic models, among others, can also contribute by allowing the investigation of the mechanisms mediating the communication between the gut microbiota and the developing brain. Such a coordinated effort may lead to the development of novel therapeutic intervention for the treatment of neurological and neuropsychiatric diseases.

7 ACKNOWLEDGEMENTS

The journey of a PhD student is a journey that you cannot travel alone. During the years of my PhD studies I received a lot of help, guidance, support, confidence, and love from so many people. I now reached the point where I have the opportunity to thank them.

I would like to begin by thanking my main supervisor **Associate Professor Rochellys Diaz Heijtz**. The moment I came to your lab it felt like home, the right place. From that start we developed an excellent personal and academic relationship. I am truly happy to have enjoyed my PhD training with you. You always showed that you are so much more than "a typical supervisor". In addition to your guidance and support, your enthusiasm for science and involvement in all the projects that we have done together over all these years are really unforgettable. I also want to thank you for encouraging me to develop scientific thinking, analyzing data critically and how to manage project from the general idea to the smallest details. Thank you again for all your support and encouragement during this PhD journey.

Professor Hans Forssberg, I am very happy you were my co-supervisor during my PhD training. You always provided excellent constructive feedback on the projects, the manuscripts, the PhD program itself, which was very helpful. During meetings and discussions, you highlighted the importance of the framework and how to emphasize a clear story. Thank you for your guidance and support!

I am grateful to our collaborators **Associate Professor Jane Foster** and **James St. Pierre** from McMaster in Canada, and **Assistant Professor Klas Udekwu** and **Spyridon Gkotzis** from Stockholm University, for the fruitful discussions and contributions to the papers.

My mentor, **Associate Professor Sara Bengtsson**, thank you for your time, encouragement and support. It was really nice to talk to you from time to time. Thank you so much.

To my great previous and current lab members of the Diaz Heijtz Lab, without you guys my PhD journey would not have been the same. **Sam**, a big thank you for all your support, teaching of techniques and contributions to my projects from day one. I want to thank all the other wonderful guys in the lab: **Mado, Teresa, Despoina, Roksana, Claire, Alfredo, Roxanne, Sophia, Henrike, Malte, Chloe** and **Dorota**. Thank you for making my years in the lab so unforgettable.

My gratitude to the former and present staff of the Department of Neuroscience and the staff at the animal facility, especially **Niklas, Ingela, Helena, Anna** and **Sandra**.

I want to take this opportunity to thank my dear friends and colleagues of the Department of Neuroscience. **Ismael, Woody, the Thursday pancake day gang (Matthijs, Josje, Manideep, Joanna, Anil), Sherez, Swapnali, Firoz, Kuba, Aileen, Dasiel, Sophia, Mansoureh, Selma** and **Konstantina** thank you for all the wonderful lunches, coffees, parties, discussions, MF visits etc.

Babak, Farshad, Arezou, Soheil, Sima, Marzieh and **Salman** I am very grateful to have such wonderful friends in Stockholm. Thanks for all the good times we have had together.

I want to thank all my friends from the Netherlands for all their support, help, fun and laughs over all the years we know each other. During my PhD, you came to visit me in Stockholm or made my trips to the Netherlands so much fun. Thank you, **David, Robert, Steph, Ellen, Timothy, Roderick, Tom, Paul, Bart, Aron, Jacco, Wouter, Erik, Michiel, Janneke, Koen S, Koen vdH** and **Joep**.

Tante Johanna, het is ongelovelijk fijn om te weten dat u mij ondersteund en nog altijd geinteresseerd bent in hetgeen waarmee ik bezig ben. Elke keer wanneer wij elkaar ontmoeten wilt u weten hoe het met promotie gaat. Ik kan vertellen dat het einde nu echt inzicht is, dus ja, ik ben bijna klaar met studeren.

Dear in-laws, **Maman** and **Ghazaleh**-e-azizam thank you so much for being so caring and supportive. It is truly wonderful to have you in my life.

Dear **Dide**, it is so wonderful that you are my sister. I want to thank you for all your support throughout the journey of this PhD. It was always so nice when you came to visit me in Stockholm. Thank you so much.

Lieve **pap** en **mam**, I am deeply grateful that you support me throughout my life. I could not have imagined to be where I am now without your help, encouragement, care and love. Ik ben jullie ontzettend dankbaar dat jullie mij altijd hebben aangemoedigd om zover mogelijk te komen. Zonder die motivatie was ik nooit op dit punt aangekomen. Dat geeft mij een ongelovelijk goed gevoel. Bedankt voor alles. Ik hou van jullie.

Dearest **Laleh**, the support and encouragement you gave me throughout the years of this PhD journey is truly unbelievable. I am extremely grateful that you stood next to me and provided me with so much love, care, support, inspiration and Persian food. Thank you so much for everything! The love you give me is indescribable.

8 REFERENCES

AAGAARD, K., MA, J., ANTONY, K. M., GANU, R., PETROSINO, J. & VERSALOVIC, J. 2014. The Placenta Harbors a Unique Microbiome. *Science translational medicine,* 6**,** 237ra65-237ra65.

ADACHI, N., NUMAKAWA, T., RICHARDS, M., NAKAJIMA, S. & KUNUGI, H. 2014. New insight in expression, transport, and secretion of brain-derived neurotrophic factor: Implications in brain-related diseases. *World J Biol Chem,* 5**,** 409-28.

ADAMS, J. B., JOHANSEN, L. J., POWELL, L. D., QUIG, D. & RUBIN, R. A. 2011. Gastrointestinal flora and gastrointestinal status in children with autism--comparisons to typical children and correlation with autism severity. *BMC Gastroenterol,* 11**,** 22.

AJSLEV, T. A., ANDERSEN, C. S., GAMBORG, M., SORENSEN, T. I. & JESS, T. 2011. Childhood overweight after establishment of the gut microbiota: the role of delivery mode, pre-pregnancy weight and early administration of antibiotics. *Int J Obes (Lond),* 35**,** 522-9.

ARENTSEN, T., QIAN, Y., GKOTZIS, S., FEMENIA, T., WANG, T., UDEKWU, K., FORSSBERG, H. & DIAZ HEIJTZ, R. 2017. The bacterial peptidoglycan-sensing molecule Pglyrp2 modulates brain development and behavior. *Mol Psychiatry,* 22**,** 257-266.

ARENTSEN, T., RAITH, H., QIAN, Y., FORSSBERG, H. & DIAZ HEIJTZ, R. 2015. Host microbiota modulates development of social preference in mice. *Microb Ecol Health Dis,* 26**,** 29719.

ASPIDE, R., GIRONI CARNEVALE, U. A., SERGEANT, J. A. & SADILE, A. G. 1998. Non-selective attention and nitric oxide in putative animal models of Attention-Deficit Hyperactivity Disorder. *Behav Brain Res,* 95**,** 123-33.

ATLADOTTIR, H. O., HENRIKSEN, T. B., SCHENDEL, D. E. & PARNER, E. T. 2012. Autism after infection, febrile episodes, and antibiotic use during pregnancy: an exploratory study. *Pediatrics,* 130**,** e1447-54.

AZAD, M. B., BRIDGMAN, S. L., BECKER, A. B. & KOZYRSKYJ, A. L. 2014. Infant antibiotic exposure and the development of childhood overweight and central adiposity. *Int J Obes (Lond),* 38**,** 1290-8.

BACKHED, F., DING, H., WANG, T., HOOPER, L. V., KOH, G. Y., NAGY, A., SEMENKOVICH, C. F. & GORDON, J. I. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci U S A,* 101**,** 15718-23.

BACKHED, F., MANCHESTER, J. K., SEMENKOVICH, C. F. & GORDON, J. I. 2007. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. *Proc Natl Acad Sci U S A,* 104**,** 979-84.

BEARFIELD, C., DAVENPORT, E. S., SIVAPATHASUNDARAM, V. & ALLAKER, R. P. 2002. Possible association between amniotic fluid micro-organism infection and microflora in the mouth. *Bjog,* 109**,** 527-33.

BENARROCH, E. E. 2015. Brain-derived neurotrophic factor: Regulation, effects, and potential clinical relevance. *Neurology,* 84**,** 1693-1704.

BERCIK, P., DENOU, E., COLLINS, J., JACKSON, W., LU, J., JURY, J., DENG, Y., BLENNERHASSETT, P., MACRI, J., MCCOY, K. D., VERDU, E. F. & COLLINS, S. M. 2011a. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. *Gastroenterology,* 141**,** 599-609, 609 e1-3.

BERCIK, P., PARK, A. J., SINCLAIR, D., KHOSHDEL, A., LU, J., HUANG, X., DENG, Y., BLENNERHASSETT, P. A., FAHNESTOCK, M., MOINE, D., BERGER, B., HUIZINGA, J. D., KUNZE, W., MCLEAN, P. G., BERGONZELLI, G. E., COLLINS, S. M. & VERDU,

E. F. 2011b. The anxiolytic effect of Bifidobacterium longum NCC3001 involves vagal pathways for gut-brain communication. *Neurogastroenterol Motil,* 23**,** 1132-9.

BERG, J. M. & GESCHWIND, D. H. 2012. Autism genetics: searching for specificity and convergence. *Genome Biol,* 13**,** 247.

BERRY, A., PANETTA, P., LUONI, A., BELLISARIO, V., CAPOCCIA, S., RIVA, M. A. & CIRULLI, F. 2015. Decreased Bdnf expression and reduced social behavior in periadolescent rats following prenatal stress. *Dev Psychobiol,* 57**,** 365-73.

BEYDOUN, H. & SAFTLAS, A. F. 2008. Physical and mental health outcomes of prenatal maternal stress in human and animal studies: a review of recent evidence. *Paediatr Perinat Epidemiol,* 22**,** 438-66.

BILBO, S. D., BIEDENKAPP, J. C., DER-AVAKIAN, A., WATKINS, L. R., RUDY, J. W. & MAIER, S. F. 2005. Neonatal Infection-Induced Memory Impairment after Lipopolysaccharide in Adulthood Is Prevented via Caspase-1 Inhibition. *The Journal of Neuroscience,* 25**,** 8000-8009.

BOERSMA, G. J., BALE, T. L., CASANELLO, P., LARA, H. E., LUCION, A. B., SUCHECKI, D. & TAMASHIRO, K. L. 2014. Long-term impact of early life events on physiology and behaviour. *J Neuroendocrinol,* 26**,** 587-602.

BONITO-OLIVA, A., MASINI, D. & FISONE, G. 2014. A mouse model of non-motor symptoms in Parkinson's disease: focus on pharmacological interventions targeting affective dysfunctions. *Front Behav Neurosci,* 8**,** 290.

BORRE, Y. E., MOLONEY, R. D., CLARKE, G., DINAN, T. G. & CRYAN, J. F. 2014. The impact of microbiota on brain and behavior: mechanisms & therapeutic potential. *Adv Exp Med Biol,* 817**,** 373-403.

BRANCHI, I., D'ANDREA, I., FIORE, M., DI FAUSTO, V., ALOE, L. & ALLEVA, E. 2006. Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. *Biol Psychiatry,* 60**,** 690-6.

BRANISTE, V., AL-ASMAKH, M., KOWAL, C., ANUAR, F., ABBASPOUR, A., TOTH, M., KORECKA, A., BAKOCEVIC, N., NG, L. G., KUNDU, P., GULYAS, B., HALLDIN, C., HULTENBY, K., NILSSON, H., HEBERT, H., VOLPE, B. T., DIAMOND, B. & PETTERSSON, S. 2014. The gut microbiota influences blood-brain barrier permeability in mice. *Sci Transl Med,* 6**,** 263ra158.

BRANTON, W. G., LU, J. Q., SURETTE, M. G., HOLT, R. A., LIND, J., LAMAN, J. D. & POWER, C. 2016. Brain microbiota disruption within inflammatory demyelinating lesions in multiple sclerosis. *Sci Rep,* 6**,** 37344.

BRAVO, J. A., FORSYTHE, P., CHEW, M. V., ESCARAVAGE, E., SAVIGNAC, H. M., DINAN, T. G., BIENENSTOCK, J. & CRYAN, J. F. 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proc Natl Acad Sci U S A,* 108**,** 16050-5.

BRETON, J., TENNOUNE, N., LUCAS, N., FRANCOIS, M., LEGRAND, R., JACQUEMOT, J., GOICHON, A., GUERIN, C., PELTIER, J., PESTEL-CARON, M., CHAN, P., VAUDRY, D., DO REGO, J. C., LIENARD, F., PENICAUD, L., FIORAMONTI, X., EBENEZER, I. S., HOKFELT, T., DECHELOTTE, P. & FETISSOV, S. O. 2016. Gut Commensal E. coli Proteins Activate Host Satiety Pathways following Nutrient-Induced Bacterial Growth. *Cell Metab,* 23**,** 324-34.

BUFFINGTON, S. A., DI PRISCO, G. V., AUCHTUNG, T. A., AJAMI, N. J., PETROSINO, J. F. & COSTA-MATTIOLI, M. 2016. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell,* 165**,** 1762-75.

BUROKAS, A., MOLONEY, R. D., DINAN, T. G. & CRYAN, J. F. 2015. Microbiota regulation of the Mammalian gut-brain axis. *Adv Appl Microbiol,* 91**,** 1-62.

CAMERON, J. & DOUCET, E. 2007. Getting to the bottom of feeding behaviour: who's on top? *Appl Physiol Nutr Metab,* 32**,** 177-89.

CAMPBELL, D. B., BUIE, T. M., WINTER, H., BAUMAN, M., SUTCLIFFE, J. S., PERRIN, J. M. & LEVITT, P. 2009. Distinct genetic risk based on association of MET in families with co-occurring autism and gastrointestinal conditions. *Pediatrics,* 123**,** 1018-24.

CAMPBELL, D. B., D'ORONZIO, R., GARBETT, K., EBERT, P. J., MIRNICS, K., LEVITT, P. & PERSICO, A. M. 2007. Disruption of cerebral cortex MET signaling in autism spectrum disorder. *Ann Neurol,* 62**,** 243-50.

CARUSO, R., WARNER, N., INOHARA, N. & NÚÑEZ, G. 2014. NOD1 and NOD2: Signaling, Host Defense, and Inflammatory Disease. *Immunity,* 41**,** 898-908.

CASTREN, E. 2014. Neurotrophins and psychiatric disorders. *Handb Exp Pharmacol,* 220**,** 461-79.

CERVI, A. L., LUKEWICH, M. K. & LOMAX, A. E. 2014. Neural regulation of gastrointestinal inflammation: role of the sympathetic nervous system. *Auton Neurosci,* 182**,** 83-8.

CHAUHAN, V. S., STERKA, D. G., JR., FURR, S. R., YOUNG, A. B. & MARRIOTT, I. 2009. NOD2 plays an important role in the inflammatory responses of microglia and astrocytes to bacterial CNS pathogens. *Glia,* 57**,** 414-23.

CHU, H. & MAZMANIAN, S. K. 2013. Innate immune recognition of the microbiota promotes host-microbial symbiosis. *Nat Immunol,* 14**,** 668-75.

CLARKE, G., GRENHAM, S., SCULLY, P., FITZGERALD, P., MOLONEY, R. D., SHANAHAN, F., DINAN, T. G. & CRYAN, J. F. 2013. The microbiome-gut-brain axis during early life regulates the hippocampal serotonergic system in a sex-dependent manner. *Mol Psychiatry,* 18**,** 666-73.

CLARKE, T. B., DAVIS, K. M., LYSENKO, E. S., ZHOU, A. Y., YU, Y. & WEISER, J. N. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med,* 16**,** 228-31.

COLLINS, S. M., SURETTE, M. & BERCIK, P. 2012. The interplay between the intestinal microbiota and the brain. *Nat Rev Microbiol,* 10**,** 735-42.

COURY, D. L., ASHWOOD, P., FASANO, A., FUCHS, G., GERAGHTY, M., KAUL, A., MAWE, G., PATTERSON, P. & JONES, N. E. 2012. Gastrointestinal conditions in children with autism spectrum disorder: developing a research agenda. *Pediatrics,* 130 Suppl 2**,** S160-8.

COX, L. M. & BLASER, M. J. 2015a. Antibiotics in early life and obesity. *Nat Rev Endocrinol,* 11**,** 182-90.

COX, L. M. & BLASER, M. J. 2015b. Antibiotics in early life and obesity. *Nat Rev Endocrinol,* 11**,** 182-190.

CRITCHFIELD, J. W., VAN HEMERT, S., ASH, M., MULDER, L. & ASHWOOD, P. 2011. The potential role of probiotics in the management of childhood autism spectrum disorders. *Gastroenterol Res Pract,* 2011**,** 161358.

CRUMEYROLLE-ARIAS, M., JAGLIN, M., BRUNEAU, A., VANCASSEL, S., CARDONA, A., DAUGE, V., NAUDON, L. & RABOT, S. 2014. Absence of the gut microbiota enhances anxiety-like behavior and neuroendocrine response to acute stress in rats. *Psychoneuroendocrinology,* 42**,** 207-17.

CRYAN, J. F. & DINAN, T. G. 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci,* 13**,** 701-12.

DESBONNET, L., CLARKE, G., SHANAHAN, F., DINAN, T. G. & CRYAN, J. F. 2014. Microbiota is essential for social development in the mouse. *Mol Psychiatry,* 19**,** 146-8.

DESBONNET, L., CLARKE, G., TRAPLIN, A., O'SULLIVAN, O., CRISPIE, F., MOLONEY, R. D., COTTER, P. D., DINAN, T. G. & CRYAN, J. F. 2015a. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain Behav Immun*.

DESBONNET, L., CLARKE, G., TRAPLIN, A., O'SULLIVAN, O., CRISPIE, F., MOLONEY, R. D., COTTER, P. D., DINAN, T. G. & CRYAN, J. F. 2015b. Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, Behavior, and Immunity,* 48**,** 165-173.

DESHMUKH, H. S., LIU, Y., MENKITI, O. R., MEI, J., DAI, N., O'LEARY, C. E., OLIVER, P. M., KOLLS, J. K., WEISER, J. N. & WORTHEN, G. S. 2014. The microbiota regulates neutrophil homeostasis and host resistance to Escherichia coli K1 sepsis in neonatal mice. *Nat Med,* 20**,** 524-30.

DIAZ HEIJTZ, R. 2016. Fetal, neonatal, and infant microbiome: Perturbations and subsequent effects on brain development and behavior. *Semin Fetal Neonatal Med,* 21**,** 410-417.

DIAZ HEIJTZ, R., WANG, S., ANUAR, F., QIAN, Y., BJORKHOLM, B., SAMUELSSON, A., HIBBERD, M. L., FORSSBERG, H. & PETTERSSON, S. 2011. Normal gut microbiota modulates brain development and behavior. *Proc Natl Acad Sci U S A,* 108**,** 3047-52.

DOMINGUEZ-BELLO, M. G., BLASER, M. J., LEY, R. E. & KNIGHT, R. 2011. Development of the human gastrointestinal microbiota and insights from high-throughput sequencing. *Gastroenterology,* 140**,** 1713-9.

DZIARSKI, R. 2003. Recognition of bacterial peptidoglycan by the innate immune system. *Cell Mol Life Sci,* 60**,** 1793-804.

DZIARSKI, R. & GUPTA, D. 2006. The peptidoglycan recognition proteins (PGRPs). *Genome Biol,* 7**,** 232.

DZIARSKI, R. & GUPTA, D. 2010. Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. *Innate Immun,* 16**,** 168-74.

DZIARSKI, R., KASHYAP, D. R. & GUPTA, D. 2012. Mammalian peptidoglycan recognition proteins kill bacteria by activating two-component systems and modulate microbiome and inflammation. *Microb Drug Resist,* 18**,** 280-5.

EGGESBO, M., MANDAL, S. & MIDTVEDT, T. 2015. Factors affecting infant gut microbiota and possible consequences for health. *Microb Ecol Health Dis,* 26**,** 28062.

ERNY, D., HRABE DE ANGELIS, A. L., JAITIN, D., WIEGHOFER, P., STASZEWSKI, O., DAVID, E., KEREN-SHAUL, H., MAHLAKOIV, T., JAKOBSHAGEN, K., BUCH, T., SCHWIERZECK, V., UTERMOHLEN, O., CHUN, E., GARRETT, W. S., MCCOY, K. D., DIEFENBACH, A., STAEHELI, P., STECHER, B., AMIT, I. & PRINZ, M. 2015. Host microbiota constantly control maturation and function of microglia in the CNS. *Nat Neurosci,* 18**,** 965-77.

FAIRLESS, A. H., DOW, H. C., KREIBICH, A. S., TORRE, M., KURUVILLA, M., GORDON, E., MORTON, E. A., TAN, J., BERRETTINI, W. H., LI, H., ABEL, T. & BRODKIN, E. S. 2012. Sociability and brain development in BALB/cJ and C57BL/6J mice. *Behav Brain Res,* 228**,** 299-310.

FINEGOLD, S. M., DOWD, S. E., GONTCHAROVA, V., LIU, C., HENLEY, K. E., WOLCOTT, R. D., YOUN, E., SUMMANEN, P. H., GRANPEESHEH, D., DIXON, D., LIU, M., MOLITORIS, D. R. & GREEN, J. A., 3RD 2010. Pyrosequencing study of fecal microflora of autistic and control children. *Anaerobe,* 16**,** 444-53.

FORSYTHE, P., BIENENSTOCK, J. & KUNZE, W. A. 2014. Vagal pathways for microbiome-brain-gut axis communication. *Adv Exp Med Biol,* 817**,** 115-33.

FORSYTHE, P. & KUNZE, W. A. 2013. Voices from within: gut microbes and the CNS. *Cell Mol Life Sci,* 70**,** 55-69.

FOUHY, F., GUINANE, C. M., HUSSEY, S., WALL, R., RYAN, C. A., DEMPSEY, E. M., MURPHY, B., ROSS, R. P., FITZGERALD, G. F., STANTON, C. & COTTER, P. D. 2012. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob Agents Chemother,* 56**,** 5811-20.

FROHLICH, E. E., FARZI, A., MAYERHOFER, R., REICHMANN, F., JACAN, A., WAGNER, B., ZINSER, E., BORDAG, N., MAGNES, C., FROHLICH, E., KASHOFER, K., GORKIEWICZ, G. & HOLZER, P. 2016. Cognitive impairment by antibiotic-induced gut dysbiosis: Analysis of gut microbiota-brain communication. *Brain Behav Immun,* 56**,** 140-55.

GAREAU, M. G., WINE, E., RODRIGUES, D. M., CHO, J. H., WHARY, M. T., PHILPOTT, D. J., MACQUEEN, G. & SHERMAN, P. M. 2011. Bacterial infection causes stress-induced memory dysfunction in mice. *Gut,* 60**,** 307-17.

GILL, S. R., POP, M., DEBOY, R. T., ECKBURG, P. B., TURNBAUGH, P. J., SAMUEL, B. S., GORDON, J. I., RELMAN, D. A., FRASER-LIGGETT, C. M. & NELSON, K. E. 2006. Metagenomic analysis of the human distal gut microbiome. *Science,* 312**,** 1355-9.

GOLDMAN, S. M., KAMEL, F., ROSS, G. W., JEWELL, S. A., MARRAS, C., HOPPIN, J. A., UMBACH, D. M., BHUDHIKANOK, G. S., MENG, C., KORELL, M., COMYNS, K., HAUSER, R. A., JANKOVIC, J., FACTOR, S. A., BRESSMAN, S., LYONS, K. E., SANDLER, D. P., LANGSTON, J. W. & TANNER, C. M. 2014. Peptidoglycan recognition protein genes and risk of Parkinson's disease. *Mov Disord,* 29**,** 1171-80.

GUAN, R., MALCHIODI, E. L., WANG, Q., SCHUCK, P. & MARIUZZA, R. A. 2004a. Crystal structure of the C-terminal peptidoglycan-binding domain of human peptidoglycan recognition protein Ialpha. *J Biol Chem,* 279**,** 31873-82.

GUAN, R., ROYCHOWDHURY, A., EMBER, B., KUMAR, S., BOONS, G. J. & MARIUZZA, R. A. 2004b. Structural basis for peptidoglycan binding by peptidoglycan recognition proteins. *Proc Natl Acad Sci U S A,* 101**,** 17168-73.

GUAN, R., ROYCHOWDURY, A., EMBER, B., KUMAR, S., BOONS, G. J. & MARIUZZA, R. A. 2005. Crystal structure of a peptidoglycan recognition protein (PGRP) in complex with a muramyl tripeptide from Gram-positive bacteria. *J Endotoxin Res,* 11**,** 41-6.

HALLMAYER, J., CLEVELAND, S., TORRES, A., PHILLIPS, J., COHEN, B., TORIGOE, T., MILLER, J., FEDELE, A., COLLINS, J., SMITH, K., LOTSPEICH, L., CROEN, L. A., OZONOFF, S., LAJONCHERE, C., GRETHER, J. K. & RISCH, N. 2011. Genetic heritability and shared environmental factors among twin pairs with autism. *Arch Gen Psychiatry,* 68**,** 1095-102.

HARRIS, N., BRAISER, D. J., DICKMAN, D. K., FETTER, R. D., TONG, A. & DAVIS, G. W. 2015. The Innate Immune Receptor PGRP-LC Controls Presynaptic Homeostatic Plasticity. *Neuron,* 88**,** 1157-64.

HEDRICK, A., LEE, Y., WALLACE, G. L., GREENSTEIN, D., CLASEN, L., GIEDD, J. N. & RAZNAHAN, A. 2012. Autism risk gene MET variation and cortical thickness in typically developing children and adolescents. *Autism Res,* 5**,** 434-9.

HOBAN, A. E., MOLONEY, R. D., GOLUBEVA, A. V., MCVEY NEUFELD, K. A., O'SULLIVAN, O., PATTERSON, E., STANTON, C., DINAN, T. G., CLARKE, G. & CRYAN, J. F. 2016a. Behavioural and neurochemical consequences of chronic gut microbiota depletion during adulthood in the rat. *Neuroscience,* 339**,** 463-477.

HOBAN, A. E., STILLING, R. M., RYAN, F. J., SHANAHAN, F., DINAN, T. G., CLAESSON, M. J., CLARKE, G. & CRYAN, J. F. 2016b. Regulation of prefrontal cortex myelination by the microbiota. *Transl Psychiatry,* 6**,** e774.

HONDA, K. & TAKEDA, K. 2009. Regulatory mechanisms of immune responses to intestinal bacteria. *Mucosal Immunol,* 2**,** 187-96.

HOOPER, L. V. & GORDON, J. I. 2001. Commensal host-bacterial relationships in the gut. *Science,* 292**,** 1115-8.

HOOPER, L. V., LITTMAN, D. R. & MACPHERSON, A. J. 2012. Interactions between the microbiota and the immune system. *Science,* 336**,** 1268-73.

HSIAO, E. Y. 2014. Gastrointestinal issues in autism spectrum disorder. *Harv Rev Psychiatry,* 22**,** 104-11.

HSIAO, E. Y., MCBRIDE, S. W., HSIEN, S., SHARON, G., HYDE, E. R., MCCUE, T., CODELLI, J. A., CHOW, J., REISMAN, S. E., PETROSINO, J. F., PATTERSON, P. H. & MAZMANIAN, S. K. 2013. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell,* 155**,** 1451-63.

HUMANMICROBIOMEPROJECT 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486**,** 207-14.

INOHARA, N., OGURA, Y., FONTALBA, A., GUTIERREZ, O., PONS, F., CRESPO, J., FUKASE, K., INAMURA, S., KUSUMOTO, S., HASHIMOTO, M., FOSTER, S. J., MORAN, A. P., FERNANDEZ-LUNA, J. L. & NUNEZ, G. 2003. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem,* 278**,** 5509-12.

IRVING, A. T., MIMURO, H., KUFER, T. A., LO, C., WHEELER, R., TURNER, L. J., THOMAS, B. J., MALOSSE, C., GANTIER, M. P., CASILLAS, L. N., VOTTA, B. J., BERTIN, J., BONECA, I. G., SASAKAWA, C., PHILPOTT, D. J., FERRERO, R. L. & KAPARAKIS-LIASKOS, M. 2014. The immune receptor NOD1 and kinase RIP2 interact with bacterial peptidoglycan on early endosomes to promote autophagy and inflammatory signaling. *Cell Host Microbe,* 15**,** 623-35.

JAKOBSSON, H. E., ABRAHAMSSON, T. R., JENMALM, M. C., HARRIS, K., QUINCE, C., JERNBERG, C., BJORKSTEN, B., ENGSTRAND, L. & ANDERSSON, A. F. 2014. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by caesarean section. *Gut,* 63**,** 559-66.

JIMENEZ, E., FERNANDEZ, L., MARIN, M. L., MARTIN, R., ODRIOZOLA, J. M., NUENO-PALOP, C., NARBAD, A., OLIVARES, M., XAUS, J. & RODRIGUEZ, J. M. 2005. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. *Curr Microbiol,* 51**,** 270-4.

JING, X., ZULFIQAR, F., PARK, S. Y., NUNEZ, G., DZIARSKI, R. & GUPTA, D. 2014. Peptidoglycan recognition protein 3 and Nod2 synergistically protect mice from dextran sodium sulfate-induced colitis. *J Immunol,* 193**,** 3055-69.

KAPARAKIS-LIASKOS, M. 2015. The intracellular location, mechanisms and outcomes of NOD1 signaling. *Cytokine,* 74**,** 207-12.

KAPPELER, S. R., HEUBERGER, C., FARAH, Z. & PUHAN, Z. 2004. Expression of the peptidoglycan recognition protein, PGRP, in the lactating mammary gland. *J Dairy Sci,* 87**,** 2660-8.

KASHYAP, D. R., ROMPCA, A., GABALLA, A., HELMANN, J. D., CHAN, J., CHANG, C. J., HOZO, I., GUPTA, D. & DZIARSKI, R. 2014. Peptidoglycan recognition proteins kill bacteria by inducing oxidative, thiol, and metal stress. *PLoS Pathog,* 10**,** e1004280.

KHARIV, V., PANG, K., SERVATIUS, R. J., DAVID, B. T., GOODUS, M. T., BECK, K. D., HEARY, R. F. & ELKABES, S. 2013. Toll-like receptor 9 deficiency impacts sensory and motor behaviors. *Brain Behav Immun,* 32**,** 164-72.

KHORUTS, A. & WEINGARDEN, A. R. 2014. Emergence of fecal microbiota transplantation as an approach to repair disrupted microbial gut ecology. *Immunol Lett,* 162**,** 77-81.

KOSTIC, A. D., HOWITT, M. R. & GARRETT, W. S. 2013. Exploring host-microbiota interactions in animal models and humans. *Genes Dev,* 27**,** 701-18.

KRUEGER, J. M., KARNOVSKY, M. L., MARTIN, S. A., PAPPENHEIMER, J. R., WALTER, J. & BIEMANN, K. 1984. Peptidoglycans as promoters of slow-wave sleep. II. Somnogenic and pyrogenic activities of some naturally occurring muramyl peptides; correlations with mass spectrometric structure determination. *J Biol Chem,* 259**,** 12659-62.

KUBO, Y., YANAGAWA, Y., MATSUMOTO, M., HIRAIDE, S. & TOGASHI, H. 2013. Enhanced depressive-like behaviors after Toll-like receptor 7 stimulation in mice. *Nihon Shinkei Seishin Yakurigaku Zasshi,* 33**,** 41-7.

LANG, M. F., SCHNEIDER, A., KRUGER, C., SCHMID, R., DZIARSKI, R. & SCHWANINGER, M. 2008. Peptidoglycan recognition protein-S (PGRP-S) is upregulated by NF-kappaB. *Neurosci Lett,* 430**,** 138-41.

LATHIA, J. D., OKUN, E., TANG, S. C., GRIFFIOEN, K., CHENG, A., MUGHAL, M. R., LARYEA, G., SELVARAJ, P. K., FFRENCH-CONSTANT, C., MAGNUS, T., ARUMUGAM, T. V. & MATTSON, M. P. 2008. Toll-like receptor 3 is a negative regulator of embryonic neural progenitor cell proliferation. *J Neurosci,* 28**,** 13978-84.

LIU, C., XU, Z., GUPTA, D. & DZIARSKI, R. 2001. Peptidoglycan recognition proteins: a novel family of four human innate immunity pattern recognition molecules. *J Biol Chem,* 276**,** 34686-94.

LIU, X., HAN, Q. & LENG, J. 2014. Analysis of nucleotide-binding oligomerization domain proteins in a murine model of pneumococcal meningitis. *BMC Infectious Diseases,* 14**,** 648.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

LUCZYNSKI, P., MCVEY NEUFELD, K. A., ORIACH, C. S., CLARKE, G., DINAN, T. G. & CRYAN, J. F. 2016. Growing up in a Bubble: Using Germ-Free Animals to Assess the Influence of the Gut Microbiota on Brain and Behavior. *Int J Neuropsychopharmacol,* 19.

MA, Y., LI, J., CHIU, I., WANG, Y., SLOANE, J. A., LU, J., KOSARAS, B., SIDMAN, R. L., VOLPE, J. J. & VARTANIAN, T. 2006. Toll-like receptor 8 functions as a negative regulator of neurite outgrowth and inducer of neuronal apoptosis. *J Cell Biol,* 175**,** 209-15.

MACFABE, D. F., CAIN, D. P., RODRIGUEZ-CAPOTE, K., FRANKLIN, A. E., HOFFMAN, J. E., BOON, F., TAYLOR, A. R., KAVALIERS, M. & OSSENKOPP, K. P. 2007. Neurobiological effects of intraventricular propionic acid in rats: possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders. *Behav Brain Res,* 176**,** 149-69.

MACFABE, D. F., CAIN, N. E., BOON, F., OSSENKOPP, K. P. & CAIN, D. P. 2011. Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder. *Behav Brain Res,* 217**,** 47-54.

MARTIN, S. A., KARNOVSKY, M. L., KRUEGER, J. M., PAPPENHEIMER, J. R. & BIEMANN, K. 1984. Peptidoglycans as promoters of slow-wave sleep. I. Structure of the sleep-promoting factor isolated from human urine. *J Biol Chem,* 259**,** 12652-8.

MAYER, E. A. 2011. Gut feelings: the emerging biology of gut-brain communication. *Nat Rev Neurosci,* 12**,** 453-66.

MAYER, E. A., TILLISCH, K. & GUPTA, A. 2015. Gut/brain axis and the microbiota. *J Clin Invest,* 125**,** 926-38.

METSALA, J., LUNDQVIST, A., VIRTA, L. J., KAILA, M., GISSLER, M. & VIRTANEN, S. M. 2015. Prenatal and post-natal exposure to antibiotics and risk of asthma in childhood. *Clin Exp Allergy,* 45**,** 137-45.

MIDTVEDT, T. 1997. Germ-free and conventional animal models for intestinal carbohydrate disposition. *Scand J Gastroenterol Suppl,* 222**,** 25-7.

MOHLE, L., MATTEI, D., HEIMESAAT, M. M., BERESWILL, S., FISCHER, A., ALUTIS, M., FRENCH, T., HAMBARDZUMYAN, D., MATZINGER, P., DUNAY, I. R. & WOLF, S. A. 2016. Ly6C(hi) Monocytes Provide a Link between Antibiotic-Induced Changes in Gut Microbiota and Adult Hippocampal Neurogenesis. *Cell Rep,* 15**,** 1945-56.

MUELLER, N. T., BAKACS, E., COMBELLICK, J., GRIGORYAN, Z. & DOMINGUEZ-BELLO, M. G. 2015a. The infant microbiome development: mom matters. *Trends Mol Med,* 21**,** 109-17.

MUELLER, N. T., WHYATT, R., HOEPNER, L., OBERFIELD, S., DOMINGUEZ-BELLO, M. G., WIDEN, E. M., HASSOUN, A., PERERA, F. & RUNDLE, A. 2015b. Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. *Int J Obes,* 39**,** 665-670.

MUELLER, N. T., WHYATT, R., HOEPNER, L., OBERFIELD, S., DOMINGUEZ-BELLO, M. G., WIDEN, E. M., HASSOUN, A., PERERA, F. & RUNDLE, A. 2015c. Prenatal exposure to antibiotics, cesarean section and risk of childhood obesity. *Int J Obes (Lond),* 39**,** 665-70.

MURPHY, R., STEWART, A. W., BRAITHWAITE, I., BEASLEY, R., HANCOX, R. J. & MITCHELL, E. A. 2014. Antibiotic treatment during infancy and increased body mass index in boys: an international cross-sectional study. *Int J Obes,* 38**,** 1115-1119.

NEUFELD, K. A., KANG, N., BIENENSTOCK, J. & FOSTER, J. A. 2011a. Effects of intestinal microbiota on anxiety-like behavior. *Commun Integr Biol,* 4**,** 492-4.

NEUFELD, K. M., KANG, N., BIENENSTOCK, J. & FOSTER, J. A. 2011b. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterol Motil,* 23**,** 255-64, e119.

OGBONNAYA, E. S., CLARKE, G., SHANAHAN, F., DINAN, T. G., CRYAN, J. F. & O'LEARY, O. F. 2015. Adult Hippocampal Neurogenesis Is Regulated by the Microbiome. *Biol Psychiatry*.

OKUN, E., BARAK, B., SAADA-MADAR, R., ROTHMAN, S. M., GRIFFIOEN, K. J., ROBERTS, N., CASTRO, K., MUGHAL, M. R., PITA, M. A., STRANAHAN, A. M., ARUMUGAM, T. V. & MATTSON, M. P. 2012. Evidence for a developmental role for TLR4 in learning and memory. *PLoS One,* 7**,** e47522.

OKUN, E., GRIFFIOEN, K., BARAK, B., ROBERTS, N. J., CASTRO, K., PITA, M. A., CHENG, A., MUGHAL, M. R., WAN, R., ASHERY, U. & MATTSON, M. P. 2010a. Toll-like receptor 3 inhibits memory retention and constrains adult hippocampal neurogenesis. *Proc Natl Acad Sci U S A,* 107**,** 15625-30.

OKUN, E., GRIFFIOEN, K. J. & MATTSON, M. P. 2011. Toll-like receptor signaling in neural plasticity and disease. *Trends Neurosci,* 34**,** 269-81.

OKUN, E., GRIFFIOEN, K. J., SON, T. G., LEE, J. H., ROBERTS, N. J., MUGHAL, M. R., HUTCHISON, E., CHENG, A., ARUMUGAM, T. V., LATHIA, J. D., VAN PRAAG, H. & MATTSON, M. P. 2010b. TLR2 activation inhibits embryonic neural progenitor cell proliferation. *J Neurochem,* 114**,** 462-74.

PARK, S. J., LEE, J. Y., KIM, S. J., CHOI, S. Y., YUNE, T. Y. & RYU, J. H. 2015. Toll-like receptor-2 deficiency induces schizophrenia-like behaviors in mice. *Sci Rep,* 5**,** 8502.

PENG, Y., HUENTELMAN, M., SMITH, C. & QIU, S. 2013. MET receptor tyrosine kinase as an autism genetic risk factor. *Int Rev Neurobiol,* 113**,** 135-65.

PETNICKI-OCWIEJA, T., HRNCIR, T., LIU, Y. J., BISWAS, A., HUDCOVIC, T., TLASKALOVA-HOGENOVA, H. & KOBAYASHI, K. S. 2009. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A,* 106**,** 15813-8.

PHILPOTT, D. J., SORBARA, M. T., ROBERTSON, S. J., CROITORU, K. & GIRARDIN, S. E. 2014. NOD proteins: regulators of inflammation in health and disease. *Nat Rev Immunol,* 14**,** 9-23.

POMARE, E. W., BRANCH, W. J. & CUMMINGS, J. H. 1985. Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood. *J Clin Invest,* 75**,** 1448-54.

PRINCE, A. L., CHU, D. M., SEFEROVIC, M. D., ANTONY, K. M., MA, J. & AAGAARD, K. M. 2015. The perinatal microbiome and pregnancy: moving beyond the vaginal microbiome. *Cold Spring Harb Perspect Med,* 5.

QIAN, Y., FORSSBERG, H. & DIAZ HEIJTZ, R. 2015. Motor Skill Learning Is Associated with Phase-Dependent Modifications in the Striatal cAMP/PKA/DARPP-32 Signaling Pathway in Rodents. *PLoS One,* 10**,** e0140974.

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F., YAMADA, T., MENDE, D. R., LI, J., XU, J., LI, S., LI, D., CAO, J., WANG, B., LIANG, H., ZHENG, H., XIE, Y., TAP, J., LEPAGE, P., BERTALAN, M., BATTO, J.-M., HANSEN, T., LE PASLIER, D., LINNEBERG, A., NIELSEN, H. B., PELLETIER, E., RENAULT, P., SICHERITZ-PONTEN, T., TURNER, K., ZHU, H., YU, C., LI, S., JIAN, M., ZHOU, Y., LI, Y., ZHANG, X., LI, S., QIN, N., YANG, H., WANG, J., BRUNAK, S., DORÉ, J., GUARNER, F., KRISTIANSEN, K., PEDERSEN, O., PARKHILL, J., WEISSENBACH, J., META, H. I. T. C., BORK, P., EHRLICH, S. D. & WANG, J. 2010. A human gut microbial gene catalog established by metagenomic sequencing. *Nature,* 464**,** 59-65.

QIN, X., FENG, J., CAO, C., WU, H., LOH, Y. & CHENG, Y. 2016. Association of peripheral blood levels of brain-derived neurotrophic factor with autism spectrum disorder in children: A systematic review and meta-analysis. *JAMA Pediatrics,* 170**,** 1079-1086.

QIU, S., LU, Z. & LEVITT, P. 2014. MET receptor tyrosine kinase controls dendritic complexity, spine morphogenesis, and glutamatergic synapse maturation in the hippocampus. *J Neurosci,* 34**,** 16166-79.

QUIGLEY, M. A., HOCKLEY, C., CARSON, C., KELLY, Y., RENFREW, M. J. & SACKER, A. 2012. Breastfeeding is Associated with Improved Child Cognitive Development: A Population-Based Cohort Study. *The Journal of Pediatrics,* 160**,** 25-32.

REHMAN, A., TAISHI, P., FANG, J., MAJDE, J. A. & KRUEGER, J. M. 2001. The cloning of a rat peptidoglycan recognition protein (PGRP) and its induction in brain by sleep deprivation. *Cytokine,* 13**,** 8-17.

REYNIERS, J. A. 1959. The pure culture concept and gnotobiotics. . *Ann NY Acad Sci. ,* 78(1)**,** 3–16.

RODRIGUEZ, J. M., MURPHY, K., STANTON, C., ROSS, R. P., KOBER, O. I., JUGE, N., AVERSHINA, E., RUDI, K., NARBAD, A., JENMALM, M. C., MARCHESI, J. R. & COLLADO, M. C. 2015. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis,* 26**,** 26050.

ROUND, J. L. & MAZMANIAN, S. K. 2009. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol,* 9**,** 313-23.

ROYET, J., GUPTA, D. & DZIARSKI, R. 2011. Peptidoglycan recognition proteins: modulators of the microbiome and inflammation. *Nat Rev Immunol,* 11**,** 837-51.

RUDIE, J. D., HERNANDEZ, L. M., BROWN, J. A., BECK-PANCER, D., COLICH, N. L., GORRINDO, P., THOMPSON, P. M., GESCHWIND, D. H., BOOKHEIMER, S. Y.,

LEVITT, P. & DAPRETTO, M. 2012. Autism-associated promoter variant in MET impacts functional and structural brain networks. *Neuron,* 75**,** 904-15.

RUSSO, A. J., KRIGSMAN, A., JEPSON, B. & WAKEFIELD, A. 2009. Decreased Serum Hepatocyte Growth Factor (HGF) in Autistic Children with Severe Gastrointestinal Disease. *Biomark Insights,* 4**,** 181-90.

SAHA, S., JING, X., PARK, S. Y., WANG, S., LI, X., GUPTA, D. & DZIARSKI, R. 2010. Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon-gamma. *Cell Host Microbe,* 8**,** 147-62.

SAMPSON, T. R., DEBELIUS, J. W., THRON, T., JANSSEN, S., SHASTRI, G. G., ILHAN, Z. E., CHALLIS, C., SCHRETTER, C. E., ROCHA, S., GRADINARU, V., CHESSELET, M. F., KESHAVARZIAN, A., SHANNON, K. M., KRAJMALNIK-BROWN, R., WITTUNG-STAFSHEDE, P., KNIGHT, R. & MAZMANIAN, S. K. 2016. Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. *Cell,* 167**,** 1469-1480.e12.

SAMPSON, T. R. & MAZMANIAN, S. K. 2015. Control of Brain Development, Function, and Behavior by the Microbiome. *Cell Host Microbe,* 17**,** 565-576.

SATO, A., MIZUGUCHI, M. & IKEDA, K. 2013. Social interaction test: a sensitive method for examining autism-related behavioral deficits.

SAVIGNAC, H. M., KIELY, B., DINAN, T. G. & CRYAN, J. F. 2014. Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice. *Neurogastroenterol Motil,* 26**,** 1615-27.

SAVIGNAC, H. M., TRAMULLAS, M., KIELY, B., DINAN, T. G. & CRYAN, J. F. 2015. Bifidobacteria modulate cognitive processes in an anxious mouse strain. *Behav Brain Res,* 287**,** 59-72.

SCHLEIFER, K. H. & KANDLER, O. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev,* 36**,** 407-77.

SCHRIJVER, I. A., VAN MEURS, M., MELIEF, M. J., WIM ANG, C., BULJEVAC, D., RAVID, R., HAZENBERG, M. P. & LAMAN, J. D. 2001. Bacterial peptidoglycan and immune reactivity in the central nervous system in multiple sclerosis. *Brain,* 124**,** 1544-54.

SEKIROV, I., RUSSELL, S. L., ANTUNES, L. C. & FINLAY, B. B. 2010. Gut microbiota in health and disease. *Physiol Rev,* 90**,** 859-904.

SEN, Z. & KARNOVSKY, M. L. 1984. Qualitative detection of muramic acid in normal mammalian tissues. *Infect Immun,* 43**,** 937-41.

SENDER, R., FUCHS, S. & MILO, R. 2016. Revised estimates for the number of human and bacteria cells in the body. *bioRxiv*.

SHARMA, R., SCHUMACHER, U., RONAASEN, V. & COATES, M. 1995. Rat intestinal mucosal responses to a microbial flora and different diets. *Gut,* 36**,** 209-14.

SHARON, G., SAMPSON, T. R., GESCHWIND, D. H. & MAZMANIAN, S. K. 2016. The Central Nervous System and the Gut Microbiome. *Cell,* 167**,** 915-932.

SHECHTER, R., RONEN, A., ROLLS, A., LONDON, A., BAKALASH, S., YOUNG, M. J. & SCHWARTZ, M. 2008. Toll-like receptor 4 restricts retinal progenitor cell proliferation. *J Cell Biol,* 183**,** 393-400.

SHENG, M. & KIM, E. 2011. The postsynaptic organization of synapses. *Cold Spring Harb Perspect Biol,* 3.

SHIAU, CELIA E., MONK, KELLY R., JOO, W. & TALBOT, WILLIAM S. 2013. An Anti-inflammatory NOD-like Receptor Is Required for Microglia Development. *Cell Reports,* 5**,** 1342-1352.

SLYKERMAN, R. F., THOMPSON, J., WALDIE, K. E., MURPHY, R., WALL, C. & MITCHELL, E. A. 2017. Antibiotics in the first year of life and subsequent neurocognitive outcomes. *Acta Paediatrica,* 106**,** 87-94.

SMITH, D. E., CLEMENCON, B. & HEDIGER, M. A. 2013. Proton-coupled oligopeptide transporter family SLC15: physiological, pharmacological and pathological implications. *Mol Aspects Med,* 34**,** 323-36.

SOMMER, F. & BACKHED, F. 2013. The gut microbiota--masters of host development and physiology. *Nat Rev Microbiol,* 11**,** 227-38.

STENSBALLE, L. G., SIMONSEN, J., JENSEN, S. M., BØNNELYKKE, K. & BISGAARD, H. 2013. Use of Antibiotics during Pregnancy Increases the Risk of Asthma in Early Childhood. *The Journal of Pediatrics,* 162**,** 832-838.e3.

STERKA, D., JR. & MARRIOTT, I. 2006. Characterization of nucleotide-binding oligomerization domain (NOD) protein expression in primary murine microglia. *J Neuroimmunol,* 179**,** 65-75.

STEVENS, B., ALLEN, N. J., VAZQUEZ, L. E., HOWELL, G. R., CHRISTOPHERSON, K. S., NOURI, N., MICHEVA, K. D., MEHALOW, A. K., HUBERMAN, A. D., STAFFORD, B., SHER, A., LITKE, A. M., LAMBRIS, J. D., SMITH, S. J., JOHN, S. W. & BARRES, B. A. 2007. The classical complement cascade mediates CNS synapse elimination. *Cell,* 131**,** 1164-78.

STILES, J. & JERNIGAN, T. L. 2010. The Basics of Brain Development. *Neuropsychology Review,* 20**,** 327-348.

STILLING, R. M., RYAN, F. J., HOBAN, A. E., SHANAHAN, F., CLARKE, G., CLAESSON, M. J., DINAN, T. G. & CRYAN, J. F. 2015. Microbes & neurodevelopment - Absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. *Brain Behav Immun*.

STOKHOLM, J., SEVELSTED, A., BONNELYKKE, K. & BISGAARD, H. 2014. Maternal propensity for infections and risk of childhood asthma: a registry-based cohort study. *Lancet Respir Med,* 2**,** 631-7.

SUDO, N., CHIDA, Y., AIBA, Y., SONODA, J., OYAMA, N., YU, X. N., KUBO, C. & KOGA, Y. 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol,* 558**,** 263-75.

TAU, G. Z. & PETERSON, B. S. 2009. Normal Development of Brain Circuits. *Neuropsychopharmacology,* 35**,** 147-168.

TOCHITANI, S., IKENO, T., ITO, T., SAKURAI, A., YAMAUCHI, T. & MATSUZAKI, H. 2016. Administration of Non-Absorbable Antibiotics to Pregnant Mice to Perturb the Maternal Gut Microbiota Is Associated with Alterations in Offspring Behavior. *PLoS ONE,* 11**,** e0138293.

TRACEY, K. J. 2009. Reflex control of immunity. *Nat Rev Immunol,* 9**,** 418-28.

TRASANDE, L., BLUSTEIN, J., LIU, M., CORWIN, E., COX, L. M. & BLASER, M. J. 2013. Infant antibiotic exposures and early-life body mass. *Int J Obes (Lond),* 37**,** 16-23.

TYAGARAJAN, S. K. & FRITSCHY, J. M. 2014. Gephyrin: a master regulator of neuronal function? *Nat Rev Neurosci,* 15**,** 141-56.

VICARIO-ABEJON, C., OWENS, D., MCKAY, R. & SEGAL, M. 2002. Role of neurotrophins in central synapse formation and stabilization. *Nat Rev Neurosci,* 3**,** 965-74.

WANG, L. W., TANCREDI, D. J. & THOMAS, D. W. 2011. The prevalence of gastrointestinal problems in children across the United States with autism spectrum disorders from families with multiple affected members. *J Dev Behav Pediatr,* 32**,** 351-60.

WHEELER, R., CHEVALIER, G., EBERL, G. & GOMPERTS BONECA, I. 2014. The biology of bacterial peptidoglycans and their impact on host immunity and physiology. *Cell Microbiol,* 16**,** 1014-23.

WILLIAMS, B. L., HORNIG, M., BUIE, T., BAUMAN, M. L., CHO PAIK, M., WICK, I., BENNETT, A., JABADO, O., HIRSCHBERG, D. L. & LIPKIN, W. I. 2011. Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances. *PLoS One,* 6**,** e24585.

WILLIAMS, S. C. 2014. Gnotobiotics. *Proc Natl Acad Sci U S A,* 111**,** 1661.

WREN, A. M. & BLOOM, S. R. 2007. Gut hormones and appetite control. *Gastroenterology,* 132**,** 2116-30.

YANO, J. M., YU, K., DONALDSON, G. P., SHASTRI, G. G., ANN, P., MA, L., NAGLER, C. R., ISMAGILOV, R. F., MAZMANIAN, S. K. & HSIAO, E. Y. 2015. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell,* 161**,** 264-76.

YEOM, C. W., PARK, Y. J., CHOI, S. W. & BHANG, S. Y. 2016. Association of peripheral BDNF level with cognition, attention and behavior in preschool children. *Child Adolesc Psychiatry Ment Health,* 10**,** 10.

ZERBO, O., IOSIF, A. M., WALKER, C., OZONOFF, S., HANSEN, R. L. & HERTZ-PICCIOTTO, I. 2013. Is maternal influenza or fever during pregnancy associated with autism or developmental delays? Results from the CHARGE (CHildhood Autism Risks from Genetics and Environment) study. *J Autism Dev Disord,* 43**,** 25-33.

ZHANG, Q., ZHANG, D., YE, K., LIU, K., SHENG, J., LIU, Y., HU, C., RUAN, L., LI, L. & TAO, F. 2016. Physiological and behavioral responses in offspring mice following maternal exposure to sulfamonomethoxine during pregnancy. *Neurosci Lett,* 624**,** 8-16.

ZHOU, L. & FOSTER, J. A. 2015. Psychobiotics and the gut-brain axis: in the pursuit of happiness. *Neuropsychiatr Dis Treat,* 11**,** 715-23.

ZIVKOVIC, A. M., GERMAN, J. B., LEBRILLA, C. B. & MILLS, D. A. 2011. Human milk glycobiome and its impact on the infant gastrointestinal microbiota. *Proceedings of the National Academy of Sciences,* 108**,** 4653-4658.

ZULFIQAR, F., HOZO, I., RANGARAJAN, S., MARIUZZA, R. A., DZIARSKI, R. & GUPTA, D. 2013. Genetic Association of Peptidoglycan Recognition Protein Variants with Inflammatory Bowel Disease. *PLoS One,* 8**,** e67393.