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THE FUNCTIONAL IMPACT OF GUT MICROBIOTA ON CNS REGULATION OF LOCAL AND SYSTEMIC HOMEOSTASIS

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The Functional Impact of Gut Microbiota on CNS Regulation of Local and Systemic Homeostasis

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To my father.

ABSTRACT

The “gut microbiota” is widely accepted as an integral part of the gut homeostasis, and is thought to contribute to the establishment of intestinal barrier. Growing body of research suggest that the influence of gut microbiota on host development and physiology reaches beyond the gastrointestinal tract, and the brain is not an exception. The brain plays a critical role in regulating systemic homeostasis through continuous monitoring of body energy state and integration of the peripheral signals. Evidences of microbiota impact on brain at different levels including development, neurobiology, and even behavior have been documented. This thesis places two aspects of central regulation of homeostasis under the spotlight, and explores the potential impact of gut microbes in this context: (i) Brain regulation of local homeostasis through the function of the blood-brain barrier (BBB). The BBB is a specialized barrier that segregates the neural tissue from the circulation and controls the provision of nutrients to the brain. An intact BBB is critical for maintaining a homeostatic environment for normal function of the brain cells. (ii) Brain regulation of systemic homeostasis in relevance to anorexia nervosa. Anorexia nervosa is a serious eating disorder with altered homeostatic function. Dysbiosis in the gut microbiota have been reported in anorectic patients.

By taking advantage of germ-free mouse model, we showed that in the absence of gut microbiota, the integrity and function of the BBB is impaired during the intrauterine period, suggesting that maternal gut microbiota mediates the development and maturation of the BBB. Impaired BBB integrity persisted into adulthood and was associated with decreased expression of endothelial tight junction proteins including occludin, claudin-5 and zona occludens-1. The alterations in structure and permeability of the BBB were restored by introducing normal gut flora into the germ-free mice, reinforcing the role of gut microbiome for the integrity of the BBB. Furthermore, we showed that short-chain fatty acids (SCFAs, bacterial metabolites of dietary fiber fermentation) improve BBB integrity in line with previous observations that SCFAs enhance the integrity of intestinal epithelial barrier. Germ-free mice monocolonized with *Clostridium tyrobutyricum* that mainly produces butyrate or with *Bacteroides thetaiotaomicron* which produces acetate and propionate exhibited decreased BBB permeability. Treatment with butyrate salt mimicked the effects. The influence of SCFAs might be mediated by epigenetic mechanisms as monocolonized and SCFA-treated germ-free mice displayed enhanced levels of histone acetylation in brain lysates.

Preterm birth is associated with impaired development and vascular fragility in the brain. During the critical early postnatal period, normal brain growth and maturation may be negatively affected by the prematurity-related factors such as nutrient deprivation or a serious infection. We used a preterm porcine model to study the effects of gestational age, early feeding, and infection on brain barriers following preterm birth. Preterm pigs spontaneously develop diet- and microbiota-related diseases including necrotizing enterocolitis. We showed that preterm piglets have impaired BBB-associated protein expression, decreased endothelial integrity, and enhanced blood-CSF barrier permeability in comparison to term counterparts.

The observed impairment in endothelial integrity measured as astroglial perivascular coverage persisted into postnatal day five independent of enteral or parenteral feeding. Next, to investigate brain barrier function in preterm piglets under inflammation, we fed a group of animals with formula which is known to increase the risk of necrotizing enterocolitis. Our results indicate that severe necrotizing enterocolitis following five day formula treatment is associated with increased systemic inflammation, impaired blood-CSF barrier, enhanced neuronal death and elevated IL-6 levels in the hippocampus.

As an example of systemic homeostasis, we hypothesized that gut microbiota has a functional relevance in anorexia nervosa, a disease with altered homeostatic function. We transplanted fecal microbiota from a female individual with anorexia nervosa and a sex-matched healthy control into female germ-free mice. Following fecal microbiota transplantation, some of the phenotypic aspects of anorexia were replicated in the recipient mice, including reduced weight gain, elevated serum corticosterone levels, and increased anxiety-like behavior measured by open-field test. This hypothesis was further reinforced by the fact that mice subjected to these transplantations display significant changes in gene-expression in the nucleus accumbens (but not in the hippocampus), a region implicated in reward and affected in patients with anorexia.

In summary, the findings in this thesis reinforce the proposed impact of gut microbiota on host homeostasis. Specifically, on local level, we showed the influences on BBB development and function, and on systemic level, we demonstrated the effects on genes involved in energy homeostasis in nucleus accumbens. Future studies will uncover the exact mechanisms underlying the impact of gut microbiota on the brain.

LIST OF SCIENTIFIC PAPERS

I. Braniste V, Al-Asmakh M, Kowal C, Anuar F, **Abbaspour A**, Tóth M, Korecka A, Bakocevic N, Ng LG, Kundu P, Gulyás B, Halldin C, Hultenby K, Nilsson H, Hebert H, Volpe BT, Diamond B, Pettersson S. The gut microbiota influences blood-brain barrier permeability in mice. *Science Translational Medicine, 2014 Nov 19, Vol. 6, Issue 263, pp. 263ra158.*

II. Brunse A*, **Abbaspour A***, Sangild PT. Brain barrier disruption and region-specific neuronal degeneration during necrotizing enterocolitis in preterm pigs. *Developmental neuroscience, 2018 Jun 6, Epub.*

III. **Abbaspour A**, Mayerhofer R, Lindskog M. Hypothesis; Gut microbiota affects brain control of metabolism and energy homeostasis in anorexia nervosa. *Manuscript in preparation.*

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RELATED PUBLICATION

Korecka A, Dona A, Lahiri S, Tett AJ, Al-Asmakh M, Braniste V, D'Arienzo R, **Abbaspour A**, Reichardt N, Fujii-Kuriyama Y, Rafter J, Narbad A, Holmes E, Nicholson J, Arulampalam V, Pettersson S. Bidirectional communication between the Aryl hydrocarbon Receptor (AhR) and the microbiome tunes host metabolism. *NPJ Biofilms Microbiomes. 2016 Aug 24, Vol.2.*

CONTENTS

LIST OF ABBREVIATIONS

ADHD	Attention-deficit hyperactivity disorder
AI-2	Autoinducer-2
ANGPTL4	Angiopietin-like 4
APOE	Apolipoprotein E
AQP-4	Aquaporin 4
ASD	Autism spectrum disorder
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BTB	Blood-testis barrier
CA	Cornu Ammonis
CCK	Cholecystokinin
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
FFAR-2	Free fatty acid receptor 2
GFAP	Glial fibrillary acidic protein
GLUT-1	Glucose transporter 1
GPCR	G protein-coupled receptor
fMRI	Functional magnetic resonance imaging
HDAC	Histone deacetylase
HPA axis	Hypothalamic-pituitary-adrenal axis
IBS	Irritable bowel syndrome
IgG2b	Immunoglobulin G2b
IL-6	Interleukin 6
JAM	Junctional adhesion molecules
MCP-1	Monocyte chemoattractant protein 1
MCT1	Monocarboxylate transporter 1
MRAP2	Melanocortin 2 receptor accessory protein 2
NEC	Necrotizing enterocolitis
NMDAR	N-methyl D-aspartate receptor

NPY	Neuropeptide Y
PCA	Principle component analysis
PDGFR-β	Platelet-derived growth factor receptor-β
PECAM	Platelet endothelial cell adhesion molecule
PET	Positron-emission tomography
POMC	Pro-opiomelanocortin
PYY	Peptide YY
SCFA	Short-chain fatty acid
SPF	Specific-pathogen-free
TLR-4	Toll-like receptor 4
VE-cadherin	Vascular endothelial cadherin
ZO-1	Zona occludens 1
5-HT	5-hudroxytrptamine or Serotonin

1 INTRODUCTION

1.1 THE GUT MICROBIOTA

Human body, similar to other multicellular organisms, is a mosaic of eukaryotic cells, prokaryotic cells, and viruses. Symbiosis of these different life forms provides an enriched gene pool that potentially enhances the ability of the organism as a whole (holobiont) for survival. The resident microorganisms in/on the body including bacteria, fungi, yeast, bacteriophages, and archea are collectively known as the "microbiota". So far, the microbiota research has been mainly focused on the bacterial component, thus the other life forms have been less investigated[1–4]. It is estimated that the number of bacterial cells in the body is roughly the same as the number of human cells[5]. Globally, various projects such as Human Microbiome Project, MetaHIT, and Asian Gut aim to identify and characterize the human microbiome. These projects commonly focus on specific body sites including the gut, nasal passages, oral cavity, skin, and urogenital tract. A recent study performed on the circulating cell-free DNA rather than looking at specific individual sites, showed that only 1% of the non-human cells in the body mapped to the existing database[6]. The results from this study suggest that the microbiota is vastly more diverse than previously known, and a large fraction of the microbiota is still uncharacterized. Nevertheless, the gastrointestinal tract represents the most heavily colonized organ, populated by more than 500 bacterial species[7]. The early colonizers of the gut are facultative anaerobes, and the neonatal gut microbiota can be characterized by low diversity and dominance by Proteobacteria and Actinobacteria phyla [8,9]. During the first years of life, the gut microbiota shifts towards a more complex and diverse adult-like community with enhanced population of strict anaerobes. By the age of 3-5 years, the gut microbiota forms a stable community that fully resembles adult microbiota predominated by Firmicutes and Bacteroidetes phyla[9,10]. Different environmental factors affect the development of the infant microbiota during the perinatal period including mode of delivery, gestational age, genetics, diet, and antibiotic treatment[11–13] (Fig. 1).

1.1.1 Early development and effects of perinatal factors: mode of delivery, gestational age and breast milk

It is thought that colonization of the body by microorganisms initiates rapidly following birth[14]. However, isolation of microbes from semen, placenta, amniotic fluid, meconium (first stool of mammalian infants), and umbilical cord blood has challenged the previously accepted already *in-utero*[11,15–17]. Nevertheless, the early life events during and shortly after birth can be influential in priming the gut microbiota[11].

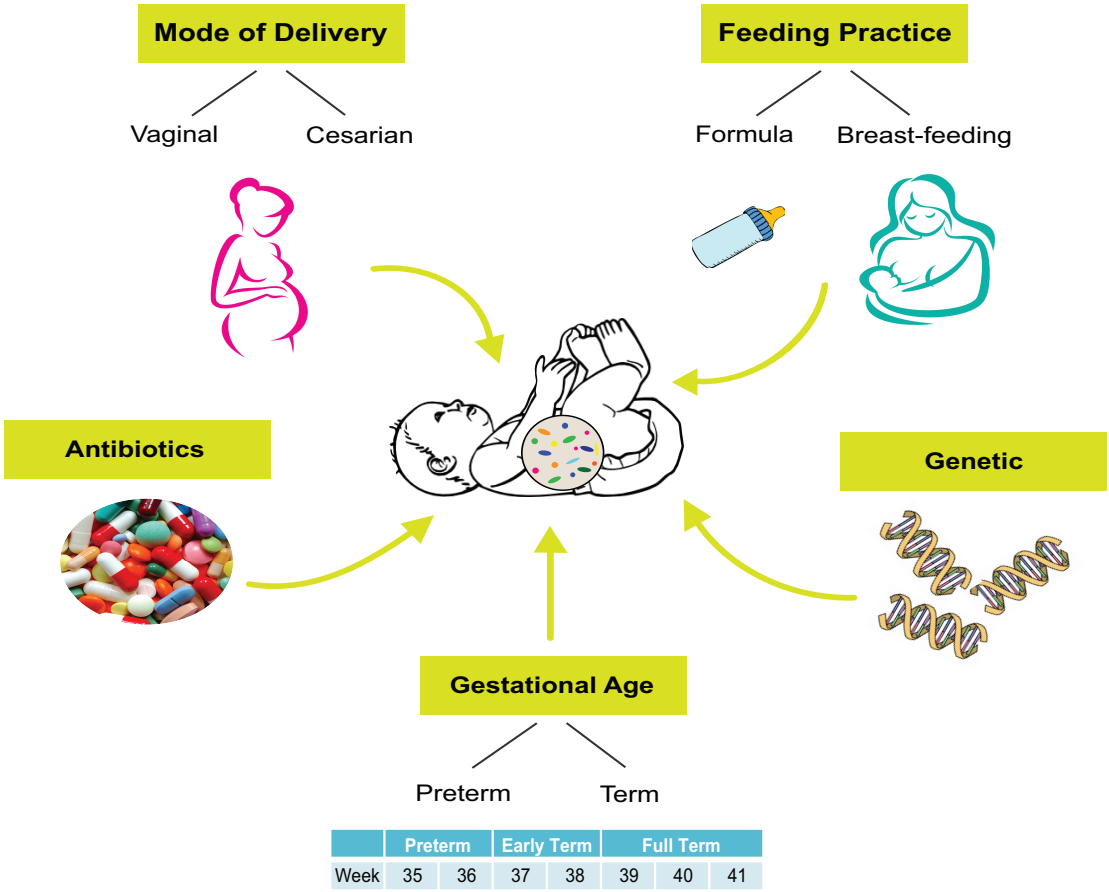


Figure 1. Graphical illustration of perinatal factors that shape infant gut microbiota.

One of these critical factors is the mode of delivery. Infants born through vaginal delivery are predominantly seeded with microbes from vaginal and fecal flora of the mother such as *Lactobacillus* and *Bifidobacterium* spp.[18]. In contrast, babies delivered by C-section are colonized by microbes that resemble the microbial members of the skin flora including *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp[18]. Compared to vaginally-delivered infants, the gut microbiota diversity is reduced in babies born by C-section. Microbiota aberrancies following C-section are associated with delayed maturation of the immune system and increased disease risk later in life[19,20].

Other sources of vertical microbial transmission from mother to the offspring are colostrum and breast milk. In addition to providing the infant with fundamental nutritional elements and bioactive molecules, colostrum and breast milk harbor distinct microbial communities[21,22]. It is estimated that 1×10^4 to 1×10^6 bacteria are passed on to the infant through consumption of ~800 mL of milk per day[23]. Furthermore, some components of human milk have prebiotic activities. Prebiotics are non-digestible food ingredients that promote the growth of beneficial

microorganisms in the intestines. The milk components with prebiotic activities facilitate the establishment of specific groups of bacteria over the others. For instance, oligosaccharides which are found abundantly in human breast milk stimulate the growth of bifidobacteria and staphylococci[24,25]. Healthy breast-fed infants are reported to have two times more *Bifidobacterium* cells in their fecal microbiota compared to formula-fed infants[26]. In fact, compared to formula feeding, exclusive breast feeding is associated with a distinct microbiota which promotes the immune system, particularly by developing populations of memory T cells and T helper 17 cells[27–29].

Gestational age is another factor that can affect the establishment of gut microbiota in newborn infants. Preterm birth, occurring earlier than 37 completed weeks of gestation, is thought to perturb the optimal development of the gut microbiota. The microbiota in preterm infants is characterized by decreased diversity and reduced number of *Bifidobacterium* and *Bacteroides* compared to full-term infants[30]. Notably, preterm birth is often confounded with C-section and antibiotic treatment which can also interfere with normal development of gut microbiota[18,31]. In a study analyzing the gut microbiota in infants following parenteral antibiotic treatment, the number of *Bifidobacterium* and *Lactobacillus* was shown to be reduced and was not fully recovered in eight weeks[31]. Furthermore, composition of the microbiota in colostrum and breast milk was suggested to be affected by the mode of delivery and gestational age. This could partly contribute to the alteration of gut microbiota in preterm-delivered infants[22,32]. Investigating the impact of gut microbiota perturbation in preterm infants could enhance our understanding of various pathologies associated with preterm birth. One of the most severe short term complications of preterm birth is necrotizing enterocolitis (NEC). It is thought that increased expression of bacterial receptor Toll-like receptor 4 (TLR-4) in the premature gut and enhanced reactivity of intestinal mucosa to microbial ligands, in part contribute to the onset of the disease[33]. Moreover, independent reports indicate that Proteobacteria is the predominant phyla in preterm infants with NEC whereas it does not account for more than 40% of total bacteria in no NEC controls[34,35]. Whether the altered gut microbiota reported in NEC is the cause or consequence of the disease remains to be further investigated.

1.1.2 Microbiota in gut-brain axis

Comorbidities between bowl diseases and alterations of emotional states have long been appreciated, and a role for “gut microbiota-brain axis” in the pathology of such diseases has been postulated[36]. An example is the irritable bowel syndrome (IBS) which often co-occurs with psychiatric conditions including anxiety and depression. Dysbiosis of the gut microbiota

has been reported in the IBS patients[37]. Increased Firmicutes: Bacteroides ratio found in some IBS patients has been correlated with anxiety and depression[38]. Other studies have suggested that treatment with *Bifidobacterium* probiotic strains or a prebiotic which stimulates the growth *Bifidobacterium* can alleviate the diseases symptoms[39–41]. Probiotics are live microorganisms which provide health benefits when consumed. Another example of co-occurring gastrointestinal and neurological diseases is autism spectrum disorder (ASD). Gastrointestinal disturbances and intestinal barrier dysfunction are frequently reported in ASD patients. In two experimental murine models showing ASD features (maternal immune activation model and *in-utero* exposure to valproic acid), alterations in postnatal development of gut microbiota have been associated with intestinal and behavioral deficits related to the disease[42,43].

The communication between the gut microbiota and the brain seems to be bidirectional as various forms of stress in the host can affect the composition and function of the gut microbiota. Mice exposed to a social stressor, were shown to have altered bacterial population in the intestines (decreased *Bacteroides,* increased *Clostridium*), and enhanced circulating levels of interleukin 6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1). Antibiotic treatment revoked stressor-induced increase in circulating cytokines in these mice, suggesting that microbiota mediates cytokine production in response to a stressor[44]. In a mouse model of maternal separation, early-life stress was shown to induce dysbiosis in the gut microbiota that persists into adulthood. Altered microbiota profile was associated with anxiety-like behavior. Interestingly, maternal separation in germ-free mice did not induce such behavior.[45]

On developmental level, the gut microbiota modulates various processes including myelination, neurogenesis, and microglia maturation[46]. Multiple independent studies have revealed that mice devoid of gut microbiota have increased myelination in prefrontal cortex[47], altered dendritic morphology[48], less responsive and immature microglia[49–51], and decreased hippocampal neurogenesis[52]. Several putative mechanisms have been proposed to mediate the integration of the signaling between the gut microbiome and the nervous system:

Neuronal signaling- The vagus nerve is a bundle of parasympathetic nerve fibers that conveys information between the periphery (including the gastrointestinal tract) and the brain. Anxiety-like behavior induced by dextran sulfate sodium-induced colitis in mice was shown to be dependent on the vagus nerve. Introduction of *B. longum* which is known to have anxiolytic effects, could not alleviate the symptoms in vagotomized mice[53]. Notably, treatment with *L. rhamnosus* and *B. infantis* was protective against colitis in both

vagotomized and control mice suggesting that gut microbiota-brain interaction via the vagus nerve might be specific to certain bacterial strains and other signaling mechanisms might be involved[54].

Endocrine and neuroendocrine communications- The enteroendocrine cells of the gut produce various hormones and peptides including gastrin, cholecystokinin, glucagon-like peptide 1, peptide YY (PYY), that are implicated in appetite regulation through communication with the brain. The lumen-projecting microvilli of enteroendocrine cells and their proximity to the gut microbiota raise the possibility of the cross-talk between the microbes and these cells. In fact, in an elegant study, Breton et al. suggested that bacterial peptides can affect host appetite through modulating the brain function. The authors showed that after food intake, *E.coli* (in its stationary phase) produces a protein that stimulate the releases of PYY by enterendocrine cells which further activates the anorexigenic neurons in the hypothalamus[55].

Bacterial metabolites- In addition to microbiota-derived neuroactive molecules, other bacterial products can potentially mediate the communication between the gut microbiota and the brain. Short-chain fatty acids (SCFAs) including acetate, propionate, and butyrate are bacterial fermentation products which are suggested to influence brain and behavior in the host[56,57]. Feeding rats with a diet rich in fermentable carbohydrates was shown to induce anxiety-like behavior and impair the memory[58]. In human, elevated levels of fecal propionic acid was associated with anxiety behavior in IBS patients[59]. Furthermore, increased substrate availability for bacterial fermentation due to carbohydrate malabsorption has been correlated to depression[60]. SCFAs also seem to modulate microglia maturation and function. Provision of SCFAs in the drinking water was shown to restore the defective microglia observed in germ-free mice. Interestingly, mice deficient in SCFA receptor FFAR-2 displayed microglia defects similar to those found under germ-free condition[61]. Another metabolic effect attributed to the gut microbiota is the modulation of circulating tryptophan availability. Tryptophan is an amino acid precursor for 5-HT, kynurenine, and indole-containing metabolites. Although germ-free and antibiotic-treated mice display elevated tryptophan levels in the plasma compared to conventional animals[62,63], kynurenine metabolism[64], circulating 5-HT and indole levels[62] were decreased indicating that gut microbiota contributes to the conversion of tryptophan into its metabolites. Introduction of gut microbiota to germ-free mice post weaning was shown to be sufficient to restore the altered levels of peripheral tryptophan and kynurenine pathway metabolism and to normalize the reduced anxiety behavior[62].

Immune system. Elevated concentrations of inflammatory cytokines such as interleukin 6 and C-reactive protein (CRPss) have been reported in psychiatric disorders including depression[65]. Manipulation of gut microbiota composition by probiotics was shown to influence the systemic cytokine levels both in experimental animal models and in human[66–68], suggesting that alterations of gut microbiota might influence the behavior through changes in cytokine levels. In rat maternal separation model of depression, treatment with probiotic *Bifidobacterium infantis* attenuated exaggerated IL-6 response and improved depression-like behavior[66], pointing to the potential therapeutic values of probiotics for psychiatric diseases.

1.2 BRAIN AND REGULATION OF HOMEOSTASIS

The brain has a key role in maintaining the body in balance in response to environmental fluctuations through homeostatic regulation of body temperature, food intake, energy expenditure, glucose metabolism, sleep, and composition of blood ions and minerals.[69] Monitoring the state of the body and integration of the peripheral signals, requires coordinated interaction between the brain and the periphery. Furthermore, CNS regulation of systemic homeostasis is dependent on a local homeostatic microenvironment which enables the brain cells to function optimally.

This thesis is devoted to two aspects of CNS regulation of homeostasis: 1) Regulation of the local homeostasis of the brain through the function of the blood-brain barrier, 2) Regulation of systemic homeostasis with a focus on anorexia nervosa, a disorder with altered homeostatic function. A potential role for the gut microbiota in these two contexts has been hypothesized and investigated.

1.3 BLOOD-BRAIN BARRIER AS A KEY COMPONENT FOR CNS HOMEOSTASIS

The molecular trafficking across the brain is tightly and selectively controlled through the function of a specialized barrier known as the blood-brain barrier (BBB). The BBB is formed by the tight junctions at the endothelium lining the microvessels of the brain. An intact BBB protects the brain from potential neurotoxic and harmful compounds while the passage of nutrients and energy substrates is facilitated by transporters at brain endothelium[70]. Unlike what the term “barrier” suggests, the properties and function of the BBB are dynamic and can be modulated in different pathological or non-pathological conditions and/or in response to CNS or circulatory factors[71,72]. Alteration of tight junction proteins and increased BBB permeability have been reported in conditions such as peripheral inflammation[73], aging[74,75], and chronic sleep deprivation[76]. Interestingly, in the latter instance, BBB integrity was shown

to be restored following sleep recovery[76]. An example of BBB alteration in response to circulating factors is the enhanced receptor-mediated transcytosis of urocortin to the brain following leptin injection[77]. Moreover, it has been suggested that the BBB can also be modulated transiently perhaps to facilitate the passage of circulating growth factors and antibodies or sampling the plasma composition or to protect the brain under conditions such as oxidative stress or hypoxia by tightening the junctional proteins[78].

1.3.1 Molecular and cellular structure of the blood-brain barrier

The tight junction proteins of the BBB are present at the apical side (facing the lumen) of the endothelial cell membrane and include occludin, claudins, and junctional adhesion molecules (JAMs). These proteins are linked to the cytoskeleton through cytoplasmic scaffolding proteins called zona occludentes (ZOs). ZOs enhance the effectiveness of the tight junctions[72]. ZO-1 is a member of the ZO family which has been implicated in the angiogenesis, and barrier formation[79]. Other transmembrane proteins present at the basal side of the endothelial membrane provide structural integrity for the cells by holding them together. These proteins, including vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule (PECAM), are known as adherens junction proteins[70] (Fig. 2). While the BBB tight junctions limit the paracellular passage of large hydrophilic molecules, smaller lipid-soluble molecules can diffuse across the lipid membrane. Metabolic products such as glucose and amino acids are actively transported, and some proteins such as insulin are taken up by receptor-mediated transcytosis[70].

The normal function of the BBB also depends on orchestrated activities of other cellular components of the neurovascular unit including astrocytes, pericytes, and nerve endings in addition to the endothelial cells (Fig. 2). Co-culturing the endothelial cells with astrocytes or astrocyte-conditioned media have been shown to enhance barrier function by decreasing the permebility[80]. More recently, pericytes and neurons were also shown to induce similar effects *in vitro*[72,81].

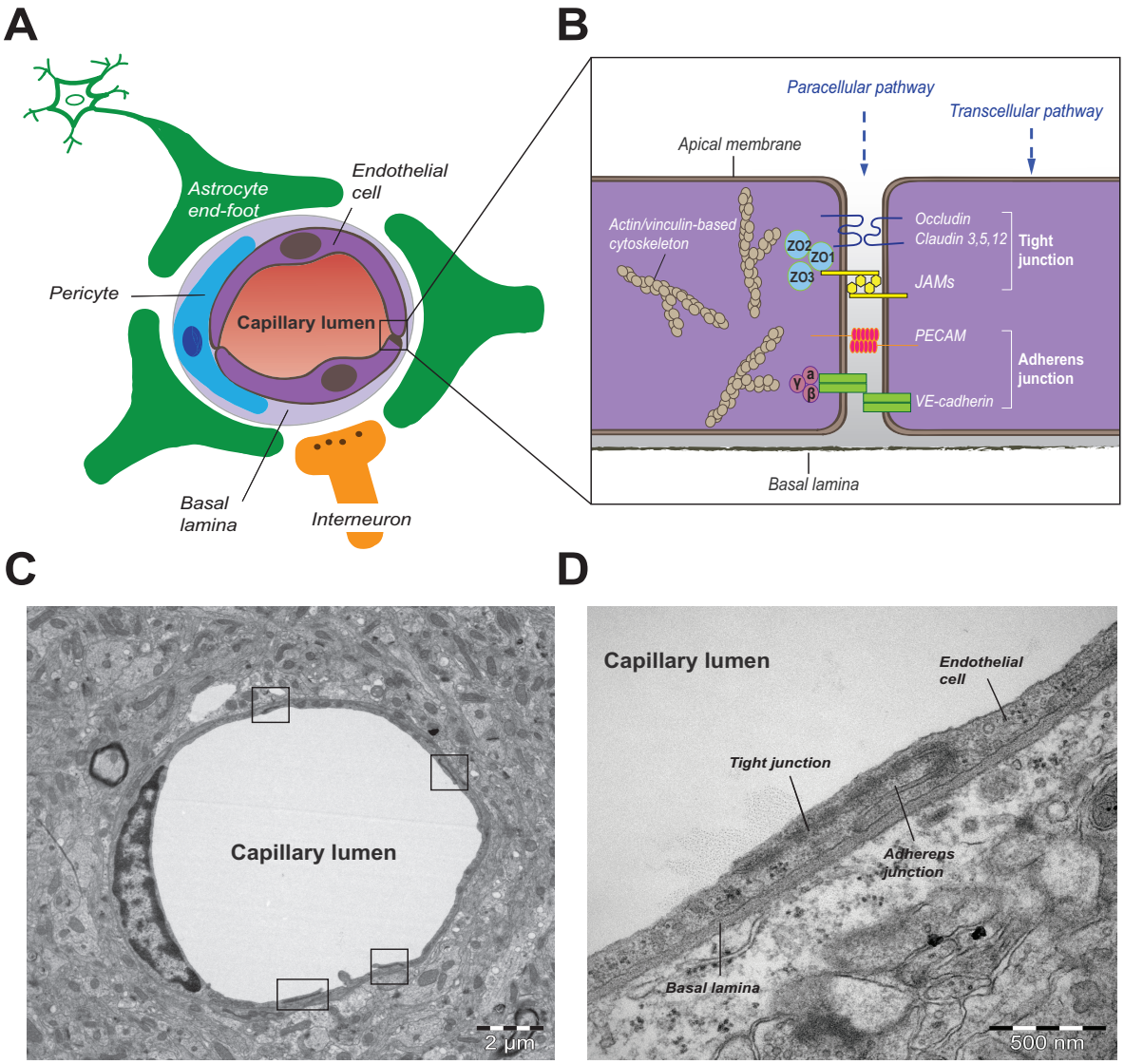


Figure 2. BBB molecular structure and cellular associations. Graphical illustrations (A,B) and transmission electron microscope images from mouse brain depicting (A,C) cross-sections of neurovascular unit, and (B,D) components of endothelial tight junctions. Tight junctions are marked by squares in (C).
JAM: Junctional Adhesion Molecule, PECAM: Platelet Endothelial Cell Adhesion Molecule, VE-cadherin: Vascular Endothelial cadherin.

Astrocytes display close physical and biochemical interactions with endothelial cells at their end-foot processes[78]. Several mechanism have been suggested for the regulatory impact of astrocyte on BBB integrity, including angiotensinogen-mediated posttranslational modification of occludin, enhancing tight junctions through secretion of sonic hedgehog, suppression of tight junction disruptive pathway, and induction of occludin phosphorylation through production of apolipoprotein E molecules APOE-2 and APOE-3 (cholesterol and phospholipid transporters)[82].

Pericytes are contractile cells that ensheath the endothelial cells of the microvessels throughout the body and in the brain. Capillary bed of the CNS is known to have the highest perycite coverage[83]. Pericytes are embedded in the basement membrane where they interact with the endothelial cells by means of direct contact or paracrine signaling. Mice deficient in platelet-derived growth factor receptor-β (PDGFR-β), a marker of the pericytes, exhibit increased BBB permeability and do not survive. Hypomorphic mutation of PDGFR-β gene results in viable mice with reduced PDGFR-β signaling and fewer pericytes than their littermates. The enhanced vascular permeability in these mice is associated with alterations in transcytosis rather than defective tight junction proteins. Pericytes are also suggested to be involved in the spatial guidance and polarization of the astrocyte end-feet[84].

1.3.2 Blood-brain barrier development

The development of the BBB begins during embryogenesis as soon as the blood vessels penetrate the brain tissue. The newly formed blood vessels express tight junction proteins and some nutrient transporters[82,85]. However, the structural and functional maturation of the BBB only occurs when the endothelial cells come into contact with pericytes and astroglia[84,86]. This leads to substantial decreases in the permeability of the BBB and the rate of transcytosis, which in mice occurs at E15-E16.5[87,88]. The involvement of astrocytes in BBB integrity begins during the first and second postnatal weeks (as marked by AQP-4 staining) in rodents, indicating that they are more involved in the maintenance of the BBB especially during the early postnatal life rather than in its induction[84].In contrast, pericytes were shown to be necessary for the formation of the BBB during embryogenesis[84]. In human, the astrocyte are already present at the last stage of gestation, suggesting that they might contribute to the functional and structural properties of the BBB in a more complex manner than in rodents[84].

1.3.3 Gut microbiota and barrier integrity

During the past few years, there has been a growing interest to understand the role of gut microbiota in regulating the intestinal barrier based on the observed alterations in gut microbiota composition and diversity (dysbiosis) in the intestinal disorders including inflammatory bowel disease and irritable bowel syndrome[89–92]. The barrier function in the gut is very complex and comprised of various layers including microbial, chemical (mucin), physical, and immunological barriers. The physical barrier is formed by a single layer of epithelial cells lining the lumen sealed by transmembrane tight junction proteins. The adherens junctions and desmosomes link the adjacent epithelial cells mechanically[93]. *In vitro* studies indicate that treating cultured intestinal epithelial cells with bacteria or bacterial products, enhance the trans-epithelial resistance of the cells and alter protein expression of the

tight junctions[94,95]. Human intestinal epithelial cell lines H-29 and Caco-2 exposed to live (but not heat-inactivated) probiotics *S. thermophilus* and *L. acidophilus* exhibited increased trans-epithelial resistance, decreased permeability and enhanced activation of ZO-1 and occludin[95]. Colonization of the germ-free mice with a common gut resident *B. thetaiotaomicron* is associated with nearly 300 fold increase in epithelial expression of small proline-rich protein-w2 which plays an important role in fortifying the intestinal epithelial barrier function[96]. Interestingly, bacterial products, namely SCFAs, were shown to improve tight junction integrity and barrier function *in vitro*[97] as well as in experimental animals[98]. Inoculation with *Bifidobacterium longum* that produces high levels of acetate enhances intestinal barrier integrity in mice devoid of bacteria[57]. So far, few mechanisms have been identified through which the SCFAs exert their effects, including inhibition of histone deacetylases (HDACs) and/or signaling via G protein-coupled receptors GPR41, GPR43, and GPR109A[57].

Dysbiosis-associated disruption of tight and adherens junction proteins has been implicated in other diseases such as obesity, and type 1 diabetes[99,100]. Prebiotic treatment of obese mice (ob/ob) in favor of *Bifidobacterium* spp is shown to be beneficial for tight junction integrity and intestinal barrier permeability[101]. In another study, the gut barrier dysfunction in a mouse model of maternal immune activation was improved following treatment with a commensal bacterium *Bacteroides fragilis.* This experimental model displays features of autism spectrum disorder accompanied by impairment in gut barrier function, a comorbidity that is observed in a subset of autistic individuals. The enhanced barrier function in mice treated with bacteria was associated with increased protein expression of tight junctions in the colon. Interestingly, autism-related behavior was also ameliorated after the treatment[42].

In addition to the microbiota effects locally on the intestinal barrier, there are some evidence suggesting that it can influence a remote barrier in the body namely blood-testis barrier (BTB). Similar to the intestinal epithelial barrier, the BTB is formed between the specialized epithelial cells of the testis by tight and adeherns junction proteins. Despite of different spatial organization, the junctional proteins of the BTB have similar molecular structure and function to those of intestinal epithelial barrier[102]. Mice devoid of gut microbiota are shown to have defective BTB permeability and cell junction proteins, suggesting that the microbiota modulate BTB integrity. Monocolonization of germ-free mice with *Clostridium Tyrobutyricum,* a butyrate producing bacterium, restores BTB integrity and cell junction protein levels[103].

1.4 NEUROBIOLOGY OF ENERGY HOMEOSTASIS AND FEEDING

The homeostatic perspective of body energy regulation was first proposed by Claude Bernard and Walter Cannon, suggesting that there is a balance between food intake and energy expenditure. They attributed the balanced internal milieu to the ability of the body to monitor the energy state and make adjustments to sustain the stability[104]. Regulation of feeding, as a complementary component of the homeostasis, is a highly complex process that involves signaling between the periphery and the central nervous system. Our current understanding of feeding control stems from two major hypotheses. The first one was proposed by Kennedy in 1950's, suggesting that proportional to the body fat content, inhibitory signals (also known as adiposity signals) are generated that act on the brain to reduce food intake[105]. Pancreatic hormone insulin, and adipocyte hormone leptin, are the two molecules identified as adiposity signaling candidates so far[106]. The effects of these hormones on the brain are shown to be mediated by distinct neuronal subpopulations and several regulatory neuropeptides in the hypothalamus[106]. In rats, microinjection of leptin into arcuate nucleus of hypothalamus was shown to inhibit food intake and reduce body weight, whereas arcuate-lesioned animals were not responsive to leptin treatment[107,108]. The second hypothesis was put forward in 1970's by Gibbs and Smith proposing that during each meal, the digestive tract produces signals that communicate to the brain to terminate the meal[109]. These signals, known as satiety signals, include information from the taste buds in the oral cavity[110], mechanical responses of stomach and small intestine during digestion[110], peptides secreted by the stomach or by enteroendocrine cells of the gut such as cholecystokinin (CCK)[111], and information related to energy metabolism in the liver[112]. The hindbrain and specifically nucleus tractus solitaries seem to be critical for the integration of the satiety signals which are received through the circulation, vagus nerve, and afferent nerves passing through the spinal cord from the gastrointestinal tract[110,111,113]. Nucleus tractus solitaries is also innervated by descending hypothalamic input.

Mounting evidence suggest that brain circuits other than the ones involved in hunger/satiety pathways might contribute to the regulation of food consumption and energy homeostasis. This includes brain circuits implicated in the reward aspects of food. Various limbic regions (nucleus accumbens, hippocampus, and amygdala), cortical areas (orbitofrontal cortex, insula, and cingulate gyrus) and neurotransmitters (dopamine, serotonin, opioids and cannabinoids) are involved in orchestrating the rewarding effects of food[114]. Dopamine is the most-studied and best-characterized neurotransmitter in the context of reward mechanism, especially dopamine projections from ventral tegmental area into the nucleus accumbens[115].

Research in experimental models suggest that disruption in dopamine synthesis, either pharmacologically or genetically, can cause profound alterations in feeding behavior[116,117]. Furthermore, dopamine plays an essential role in reinforcement of food-seeking behavior[118–120], and can be modulated by food availability cues and appetite-related hormones[121].

1.4.1 Anorexia nervosa and regulation of energy homeostasis and feeding

Anorexia nervosa is a complex eating disorder characterized by extreme preoccupation with dieting, significantly low body weight, and intense fear of weight gain[122]. Anorexia is sex- and age-linked, and adolescent females are the most affected group. Nevertheless, the disease also affects males and other age groups[122,123]. Two subtypes of anorexia have been identified: the restrictive subtype marked by restricted energy intake, and the binge-eating/purging subtype which engage in recurrent episodes of binge-eating and purging[122].

Anorexia is often accompanied by severe neuropsychiatric symptoms including depression, anxiety and obsessive-compulsive disorder[124–126].As the disease progresses, anorectic patients exhibit various clinical complications including hypothermia, physical hyper-activity, and systemic endocrine deregulation such as impaired hypothalamic-pituitary-adrenal (HPA) axis and altered appetite-regulating hormone levels[127,128]. These complications are suggested to be adaptive responses to chronic starvation[128–130], however, they might further contribute to the development and maintenance of the disease. Although eating normalization is shown to improve the weight gain in anorexic patients, still little is known about the etiology of the disease.

As mentioned earlier, the hypothalamus has received significant attention in the context of feeding behavior regulation. However, evidence suggesting a critical role for the hypothalamic peptides in the neurobiology of anorexia is limited[131]. Progress in brain imaging techniques has led to the recognition of other involved neural circuits[132,133]. Dysfunction in these circuits are related to altered dopamine and serotonin metabolism. Brain fMRI scans from anorectic patients indicate elevated activity of the nucleus accumbens, a brain region densely innervated by dopaminergic neurons[134–136]. The dopamine system, and especially the projections to nucleus accumbens, are implicated in many brain functions that may be affected in anorexia nervosa including reward, punishment, satiety, habit formation and addiction[137].

1.4.2 Gut microbiota in anorexia nervosa

The gut microbiota is recognized as an important modulator of the host metabolism[138–141] and appetite[55]. Various mechanisms have been proposed for the functional impact of gut microbiota on host metabolism including promotion of energy harvest capacity from the diet, modulation of polysaccharides and bile acid metabolism through microbial enzymatic activities, and enhancement of triglyceride production and transport[142,143]. Furthermore, following nutrient provision, bacterial peptides can stimulate hypothalamic pro-opiomelanocortin (POMC) expressing neurons directly and/or through stimulation of gut hormones, and thus can regulate host satiety[55]. Moreover, accumulating data suggest that behavior and brain neurochemistry can be influenced by gut microbiota[37,144]. In the absence of gut microbes, mice show elevated turn-over of neurotransmitters, including dopamine, noradrenaline, and serotonin, as well as reduced expression of genes related to synaptic transmission[144]. In mice devoid of gut microbes, strain-dependent alterations in anxiety-like behavior and locomotor activity have been reported[45,64,144–147]. Interestingly, there is evidence that the gut microbiota can influence the reward-mediating systems of the host. Depletion of gut microbiota enhances the sensitivity to cocaine reward and locomotor sensitization to repeated dose of cocaine[148], and a study in ADHD patients shows that alterations in the gut microbiota is associated with reduced ventral striatal responses measured by fMRI during reward anticipation[149].

So far, few studies have reported dysbiotic gut microbiota in anorectic patients. Using a culture-based approach, 19 previously unknown species were identified from a single anorexia patient[150]. More in-depth culture-independent studies suggest that fecal microbiota diversity in patients with anorexia is reduced compared to healthy controls.[151,152] Notably, levels of depression and anxiety were shown to be associated with composition and diversity of the intestinal microbiota[151]. In another study comparing the fecal profiles of obese, anorectic and normal individuals, anorectic patients displayed elevated levels of the archaeon *Methanobrevibacter smithii which is associated with* efficient microbial fermentation and increased energy yield[153]. The levels of mucin-degraders *Verrucomicrobia* and *Bifidobacteria* were also reported to be increased in anorectic patients in comparison with normal weight participants. It was previously shown that the abundance of *Akkermansia muciniphila,* a mucin-degrader bacterium, is inversely correlated to body weight[154,155]. Furthermore, anorectic patients exhibited reduced levels of *Roseburia spp.,* a SCFA producing subspecies. After weight gain, microbial diversity was increased but the perturbations in the intestinal microbiota, SCFA profile and several gut symptoms were not improvd[156]. Whether the

alterations in the gut microbiota of anorectic patients precede the onset of symptoms or they appear during the illness as secondary effects is not known. Nevertheless, calorie restriction[157] and endurance exercise[158,159] were shown to modulate the diversity and composition of gut microbes, therefore dieting and excessive exercise in anorectic patients could leave their imprints on the gut microbiota.

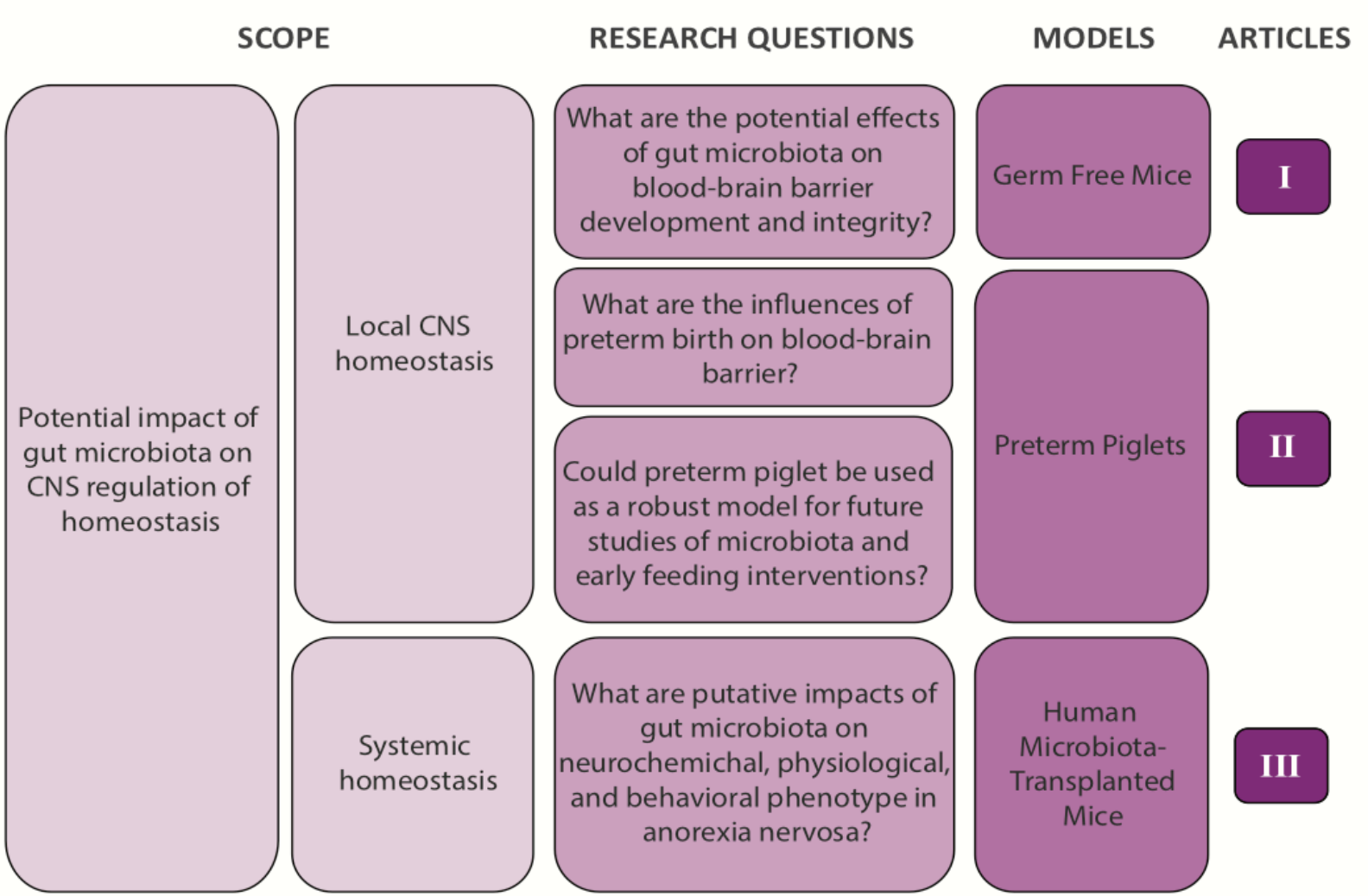
2 AIMS

Inspired by what is known about the ability of the gut microbiota to modulate tissue barriers and based on the existence of a gut microbita-brain axis, we aimed to identify the potential role of gut microbiota on CNS regulation of local and systemic homeostasis. The specific aims of each paper were:

Paper I: To assess the influence of gut microbiota on intrauterine development of the BBB as well as on its integrity and function during adulthood using germ-free mouse model.

Paper II: To characterize the BBB in a porcine model of preterm birth for further investigation of potential beneficial effects of microbiota and diet interventions on brain development and maturation under preterm conditions.

Paper III: To investigate the relevance of gut microbiota for physiological, behavioral, and neurochemical phenotype in anorexia nervosa.



Overview of the research framework

3 METHODOLOGICAL HIGHLIGHTS

3.1 ANIMAL MODELS

3.1.1 Germ-free or axenic mice

"Germ-free" or "axenic" mice are microbiologically sterile animals (as determined within the limitations of the available detection methods) that are raised in isolators under very strict handling procedures. The use of germ-free animals is a valuable approach which allows for investigation of microbe-microbe and microbe-host interactions. Germ-free animals can be selectively colonized by one or more bacterial species. Once inoculated with known microbial population the animals are referred to as "gnotobiotic". "Gnotobiotic" is derived from the greek "gnotos" (known), and "bios" (life). The terms "germ-free" and "gnotobiotic" are sometimes used interchangeably.

In **project I**, we used germ-free mice obtained from Core Facility for Germ Free Research (CFGR) at Karolinska Institutet. All animals had ad libitum access to autoclaved R36 Lactamin chow and sterile water and maintained under 12h light/dark cycles. As controls, specific-pathogen-free (SPF) adult mice were used. The SPF mice possess commensal bacteria but are free from known pathogens that causes clinical and subclinical infections[160]. The SPF mice are regularly screened for pathogens (3 or 4 times a year) as recommended by Federation of Laboratory Animal Science Associations. In order to confirm that the alterations observed in our experiment were mediated by microbiota and/or microbial metabolites we conventionalized (CONV) a group of germ-free adult mice with fecal samples from the SPF mice. Another group was treated with bacterial strains that produce SCFAs, and a third group recieved the sodium salt of butyrate.

Emergence of germ-free animals

In 1885, Louis Pasteur proposed that animals devoid of bacteria would not be able to survive[161], pointing out to the importance of the symbiosis between the microbes and the host. About ten years later, for the first time, Nuttall and Thierfelder reported successful rearing of germ-free derived guinea pigs for more than one week[162]. Today, thanks to the advances of germ-free technology, we know if proper environmental conditions are provided, animals could survive in the absence of co-habiting microbes, albeit with certain physiological and behavioral alterations[161]. Germ-free technology flourished about a century after the generation of the first germ-free mammals through the work of three independent research groups. James Reyniers and the coworkers at the University of Notre Dame were the

pioneers to generate germ-free rodents[163]. In parallel, a group led by Bengt Gustafsson at Lund University in Sweden began a germ-free research program and succeeded to design novel stainless steel germ-free rearing isolators (Fig. 3)[164]. The third group was headed by Masasumi Miyakawa at the University of Nagoya in Japan[165].

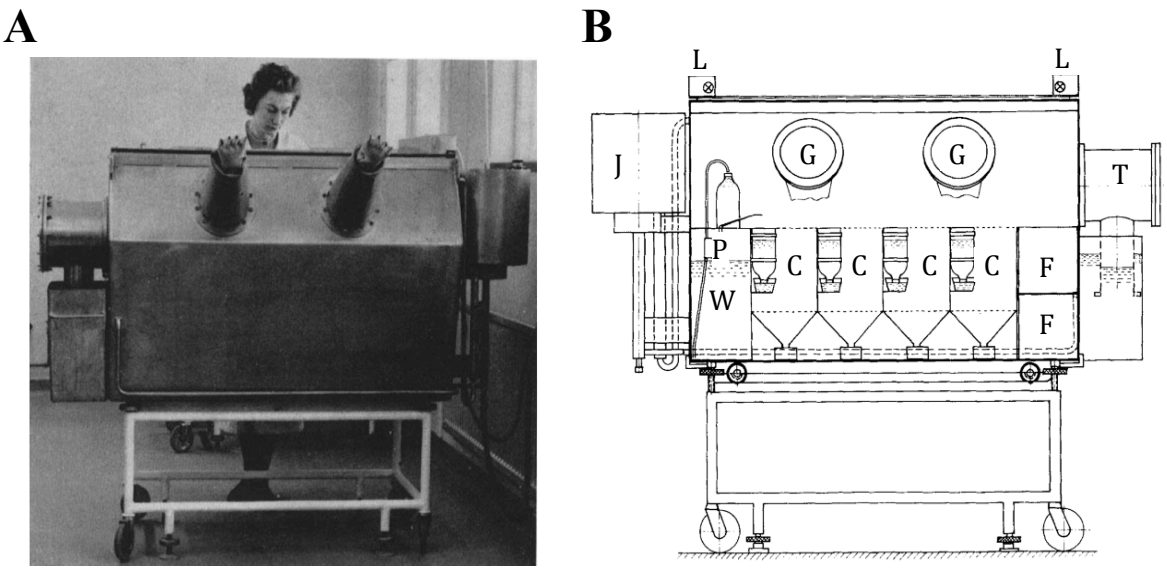


Figure 3. Gustafsson's stainless steel germ-free isolator. (A) Exterior of germ-free isolator. (B) Cross-section of germ-free isolator. L. lighting frame, J. air sterilizer, W. water tank, P. water pump, C. cages, G. glove ports, F. food canisters, T. Food autoclave and transfer unit. Adopted from Gustafsson BE, Ann N Y Acad Sci, 1959. Copyright 2006. With permission from Wiley Publications.

Derivation and maintenance of germ-free animals

The methodological approach to derive germ-free or axenic animals has not been changed drastically since the generation of the first germ-free rodents by Reyniers. In order to initiate the first colony, the pups are delivered by C-section in a sterile manner to avoid acquisition of microbes from the environment, mother's vagina or mother's skin. Following birth, the pups are transferred into sterile isolators and hand-reared1[58–160]. Thereafter, the next colonies could be interbred and born inside the isolators. An alternative method is to transfer embryos at 2-cell stage into pseudo-pregnant germ-free recipients. This method eliminates contaminations associated with vertical transmission in C-section method[161,162]. The germ-free mice receive sterile food and water. Any other material that is brought into the isolators including bedding and experimental tools should be devoid of microbes. The cages are regularly swabbed and the feces samples are analyzed to assure that the animals remain germ-free inside the isolators[157–160].

Characteristics of germ-free mice

Germ-free mice deviates from conventional mice in various anatomical, physiological and behavioral aspects. Some of the characteristics of germ-free mice are summarized in Table 1.

Table 1. Characteristics of germ-free mice

No.	Category	Phenotype
1	**Anatomy**	Enlarged cecum[166] Smaller heart, liver, and lungs[167]
2	**Intestinal morphology**	Thinner intestinal wall[168] Decreased number of villi[167] Fewer and smaller Peyer's patches[168] Thinner mucus layer[169] Fewer number of goblet cells[168]
3	**Enteric neural network**	Reduced myenteric neurons[170,171] Decreased enteric neural network[170,171]
4	**Metabolism**	Decreased basal metabolic rate, body fat percentage, circulating levels of adiposity hormones and glucose[142] Resistance to diet-induced obesity[172,173]
5	**Behavior**	Strain-specific alterations in anxiety-like behavior[45,64,144–147] Strain-specific alterations in locomotor activity[144,146] Contradictory data on social preference[145,174]
6	**Central neurochemical changes**	Elevated turn-over of monoamine neurotransmitters in striatum[144] Region-specific alterations in BDNF transcription[144,175]
7	**Immune system**	Impaired development of gut-associated lymphoid tissues[176,177] Defective antibody production[168] Deficient expression of antimicrobial proteins[168]

One of the anatomical hallmarks of the germ-free mice is the enlarged cecum (Fig. 4). The cecum in germ-free mice contains considerably higher amount of liquid content[166,178]. Cecum enlargement has been associated to osmosis caused by accumulation of dietary fibers, undegraded mucus, and sulfate-containing glycoproteins.

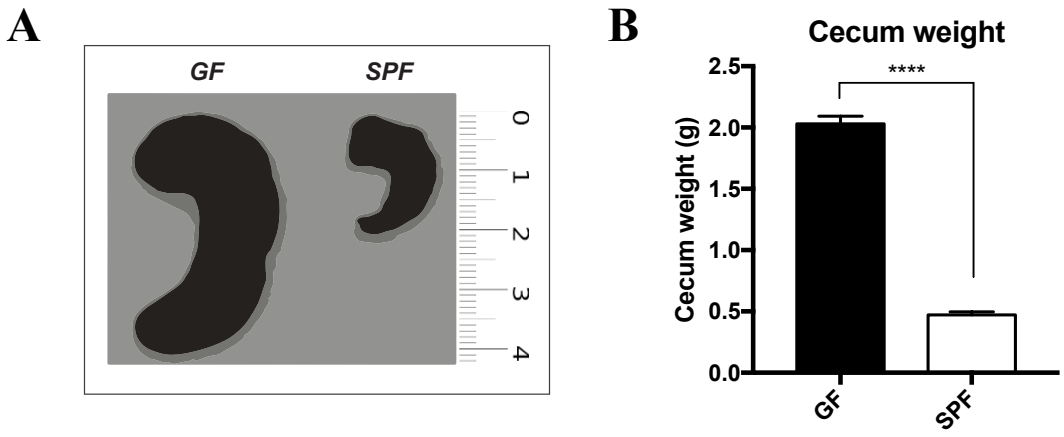


Figure 4. Enlarged cecum in germ-free mice. (A) Representative macroscopic illustration and (B) weight measurement of the cecum in germ-free (GF) mice in comparison to specific pathogen-free (SPF) controls. n= 12, **** P< 0.0001, Error bar represents S.E.M.

Limitations of germ-free animals

Similar to any other experimental tool, germ-free model systems are required to be thoroughly understood to be able to use them for suitable purposes. Notably, the conditions inside the sterile isolators in which laboratory germ-free animals are bred and maintained are far from the conditions in the outside world where animals (and human) have intimate relationship with environmental microorganisms as well as with their own resident microbes. Rare exceptions were two patients maintained in sterile hospital rooms due to severely compromised immune system: David Vetter who became known as the "bubble boy" (Fig. 5), and Ted DeVita[179,180]. An alternative method to germ-free animals, is the use of antibiotics to deplete the gut microbiota. However, antibiotic treatment has its own limitations as some antibiotics could confer direct effects on the host.

Another limitation of working with germ-free animals is that it burdensome to perform procedures that require a lot of handling or specific tools inside the isolators. Usually, such procedures should be planned at the end of the experiment when the animals could be taken out of the isolators. Despite the limitations, germ-free animals are proven to be valuable experimental models to investigate microbe-host interactions and contributed enormously to the knowledge we have today about the cross-talk between the microbes and the host.

A

B

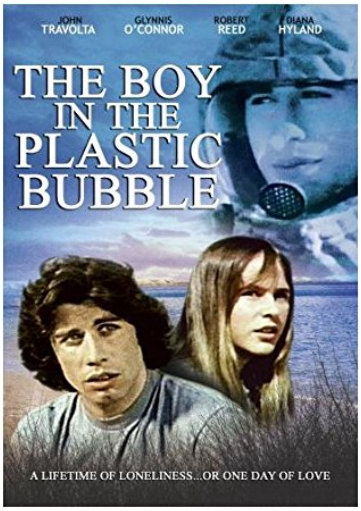


Figure 5. The boy who was raised inside an isolator. (A) David Vetter (left) and immunologist Rafael Wilson (right) who created the isolator to keep the newborn germfree until bone-marrow transplantation could be performed. Photo: Courtesy Baylor College of Medicin Archives. (B) public recognition of the "bubble boy" in a movie directed by Randal Kleiser (1976).

3.1.2 Porcine model of preterm birth

In **paper II**, we took a step forward into an experimental animal model, which in comparison to rodents, shows greater physiological and developmental similarities to human. Pigs are monogastric animals and have a digestive system that highly resembles to human. Therefore, they are widely accepted as model for nutritional and gastrointestinal studies. Moreover, pig brain is convoluted, and has higher connectivity and complexity compared the lissencephalic brain in rodents. Another advantage of the pig model is that due to their larger body size compared to rodents, it is relatively easy to handle and perform surgical experimentations on them. Collectively, these properties make pig a translational model for our study concerning BBB maturation under preterm condition and in response to early feeding.

Our preterm pigs were delivered at 90% of gestation (day 106) by caesarean section. A group of animals were sacrificed within eight hours after birth, and another group was reared for five days at the Neonatal Pig Research Center, Copenhagen University. Compared to term piglets, these animals display signs of immaturity in different organs. Due to respiratory distress, enteral food intolerance, impaired thermoregulation, and poor locomotion, preterm piglets need to be housed in intensive care units[181]. Similar to preterm infants, preterm piglets spontaneously develop microbiota- and diet-associated disease i.e. necrotizing enterocolitis (NEC)[182]. Previous investigations of the luminal bacteria indicated that gut

colonization in preterm piglets is distinct compared to the term controls and is marked by decreased abundance of *Lactobacilli* spp.[183]

Parenteral and enteral feeding

Infants who survive NEC were shown to have higher risk for neurodevelopmental impairments[184–186]. Previous studies suggest that enteral feeding with infant formula predisposes to NEC in human preterm infants[33,187], as well as in preterm pigs[182], whereas early gradual feeding with bovine colostrum is protective against NEC in preterm piglets[188]. To assess the potential effects of feeding on the development of the brain barriers in preterm pigs, we divided the animals into two groups: one received total parenteral nutrition (PAR, n = 10 per group) through vascular catheters (4Fr, Portex) in the umbilical cord, and the other group received enteral feeding via orogastric tubes (6Fr, Portex, Kent, UK) + supplementary parenteral nutrition (ENT, n = 10 per group). The PAR groups received 96 ml/k/d of modified Kabiven intraarterially (Fresenius-Kabi, Bad Homburg, Germany) on day 1 increasing to 144 ml/kg/d on day 5. The ENT groups received 16 ml/kg/d of intragastric bovine colostrum (Biofiber Damino, Gesten, Denmark) on day 1 slowly increasing to 64 ml/kg/d on day 5 with decreasing supplements of intra-arterial Kabiven to ensure total isoenergetic levels throughout the five days. In another experiment, the animals were fitted with vascular catheters and oragastric feeding tubes. Enteral feeding over the first five days after birth consisted of rapidly increasing volumes of infant formula, containing maltodextrin as a main carbohydrate source. The first 48 h, pigs were fed 24-48 ml/kg/d of enteral nutrition + 32-48 ml/kg/d of intra-arterial Kabiven (Fresenius-Kabi). Subsequently, parenteral nutrition was discontinued and piglets were fed increased volumes of enteral nutrition (80-120 ml/kg/d) until euthanasia.

Limitations of porcine models of preterm birth

Long gestational period in pigs is a limiting factor for the design of the experiments. Full term gestation takes 117 days in Danish production herds. In case of preterm pigs, due to immaturity, a lot of care and a dedicated facility is required during the first days following birth. This includes transfer into oxygenated and thermo-regulated incubators, activity monitoring, and fitting umbilical catheters and orogastric tubes for feeding.

Previous observations have led to the estimation that preterm piglets delivered at 90% gestation correspond to preterm infants born at 75% gestation[189]. However, this estimation is based on gastrointestinal characteristics and do not apply to other tissues such as the brain. In fact, the brain in preterm piglets is more mature relative to preterm human infants[181].

Therefore, the interpretation of the results in neurodevelopmental studies in preterm piglets should be made with careful consideration.

3.1.3 Human fecal microbiota-transplanted mice

In animals, the use of germ-free rodents has largely contributed to unraveling the causality of microbiota in certain diseases. However in human, microbiota research is mainly limited to studies of alterations in diversity and composition which only illustrate correlations rather than causality. To tackle this problem, human microbiota-transplanted (HMT) mice are being used more commonly. The HMT mice are obtained through transplantation of human fecal microbiota into germ-free or antibiotic-treated mice (with depleted gut microbiota). Whether features of pathophysiological phenotype observed in the donor could be recapitulated in the recipient mice is then investigated in comparison to mice inoculated with microbiota from healthy controls. Analysis of human microbiota before and after the transplantation indicates that approximately 85% of the microbiota at the genus level could be successfully transferred into the recipient mice[190]. Furthermore, various studies have reported that behavioral and pathophysiological phenotype of the human donor could be reproduced in the recipient mice through microbiota transplantation. This includes obesity[138], childhood asthma[191], and pregnancy metabolic syndrome[192]. Gordon and coworkers were pioneers in using microbiota transplantation method in combination with dietary regimens to understand the role of the gut microbiota in defining the host's nutritional status, especially under conditions such as obesity and under-nutrition which impose considerable burden on global health. In their approach, they inoculate germ-free mice with microbiota collected from the donors sharing the characteristics of interest, and feed them with the diet consumed by the corresponding donor or derivatives of those diets. The features of the donor's phenotype that could be transmitted are then investigated and the metabolic and signaling networks as wells as the effect of dietary regimens on microbe-host and microbe-microbe interactions are identified[138,139].

In **paper III** we used HMT mice to investigate the relevance of gut microbiota for disease phenotype associated with anorexia nervosa. We transplanted fecal microbiota from one anorectic patient and one healthy control into germ-free mice by oral gavage. Following fecal transplantation, we housed the two groups in separate isolators for 10 weeks. During this period we monitored food intake and weight gain. At the end of the experiment we assessed anxiety-like behavior and locomotion with open-field test. Afterwards, different tissues were harvested and stored for analysis.

Limitations of HMT mice

When working with HMT mice, it should be considered that microbiota transplantation from human individuals, only captures a snapshot of the microbiota in a fixed point of time under certain conditions. This snapshot can be possibly affected by various factors such as diet, antibiotic treatment, and age of the subject, as well as sampling and storage methods[193], which can complicate the interpretation of the outcomes. Therefore, careful consideration should be given in terms of potential confounding factors.

Moreover, the coevolution of the mammalian host and the microbiota favors genetic and physiologic adaptations that maximize the efficiency of the symbiotic relationship and leads to development of host-specific microbial communities and mechanisms [194–197]. An example of such mechanisms is the ability of the bacteria to form epithelial biofilm which is strictly dependent on the host origin. Monocolonization of the mice with strains of *L. reuteri* only induces epithelial adherence and biofilm formation if the strain is isolated from murine host[194]. Moreover, metagenome analysis comparing human and murine microbiome suggest that they only share ~10% of the microbiome at genus level and 14.2% at species level[198]. Germ-free mice colonized with human microbiota exhibit lower levels of innate immune cells, declined expression of antimicrobial peptides, and overall less mature intestinal immune response relative to germ-free mice colonized with murine microbiota[196].

Despite the substantial differences between human and mice microbiota at species level, they still share great similarities at higher taxonomic levels with Firmicutes, Bacteroidetes, and Proteobacteria being the predominant phyla in both hosts.[198,199]. In addition, the 20 most abundant core bacterial genera in mice, shows 65% similarity to that of human[198] (Fig 6). Therefore, despite the limitations of the HMT mice, this model can still serve as one of the best models to study dybiosis-associated diseases.

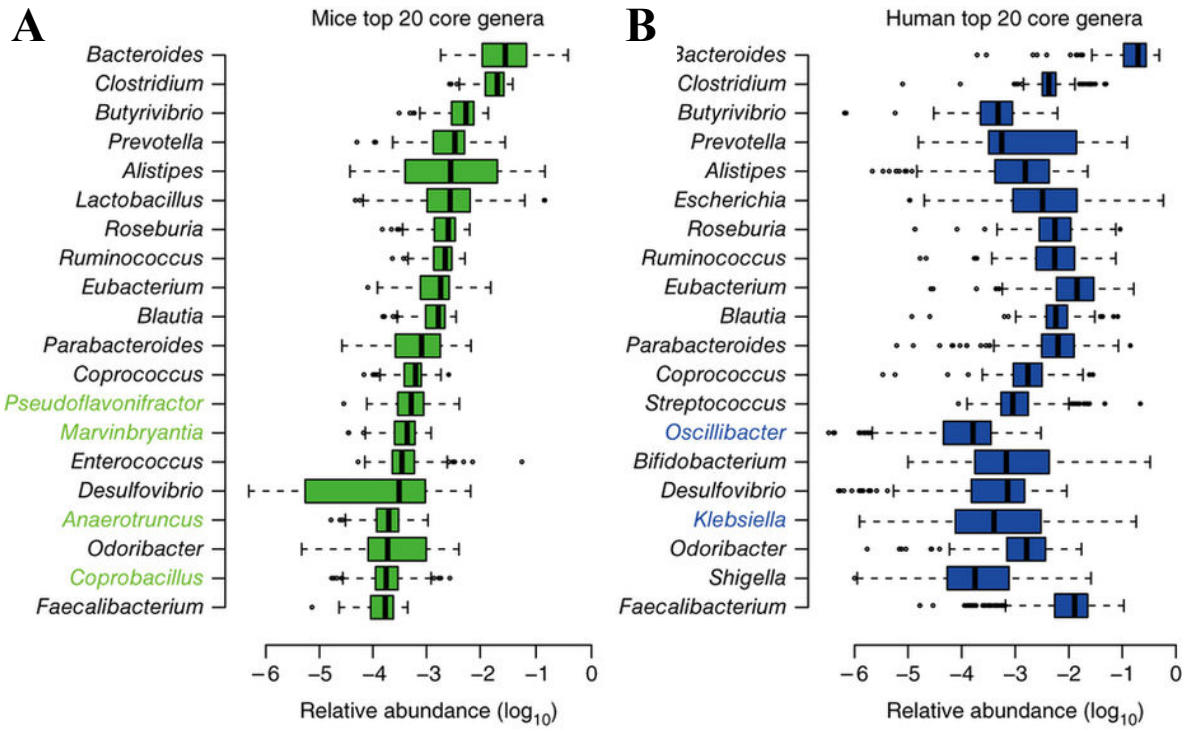


Figure 6. Top 20 core bacterial genera in mouse and human microbiota. Adopted from Xiao et al., Nature Biotechnolog, 2015. Copyright 2015. With permission from Wiley Publications.

3.2 PERMEABILITY ASSESSMENT OF THE BRAIN BARRIERS

In **Paper I**, we used three different methods to assess BBB permeability in germ-free mice compared to mice with normal flora: (i) Evans blue perfusion, (ii) Positron emission tomography imaging with [11C] raclopride, and R4A antibody injection. In **paper II**, we assessed blood-CSF barrier permeability by measuring CSF:blood ratio of endogenous protein (albumin) and an exogenous tracer (raffinose).

Evans blue perfusion

Evans blue is a an azo dye with high affinity for serum albumin and is commonly used as a chemically inert tracer for the assessment of BBB permeability. It fluoresces with excitation peaks at 470 and 540 nm and an emission peak at 680 nm. Extravasation of Evans blue into brain parenchyma is thought to reflect albumin leakage and increased BBB permeability. Albumin has a high molecular weight (66.5 KDa) and is poorly transported cross the BBB under physiological conditions. Evans blue perfusion is a reliable and inexpensive method to visualize disruption in the BBB and it has been used since the discovery of BBB by Ehlrich

and Goldmann. We utilized this method with a controlled perfusion rate to avoid damaging the capillaries.

Positron emission tomography (PET) imaging with [11C]raclopride

Raclopride is a synthetic compound that acts on dopamine D2 receptors as an antagonist. Radiolabelled raclopride is used in *in vivo* PET imaging, primarily for the assessment of binding capacity of dopamine D2 receptors, useful for diagnosis of movement disorders. But here, we used raclopride as a tracer to assess BBB permeability. Following intravenous injection of the tracer, we measured the regional tissue radioactivity concentrations. Concentrations in the initial flow phase represent the presence of the radioligand in the whole brain due to BBB permeability as opposed to concentrations in the later phase of the activity curve which indicate binding capacity to dopamine D2 receptors.

R4A antibody

R4A is an anti-DNA antibody which cross-reacts with N-methyl D-aspartate receptor (NMDAR) and mediate neuronal death only if the BBB is breached[200,201]. We assessed the morphology and number of neurons in the hippocampus following intravenous injection of R4A to germ-free versus specific-pathogen free mice.

CSF:blood raffinose

Raffinose is a small (504 Da) hydrophobic plant trisaccharide. Since human and monogastric animals including pigs do not possess the enzyme (α-galactosidase) to breakdown raffinose, we used it as an inert tracer to measure blood-CSF barrier permeability in pigs. Raffinose concentrations in collected plasma and CSF samples were quantified by liquid chromatography tandem mass spectrometry.

3.3 MICROARRAY

Microarray is a robust and reproducible high through-put method for detecting relative gene expression levels. In this method, mRNA molecules isolated from both experimental and control samples are reverse-transcribed into complementary DNA (cDNA) and differentially labeled with fluorescent dyes. The samples are then applied to a DNA chip which contains large number of DNA hybridization probes at defined positions. Following the hybridization step, the chip is scanned to measure the expression of each gene. We used Mouse Gene 2.1 ST Array Plate (Affymetrix, 902140) to compare expression profiles between mice harboring anorexic microbiota and mice transplanted with healthy microbiota in micro-dissected

nucleus accumbens and hippocampus samples. Following RNA isolation, Agilent RNA ScreenTape assay in combination with the 4200 TapeStation system was used for quality control. Raw data were processed in Affymetrix Expression Console software (v.1.4.1) using the RMA analysis method. The data was then transferred to Qlucore Omics Explorer 3.3 (Qlucore AB, Lund, Sweden) for further analysis. Heatmaps and PCA plots were generated following statistical filtering.

Despite the comprehensive sequencing information derived by microarray analysis, this method cannot be used for detection of structural variations and isoforms, and discovery of novel transcripts, because the design of the hybridization probes is based on prior sequencing knowledge. Nevertheless, it is a cost-efficient and widely-used method, suitable for comparative gene expression.

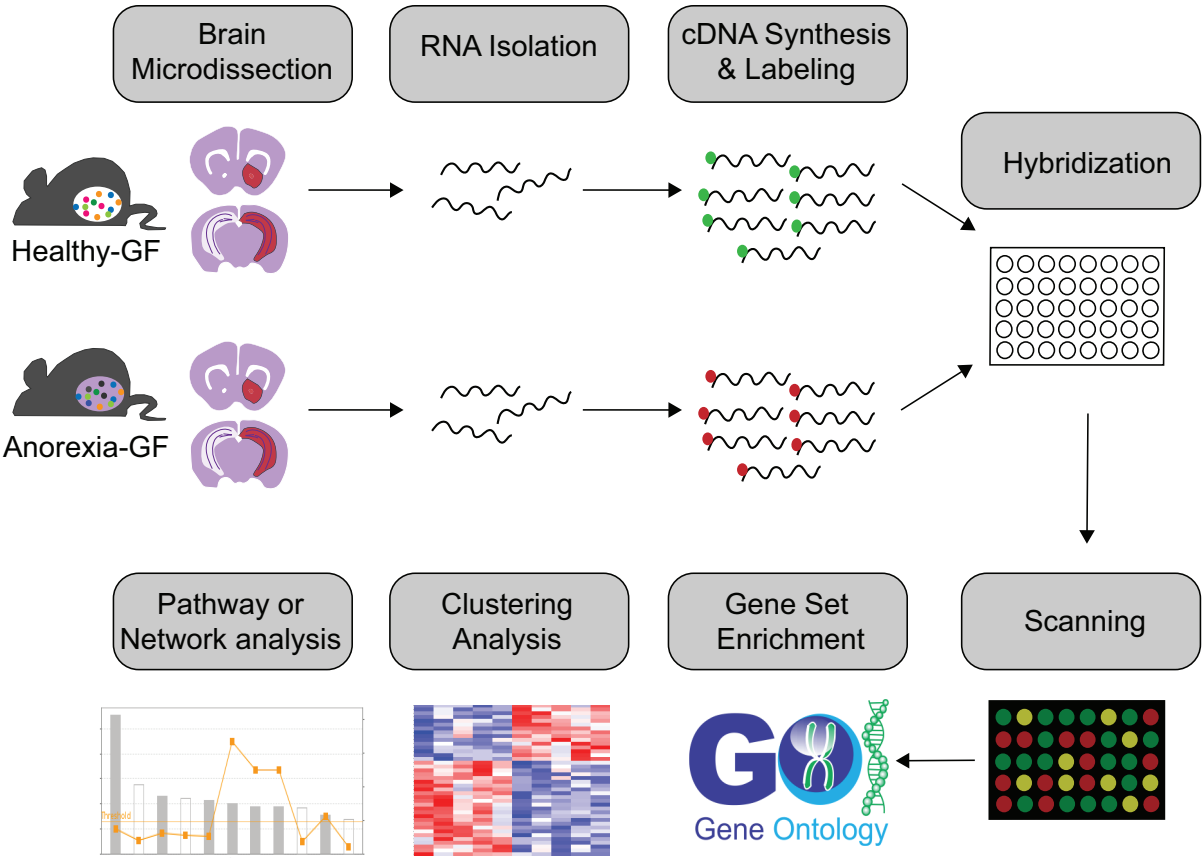


Figure 7. Schematic illustration of microarray workflow.

4 RESULTS AND REFLECTIONS

More detailed description of the results of each study can be found in the respective article/manuscript. Here, the main findings are presented briefly.

4.1 Maternal gut microbiota mediates intrauterine development of blood-brain barrier

Previous observations suggest that the gut microbiota modulates barrier permeability and function in the intestines[98,101] and in testis[103], but the putative effects on the BBB were not investigated. In paper I, we assessed the impact of gut microbiota on the development and function of the BBB, using the germ-free mouse model. The development and maturation of the BBB begins during intrauterine stage and continues progressively into early postnatal period[82]. Based on the evidence that the fetus is exposed to maternal microbiota and microbial metabolites[15,16], we explored the impact of maternal gut microbiota on fetal BBB development. We injected an immunoglobulin G2b (IgG2b) antibody labeled with infrared dye into dams during timed pregnancies and measured the presence of the antibody in the brains of the embryos. First, we showed that in embryos of dams with normal gut flora, starting around E15.5-E17.5, the antibody was confined to the developing vasculature and did not penetrate into the brain parenchyma. This was in agreement with previous observations showing that BBB becomes functional during the late stage of intrauterine life[84,88]. In contrast, the antibody was diffused into brain parenchyma in E16.5 embryos of germ-free dams. Furthermore, expression of tight junction protein occludin was significantly lower in germ-free embryos compared to controls. Our results suggest that coinciding with increased nutritional demand in late pregnancy, maternal gut microbiota contributes to the maturation of an intact BBB in order to strictly control molecular trafficking to the brain in the developing offspring.

4.2 Gut microbiota influences blood-brain barrier functional permeability and expression of tight junction proteins in adult mice

In order to assess whether impairment of BBB in the absence of gut microbiota persists into adulthood, we measured BBB permeability in adult germ-free mice in comparison to mice with normal flora. Using Evans blue technique, we showed increased BBB permeability in hippocampus, striatum, and prefrontal cortex of germ-free mice, indicated by penetration of Evans blue into the parenchyma. The increased BBB permeability was also displayed as elevated [^{11}C] raclopride uptake in the brain by *in vivo* PET imaging, and increased neuronal death caused by penetration of intravenously injected R4A antibody. We next investigated the expression and topography of BBB tight junction proteins by western blot,

immunofluorescence staining, and transmission electron microscopy in our animal model. Our data indicated that the increased BBB permeability in germ-free mice was accompanied by disruption in occludin and claudin-5.

To confirm whether the alterations observed in BBB are mediated by the gut microbiota and bacterial products, we introduced either active or heat-inactivated fecal microbiota into the germ-free mice through a single oral gavage. The mice were left for 14 days before being sacrificed. Then, we assessed albumin leakage into parenchyma following Evans blue permeability test. The active microbiota induced reduction in the BBB permeability, whereas Evans blue could still be detected in the brain parenchyma of the recipients of inactive flora (Fig. 8). Moncolonization of the germ-free mice with bacteria known to produce SCFAs or treatment with butyrate salt, one of the three SCFAs, also induced effects similar to colonization with active flora. SCFAs were previously shown to enhance the integrity of intestinal epithelial barrier[202,203].

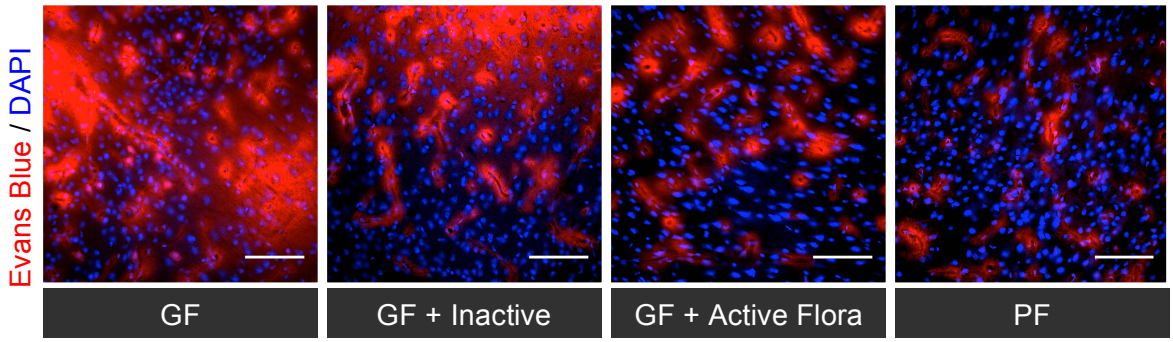


Figure 8. Colonization of germ-free mice with active flora alleviates impaired BBB permeability. Representative images of Evans blue dye (shown in red) in the prefrontal cortices of germ-free (GF), GF colonized with heat-inactivated microbiota (GF + Inactive Flora), GF colonized with active flora, and pathogen free (PF) mice. Cell nuclei were stained with DAPI (shown in blue). Scale bars, 50 μm.

4.3 Preterm birth is associated with structural and functional deficits of developing brain barriers in pigs

Preterm birth is associated with impaired development and maturation of many organs including the brain[204]. During the critical early postnatal period, normal brain growth and maturation may be negatively affected by the prematurity-related factors such as nutrient deprivation or a serious infection[205]. In **paper II,** we used a preterm porcine model to study the effects of gestational age, early feeding, and infection on brain barriers following preterm birth. First, to characterize BBB structure in our model, we investigated BBB molecular and cellular components. Our protein analysis data indicated that, at birth, BBB-associated

proteins including occludin, ZO-1, VE-cadherin, GLUT-1 and MCT-1 were differentially regulated in striatum and hippocampus of preterm piglets compared to term controls.

Astroglial end-feet provides structural support to the endothelial cells. We analyzed the astrocyte perivascular coverage by measuring the percentage of overlap between astrocytes (stained with anti-GFAP antibody) and blood vessels (stained with anti-laminin antibody) in microscopic images (Fig. 9A). Our data indicate that astrocyte-vessel overlap was approximately 10% decreased ($p < 0.0001$, Fig. 9B) in the hippocampus (not in striatum) of preterm piglets relative to term controls. The decreased astroglial perivascular coverage was not due to differences in capillary density as the laminin-stained areas were similar among the groups (data not shown). In agreement with the immunofluorescent staining data, western blot analysis of auqaporin-4 (AQP-4), a water channel protein which is highly expressed by astrocyte end-feet[206], indicated decreased protein expression in the hippocampus of preterm piglets ($P<0.05$, Fig.9 C-D).

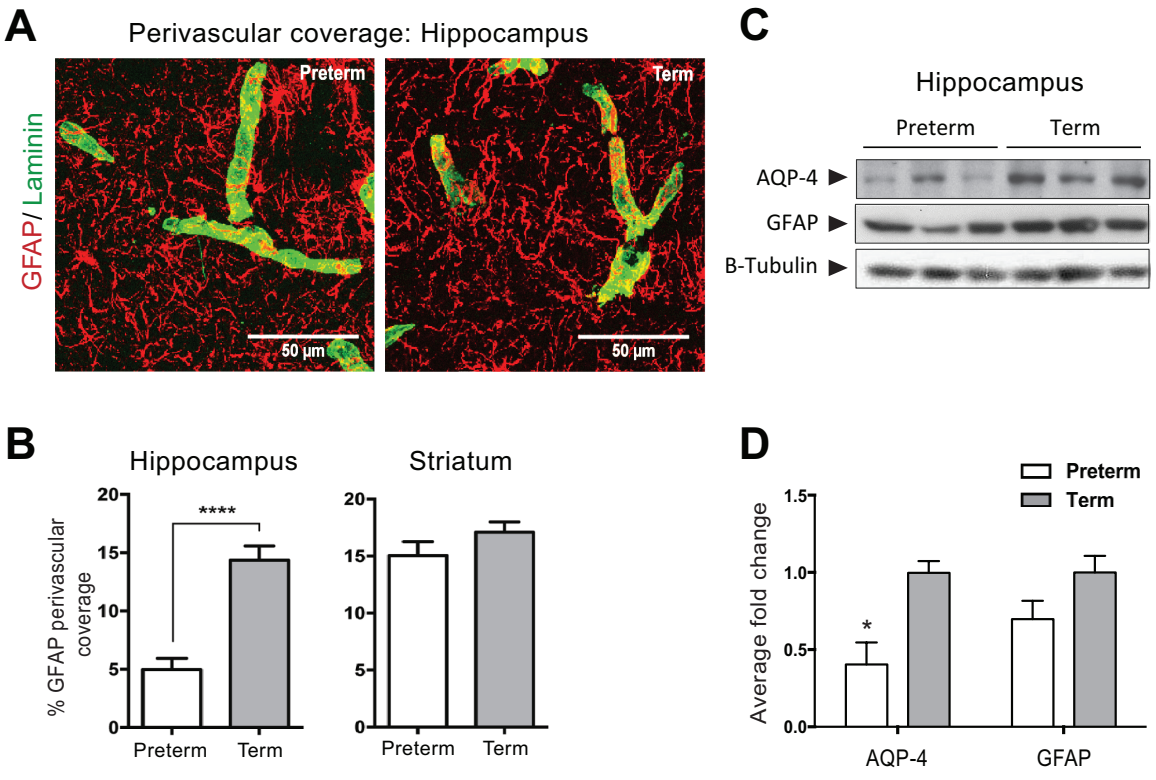


Figure 9. Decreased astroglial perivascular coverage in the hippocampus of preterm piglets. (A) Representative images of astrocyte (GFAP, red) and blood vessel (laminin, green) co-stained hippocampal coronal tissue sections from preterm and term newborn pigs.(B) Quantification of GFAP and laminin overlay as a measure of perivascular astrocyte coverage (n = 5–10 per group). (C) Representative Western blots and (D) densitometric quantifications of AQP-4 and GFAP in the

hippocampus of preterm and term newborn pigs (n = 5–10 per group). Values are normalized to the reference protein β-tubulin. Mean term levels are assigned a value of 1 and preterm levels scaled accordingly. All data are presented as means with standard errors. * $p < 0.05$, **** $p < 0.0001$. Modified from paper II. Copyright 2018. With permission from S. Karger AG, Basel.

We then assessed blood-CSF barrier permeability by measuring CSF-plasma ratio of albumin as well as CSF-plasma ratio of raffinose following intra-arterial administration of 5 mL/Kg body weight of raffinose. Preterm piglets displayed elevated albumin and raffinose ratios compared to term controls suggesting increased nonselective transcellular transport across blood-CSF barrier.

Collectively, these data suggest that there is a deficit in the brain barriers following preterm birth which might enhance the vulnerability of the developing brain to inflammation and nutrient deprivation associated with preterm birth. One of the severe complications of preterm birth is necrotizing enterocolitis (NEC, inflammation in the intestines) which affects up to 7% of preterm infants[207]. The onset of the disease is in part due to enhanced reactivity of the premature intestinal mucosa to microbial colonization during the first days following birth[33]. Notably, the survivors of the NEC display an increased risk of neurodevelopmental impairments[184–186].

In an independent experiment, we fed preterm-born piglets with infant formula for five days. Formula feeding was previously shown to predispose to NEC in human preterm infants[33,187] as well as in preterm pigs[182]. We evaluated NEC-like lesions in harvested colon and intestine samples and divided the animals into three groups based on their NEC scores (Fig. 10): 1. Healthy (highest NEC score 1–2, $n = 49$), 2. Moderate NEC (highest score 3–4, $n = 39$), or 3. Severe NEC (highest NEC score 5–6, $n = 43$). Assessment of the inflammatory markers in the intestinal tissue and in circulation showed that sever NEC is associated with local and systemic inflammation. The levels of IL-8 in the small intestine and IL-6 in the plasma were significantly increased in piglets with sever NEC compared to unaffected counterparts.

We then evaluated blood-CSF barrier permeability by measuring CSF-plasma ratio of albumin and raffinose. Our data indicated that NEC severity is associated with increased blood-CSF barrier permeability. Furthermore, with histopathalogical analysis, we identified a characteristic pattern of pyramidal neuron degeneration in the hilus and CA regions of the hippocampus in piglets with sever NEC. We also showed increased IL-6 levels in the hippocampus in line with the elevated levels in the circulation. Taken together, we propose

that sever NEC in preterm piglets is associated with brain barrier dysfunction, inflammation and neural damage. Interestingly, in another experiment, we showed that in the absence of NEC, preterm piglets treated with either partial enteral or total parenteral nutrition for five days had normalized expression levels of endothelial tight junctions (except ZO-1) and transport proteins compared to the term controls, while the astroglial perivascular support remained lower in the hippocampus of preterm pigs.

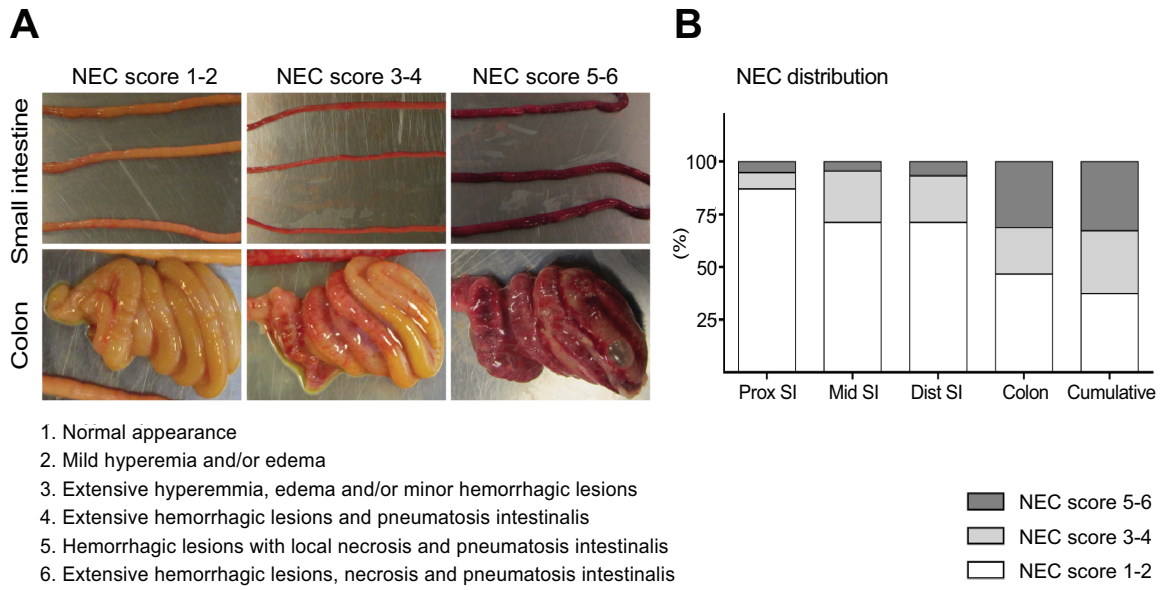


Figure 10. NEC lesion evaluation in formula-fed preterm piglets. (A) Representative necropsy pictures of small intestine and colon in animals with No (score 1-2), moderate (score 3-4), or sever (score 5-6) NEC lesions (upper). Scoring system consists of 6 grades of increasing pathology (lower). (B) Proportion of pigs with NEC distributed by lesion severity across gut segments and accumulated (n = 39–49 per group). Modified from paper II. Copyright 2018. With permission from S. Karger AG, Basel.

4.4 Microbiota transplantation from an anorectic individual induces anorexia-like symptoms in germ-free recipient mice

The gut microbiota is capable of secreting or modulating the production of molecules that affect both energy balance and energy stores[142,173,175,208]. Most of the studies that have investigated the role of gut microbiota in the context of energy homeostasis and metabolism have focused on obesity and diabetes, probably due to the profound and pervasive effects of these conditions on global health. Other diseases with altered metabolism and homeostatic function have received less attention. In paper III, in order to explore the impact of gut microbiota on host homeostasis on a systemic level, we focused on anorexia nervosa, a

serious eating disorder characterized by restricted energy intake, significantly low body weight, fear of gaining weight, and distorted perception of one's body. Microbiota dysbiosis has been documented in anorectic patients[150–153,156]. In a pilot study, we transferred gut microbiota from an anorectic patient and a sex-matched healthy individual into germ-free mice (n=10 per group). Fecal microbiota transplantation was performed through a single oral gavage, and following the transplantation, animals were maintained in isolators for 10 weeks. During this period, body weight and food intake were recorded twice per week. At the end of 10 weeks, mice were taken out of the isolators and an open-field test was performed. The mice were then sacrificed and various tissues were collected for further analysis.

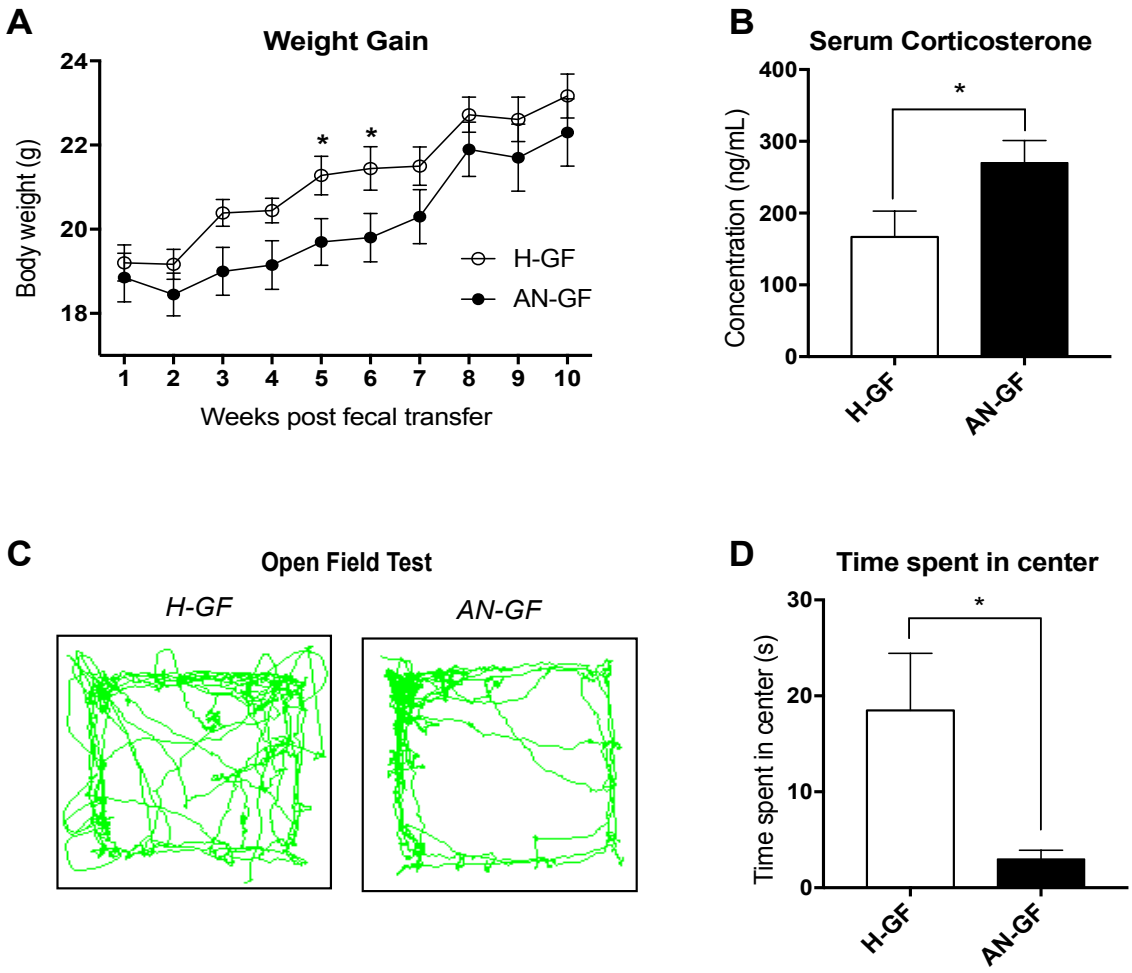


Figure 11. Changes in body weight, anxiety-like behavior and HPA axis. (A) Body weight gain was measured in mice harboring anorexic (AN-GF) versus healthy (H-GF) human gut microbiota over 10 wk following fecal transplantation. All mice were given ad libitum access to food and water during the experiment. At week 10 post transplantation open-field test was performed for 5 min. (B) shows corticosterone levels in the serum following the open field challenge. (C) displays schematic of zones in the open-field arena and representative traces of mouse movement during an open-field test. (D) illustrates the total time spent in the center of the arena. Statistical significance was measured by two-way ANOVA with Sidak's test for body weight measurements, and with t test for open-field test and

corticosterone quantification. n= 10; *P < 0.05. All error bars represent S.E.M.

In line with what is known from anorectic patients, microbiota transfer resulted in reduced weight gain, substantial elevation in the corticosterone levels, as well as increased anxiety-like behavior in recipient mice compared to mice with healthy microbiota (Fig. 11). Despite the decreased weight gain, no differences were observed in food intake, weight of visceral white adipose tissue, and circulating levels of leptin between the groups. This might be due to difference in efficiency of energy harvest between the anorectic and healthy microbiota. Previous studies suggest that gut microbiota play a critical role in energy harvest, storage and expenditure from the diet by mechanisms such as breaking down the SCFAs, stimulation of gut peptide production, affecting gut motility, and regulating the expression of angiopietin-like 4 (*ANGPTL4*)[209] which encodes a protein that is directly involved in regulating lipid metabolism.

4.5 Gene transcription is altered in the nucleus accumbens of mice harboring anorexic microbiota

Nucleus accumbens, an area densely innervated by dopaminergic neurons, regulates many functions potentially affected in anorexia nervosa, including reward, punishment, satiety, habit formation and addiction[134,136]. Increased neuronal activity in nucleus accumbens have been reported in anorectic patients[135]. We performed microarray analysis on brain homogenates from mice harboring either anorectic or healthy microbiota. Our data indicated pronounced changes in gene expression in the nucleus accumbens, while very few transcripts were altered in the hippocampus. In total, 157 transcripts in nucleus accumbens were differentially expressed ($P \leq 0.01$, $-1.5 \geq$ fold change ≥ 1.5). Next, we analyzed the genes that were either up- or down-regulated in the following selected pathways: feeding behavior, ion transport, synaptic transmission, inflammatory response, and temperature control. Of particular interest was the altered expression of melanocortin 2 receptor accessory protein 2 (MRAP2), a metabolic regulator implicated in leptin-melanocortin pathway. Mice lacking a functional copy of MRAP2 develop sever obesity, and rare variants of MRAP2 may be associated with obesity in humans[210]. The role of MRAP2 in regulation of energy expenditure and food intake has been explained in the hypothalamus but its function in the nucleus accumbens has not been investigated.

Interestingly, among all the selected pathways, the ion transport pathway had the highest number of altered genes in the nucleus accumbens. This might indicate a perturbed neuronal

function. It was shown that open-probability of ATP-dependent potassium channels of dopaminergic midbrain neurons is enhanced under food restriction and is associated with exploratory behavior, supporting the tie between starvation and motivational value[134,211].

Ingenuity Pathway Analysis (IPA) of the microarray data indicated that eleven canonical pathways were significantly altered, including pathways involved in appetite and body weight regulation such as cholecystokinin/gastrin-mediated signaling. Cholecystokinin (CCK) is the first gut peptide released during/after each meal to reduce food intake[212] and was shown to be modulated by gut microbiota[213]. Obese rodents, which are known to have drastic changes in the gut microbiota, display reduced CCK signaling in the brain[212,214]. In addition, dopamine, serotonin, glutamate and catecholamine pathways were also affected in the nucleus accumbens of mice with anorectic microbiota, as shown in the IPA analysis. The impact of anorectic microbiome on the dopamine system was confirmed by qPCR and immunohistochemistry analysis showing elevated dopamine producing enzyme tyrosine hydroxylase in the nucleus accumbens.

5 CONCLUDING REMARKS AND PERSPECTIVE

Traditionally, the view over the pathophysiology of neurological and psychiatric disorders was a "germ-free" view. The observation that the parasite *Toxoplasma gondii* is able to hijack the brain mechanisms to alter the behavior in the rodent host in favor of its own reproduction[215–217], provided a remarkable evidence for microbial modulation of brain function and behavior. Over a decade of research on microbial residents of the gut suggest that manipulation of host behavior and brain function is not limited to the parasites. The contribution of the gut microbiota to the basic developmental process of the CNS such as neurogenesis, myelination, and microglia maturation has been implicated[46]. However, the mechanisms underlying the gut microbiota-brain interaction remain to be further understood.

In paper I, we showed that the gut microbiota mediates the development of the BBB and plays an important role for maintaining BBB integrity and function. Through monocolonization of germ-free mice with bacteria that produce SCFAs or treatment with sodium butyrate, we further proposed that bacterial metabolites are involved in the BBB modulation by gut microbiota. Our findings were confirmed in a later study where antibiotic treatment was used as a mean to deplete the gut microbiota. Fröhlch et al. showed that short-term intragastric treatment of mice with a mix of non-absorbable antibiotics was associated with gut microbiota dysbiosis and altered protein expression of tight junctions in the hippocampus and amygdala. Antibiotic-treated mice displayed impaired novel object recognition memory and neurochemical changes including differential expression of brain-derived neurotrophic factor (BDNF) and serotonin transporter, alteration in neuropeptide Y (NPY) system, and changes in corticosterone levels[218] which were previously reported in germ-free animals. We acknowledge that "all out" or single species colonization approaches do not address the complex interspecies cross-talk among different microbes and microbial sub-populations within the gut. Future studies investigating microbiota-derived impacts on the BBB by targeted manipulation of the gut microbiota could lead to discovery of novel therapeutic approaches for the treatment of neurological diseases with BBB impairments. Currently, strategies such as targeting quorum–sensing (a bacterial cell-cell communication process) and engineering phages (viruses that infect bacteria) in order to manipulate the gut microbiota are being increasingly explored[219]. With the advancement of synthetic biology smart microbes with novel functionalities could be developed. For instance, engineered *E.coli* bacteria which overproduce interspecies quorum sensing molecule autoinducer-2 (AI-2) was used in a study where dysbiosis of microbiota was induced by streptomycin treatment of the mice and was shown to rebalance the observed alteration in Firmicutes/Bacteroidetes ratio[220].

An intact and functional BBB is specially important in the early postnatal period which coincides with microbial colonization and introduction of enteral feeding. This was supported by our observation in paper II that the impaired brain barrier integrity and function in preterm piglets with sever NEC, was associated with neuronal damage and increased levels of inflammatory cytokines in the brain. Deficiencies in brain barriers could potentially contribute to the increased susceptibility to neurodevelopmental diseases in preterm infants. In a way, the BBB functions as a double-edged sword. On the one hand, an intact BBB is favorable and required for normal neuronal function and development, and on the other hand, it is an obstacle for the drug delivery to the brain. In fact, the BBB is one of the main reasons behind the lack of effective treatments for the majority of the CNS diseases. Almost all the large-molecule drugs and most of small-molecule drugs (> 98%) are excluded by the BBB and only limited number of small lipophilic drugs with molecular mass below 400 Da may cross[221]. Despite the advancement in the strategies such as chemical modification of the drugs, use of drug vectors, "temporary" BBB disruption, transnasal and transcranial drug delivery to breach the BBB, it still remains a major bottleneck in neurotherapeutics. Understanding the mechanisms behind microbiota-mediated modulation of the BBB could be beneficial for the development of new BBB delivery solutions.

Previous investigations of the impact of gut microbiota on brain mechanisms involved in regulation of energy homeostasis have proposed alterations in the hypothalamus and brain stem gene expression[55,175]. In paper III, we documented transcriptional changes in nucleus accumbens in formerly germ-free mice colonized with fecal microbiota from an anorectic patient. Interestingly, many of the genes affected in our pilot study are known to regulate feeding in the hypothalamus. Whether this suggests that the nucleus accumbens is directly involved in feeding behavior, or if these genes regulate different functions in the nucleus accumbens compared to the hypothalamus, remains to be investigated. Nevertheless, we are just beginning to understand the potential relevance of gut microbiota for anorexia nervosa, and up to this date, there are only few studies of microbiome profile in anorectic patients with small sample sizes. Future studies in larger cohorts aimed to differentiate the causal versus effectual role of gut microbiota in anorexia nervosa might lead to a better understanding of the etiology of the diseases.

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