Dissertation

Analysis of Gut Microbiota-Brain Communication

submitted by

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Declaration

I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of "Good Scientific Practice".

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Abbreviations and Definitions

ANOVA	analysis of variance
ANS	autonomic nervous system
AUC	area under the curve
BBB	blood-brain barrier
BDNF	brain-derived neurotrophic factor
BM	Barnes maze
CAT	catalase
CCL2	chemokine (C-C motif) ligand 2
CLDN1	claudin 1
CLDN5	claudin 5
EPM	elevated plus maze
GABA	gamma-aminobutyric acid
GF	germ-free
GRIN2B	glutamate receptor, ionotropic, NMDA2B (epsilon 2); N-methyl-D-aspartate receptor subunit 2B
GSR	glutathione reductase
HPA	hypothalamic-pituitary-adrenal
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFN	interferon
IL	interleukin
i.p.	intraperitoneal

LD	light-dark
LLOQ	lower limit of quantification
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
MAMP	microbe-associated molecular pattern
MVA	multivariate analysis
NORT	novel object recognition test
NPY	neuropeptide Y
NPY1R	neuropeptide Y receptor Y1
NPY2R	neuropeptide Y receptor Y2
NPY5R	neuropeptide Y receptor Y5
NR3C1	nuclear receptor subfamily 3, group C, member 1; glucocorticoid receptor
OCLN	occludin
OFT	open field test
OTU	operational taxonomic unit
PAMP	pathogen-associated molecular pattern
PC	phosphatidylcholine
PCA	principal component analysis
PRR	pattern recognition receptor
SCFAs	short-chain fatty acids
SIT	social interaction test
SLC6A4	solute carrier family 6 (neurotransmitter transporter), member 4; serotonin transporter

TJP1	tight junction protein 1
TLR	toll-like receptor
TNF	tumor necrosis factor
TST	tail suspension test
UVA	univariate analysis

Abstract in German

Die Darm-Gehirn-Achse beschreibt die bidirektionale Kommunikation zwischen Darm und Gehirn, die für die Gesundheit sowohl des Gastrointestinaltrakts als auch des Zentralnervensystems eine Rolle spielt. Dieses Konzept wurde kürzlich noch erweitert durch die Mikrobiota als einen ausschlaggebenden Knotenpunkt in der Kommunikation zwischen beiden Organen. Der Austausch zwischen Darm und Gehirn findet über viele Wege statt, wobei speziell neuronale, hormonale und immunologische Bahnen zu nennen sind. Manche mikrobiellen Komponenten, wie zum Beispiel Lipopolysaccharid, aktivieren das Immunsystem und können dadurch Angststörungen und Depression auslösen und das Sozialverhalten verändern. Klinische Studien konnten zeigen, dass das Profil der Darmmikrobiota in vielen neurologischen und psychiatrischen Erkrankungen verändert ist. Dies führte zur Hypothese, dass eine gestörte Mikrobiota (Dysbiose) ein pathogenetischer Faktor bei diesen Krankheiten ist. Antibiotika-induzierte Dysbiose-Modelle erlangten zunehmend Bedeutung für die Erforschung von Kausalzusammenhängen in der Mikrobiota-Wirt Interaktion. Es gibt jedoch bis jetzt kein etabliertes und validiertes Antibiotika-induziertes Dysbiose-Modell. Überdies wurden die verschiedenen Modelle nicht eingehend charakterisiert, da die Studien sich meist auf einzelne Aspekte der mikrobiellen Veränderungen konzentriert haben.

Um die Kommunikation zwischen Darmmikrobiota und Gehirn genauer zu untersuchen, wurde ein Antibiotika-induziertes Dysbiose-Modell in adulten männlichen C57BL/7N Mäusen entwickelt. Dieses wurde im Hinblick auf die Zusammensetzung des Darmmikrobioms, das Metabolitenprofil in Kolon und Plasma, Veränderungen in den mRNA-Expressionsmustern in Kolon und Gehirn sowie emotionale, affektiven und kognitiven Verhaltensänderungen genau charakterisiert. Da Verhaltensänderungen durch das bakterielle Protein Flagellin noch nicht untersucht sind, wurde das Angstverhalten nach Verabreichung von Flagellin an adulte männliche C57BL/7N Mäuse analysiert.

Eine Antibiotika-induzierte Dysbiose wurde durch die orale Gabe von 5 Antibiotika über 11 Tage induziert. Die Antibiotika-induzierte Dysbiose zeigte sich in einer markanten Veränderung der mikrobiellen Zusammensetzung und einer deutlichen Reduktion der bakteriellen Masse. Darüber hinaus sorgte die Dysbiose für ein verändertes Metabolom-Profil im Kolon und Plasma ohne Entwicklung einer offensichtlichen Entzündung. Die Antibiotika-induzierte Dysbiose führte zu einer Beeinträchtigung des Gedächtnisses für Wiedererkennung, während räumliches Erinnerungsvermögen sowie Ängstlichkeit und

Depressionsverhalten unverändert blieben. Zusätzlich war die Expression von neuronalen Signalmolekülen im Gehirn der Antibiotika-behandelten Mäusen deutlich verändert.

Das Darmmikrobiom stellt die größte Quelle für bakterielle Komponenten im Körper dar. Manche dieser Moleküle können an Rezeptoren des angeborenen Immunsystems binden und dadurch eine Immunreaktion auslösen. Da eine Aktivierung des Immunsystems in Zusammenhang mit psychiatrischen Erkrankungen wie etwa Angststörungen steht, wurde außerdem untersucht, ob Flagellin einen Einfluss auf das Verhalten hat. Die intraperitoneale Gabe von Flagellin (200µg/kg) aus drei verschiedenen Bakterienarten (*Bacillus subtilis*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) führte jedoch zu keinen konsistenten Veränderungen der Ängstlichkeit.

Zusammenfassend konnte die vorgelegte Dissertation die Kommunikation zwischen Darmmikrobiota und Gehirn auf mehreren Ebenen genauer analysieren. Die Ergebnisse zeigen insbesondere, dass eine Antibiotika-induzierte Dysbiose die Kognition der Mäuse beeinträchtigt, wobei ein verändertes Metabolitenprofil im Plasma und neurochemische Veränderungen in kognitionsrelevanten Gehirnarealen eine Rolle zu spielen scheinen. Die Veränderungen, welche in Antibiotika-behandelten Mäusen beobachtet wurden, decken sich teilweise mit Daten aus keimfreien Mäusen.

Abstract in English

The concept of the gut-brain axis, the bidirectional communication between gut and brain, has been supported by many experimental and clinical studies. This model has recently been expanded by including the gut microbiota as a critical node in the communication between the gut and brain. The communication between gut and brain involves neuronal, hormonal and immune routes. Some microbial components, like lipopolysaccharide, have been shown to activate the immune system, leading to alterations in anxiety, depression and social behavior. Clinical observations that the intestinal microbiota profile is altered in many neurologic and psychiatric disorders and diseases have given rise to the hypothesis that a disordered microbiota (dysbiosis) is a pathogenetic factor in these pathologies. As animal models raised in the absence of germs (germ-free) exhibit physiological differences compared to their colonized counterparts, antibiotic treatment gained appreciation as an approach for studying dysbiosis and causality in microbiota-host interactions. However, so far no standardized model of antibiotic-induced dysbiosis has been established. Moreover, the different models have not been characterized in detail, mostly concentrating on single aspects of changes in microbial composition.

To shed more light on the communication between intestinal microbiota and brain function, an antibiotic-induced dysbiosis model was established in adult male C57BL/7N mice and characterized in great depth, including microbiome analysis of colon tissue and luminal content, metabolome analysis of colonic luminal content and plasma, mRNA expression pattern in colon and brain tissue, histologic evaluation of the small and large intestine, and assessment of emotional, affective and cognitive behavior. In addition, as behavioral sequelae of flagellin have been little studied, the effects of this bacterial protein with regard to anxiety-like and social behavior were investigated in adult male C57BL/7N mice.

In the antibiotic regimen, mice were treated for 11 days with an antibiotic mix consisting of five antibiotics. This treatment induced marked dysbiosis that was associated with a significant disruption of the microbial community structure and load in the gut. Furthermore, dysbiosis altered the metabolite profile of the colonic content and plasma without inducing overt inflammation. Whereas anxiety- and depression-like behavior remained unaltered, antibiotic-induced dysbiosis impaired novel object recognition memory but had no impact on spatial memory. In addition, expression of neuronal signaling molecules was markedly altered in the brain of antibiotic-treated mice.

The intestinal microbiota is the main source of bacterial components in the body. Some of these molecules can bind to receptors of the innate immune system and thereby elicit immune responses. As activation of the immune system has been shown to be involved in the pathogenesis of psychiatric disorders like anxiety, I was interested to know whether administration of flagellin would elicit behavioral alterations in mice. However, intraperitoneal injection of flagellin (200µg/kg) from *Salmonella typhimurium* had no consistent impact on anxiety-like and social behavior.

In summary, these results add important information on the communication between the gut microbiota and the brain at several levels of the gut-brain axis, using distinct molecular, biochemical and neurobiological methods. In particular, the data of this dissertation show that antibiotic-induced dysbiosis in mice impairs cognition, an effect in which alterations of the metabolite profile and changes in the cerebral expression of signaling molecules may play an important role. In addition, several molecular and functional alterations of antibiotic-induced dysbiosis are to some extent similar to those found in germ-free mice.

1 Introduction

1.1 The gut-brain axis

In-depth research has shown that the popular statement to "trust your gut feeling" actually has a neurobiological basis, namely the gut-brain axis. The interaction between brain and gut in health and diseases has in fact been suggested for many centuries (Mayer 2011). It took much research effort in the past decades until this link was not only considered an "ancient wisdom" passed on from generation to generation but actually gained scientific confirmation and acknowledgement. With the comfort of sharing information globally came the advantage that clinicians and scientists could share and compare their observations and experience. This enabled them to study much larger groups of patients to observe clinical manifestations and comorbidities of diseases. In the 1990s several studies and reviews were published showing a comorbidity of gastrointestinal disorders and psychiatric symptoms (Walker et al. 1992, Gautam 2010). From that point on, this topic gained great interest and became a mainstay topic considered in most gut-brain axis papers. In fact, the observations back then helped understand the link between the two organs, which topographically are far away from each other. Patients with gastrointestinal disorders (e.g. irritable bowel syndrome (IBS), inflammatory bowel disease (IBD)) often also have mental diseases like anxiety and depression (Shah et al. 2014). Animal models and treatment with diets and/or antibiotics helped to elucidate that in fact there was a complex bidirectional communicating system at work (the gut-brain axis) (Romijn et al. 2008). The anatomy and physiology of these two organs differs tremendously, given their different functions for the host, which will be explained in detail in the following chapters, as well as the various pathways used for communication between gut and brain.

1.1.1 The gut – a multifaceted organ

The human gut includes the stomach, small (duodenum, jejunum, ileum) and large (cecum, colon, rectum) intestine (Howell, Wells 2011). In humans the average length and surface ratio of small intestine: colon is 7 and 400, whereas in mice this ratio is only 2.5 and 18, respectively (Casteleyn et al. 2010, Nguyen et al. 2015). In contrast to mice, the human cecum is relatively small and has an attached appendix (Nguyen et al. 2015). Interestingly, recent research suggested that the appendix plays a role in re-colonization of the intestine

after depletion of the normal flora (e.g. via antibiotics) (Smith et al. 2013a, Nguyen et al. 2015).

The wall of the small and large intestine is composed of functionally diverse layers. The outer layer consists of the serosa and musculature (with taenia coli in the colon). In the middle layer the submucosa and muscularis mucosae is situated. The inner layer is composed of the laminar propria and a columnar epithelium (Howell, Wells 2011). This simple intestinal epithelium lines the luminal surface. In the small intestine the surface is shaped into villi (finger-like projections) and crypts. In contrast, in the large intestine villi are absent, which is also a reason for the reduced surface area of the colon compared to the small intestine (Stipanuk, Caudill 2013). Near the bottom of the epithelial crypts intestinal stem cells reside and give rise to new epithelial (progenitor) cells. Thereby the lining of the intestine is continuously renewed. Enterocytes and colonocytes are the dominant intestinal epithelial cell type in the small and large intestine, respectively. Their main function is the absorption of nutrients and water. Paneth cells secrete lysozymes and anti-microbial peptides, reside near the base of the crypts and have been shown to be important for the intestinal stem cells, as they are involved in the provision of the microenvironmental niche in the base of crypts (Howell, Wells 2011). Goblet cells are responsible for mucus secretion and are more abundant in the large than in the small intestine. Although enteroendocrine cells (e.g. enterochromaffin cells) make up less than 1% of all intestinal epithelial cells, they are an important endocrine organ as they secrete gastrointestinal hormones (Howell, Wells 2011, Mayer 2011, Stipanuk, Caudill 2013). In terms of immunity the microfold or M cells need to be mentioned. They are associated with the gut-associated lymphoid tissue and mucosa-associated lymphoid tissue. M cells are responsible for the uptake and transport of antigens present in the luminal content to lymphoid tissues (Corr, Gahan & Hill 2008, Stipanuk, Caudill 2013). The connection between epithelial cells involves tight junctions near the apical ends of the epithelial cells, which controls the paracellular transport through a semipermeable epithelium (Ulluwishewa et al. 2011, Stipanuk, Caudill 2013).

A coordinated interplay between the intestinal layers is important to execute the physiologic tasks of the intestine (Howell, Wells 2011). The gastrointestinal tract fulfills many functions including but not limited to digestion, secretion, absorption, mixing, peristalsis and defense (Uesaka et al. 2016).

1.1.2 The brain – the restricted area

Together with the spinal cord the brain forms the central nervous system. Three compartments build the brain tissue: neural cells (e.g. neurons, glial cells), the vascular system and interstitial system (Fumagalli, Ortolano & De Simoni 2014). The latter two form the brain microenvironment, providing the living environment for the neural cells, and have been recognized to play also an active role in brain function (Lei et al. 2016). Neurons are connected to other neurons by axons, dendrites and synapses and thereby process and transmit information over wide ranges. Despite the prominent role of the neurons in the brain, they are actually outnumbered by glial cells (Pelvig et al. 2008, Lei et al. 2016). Glial cells in the brain (astrocytes, oligodendrocytes and microglia) have diverse functions including defense against pathogens and support of neuronal metabolism. To this end they are in continuous reciprocal communication with neurons (Parpura et al. 2012, Jha et al. 2013). Due to the importance of the brain for our health, homeostasis must be strictly maintained. Therefore, the transport of substances and ions between the blood and brain is tightly regulated (Serlin et al. 2015) by a unique barrier, the blood-brain barrier (BBB). The BBB is formed by a specialized and polarized endothelial cell layer and its basement membrane, which separates the blood and brain interstitial fluid (Goldstein et al. 1986, Serlin et al. 2015). Furthermore, the intercellular clefts of the endothelial cells are sealed with tight junctions and the endothelium is surrounded by pericytes and astrocyte endfeet (Bentivoglio, Kristensson 2014, Serlin et al. 2015). The physiologic development of the BBB in the early fetal life is crucial to protect the fetus against harmful substances and microorganisms (Ballabh, Braun & Nedergaard 2004, Knudsen 2004, Abbott et al. 2010). Through an intact BBB the transport of large molecules is tightly restricted, which is both blessing and curse for the action of drugs (Pardridge 2012). A properly functioning BBB is crucial throughout life as disruptions of the BBB make the brain vulnerable to damage and have been shown to be implicated in various neurological pathologies (Ballabh, Braun & Nedergaard 2004, Tenreiro et al. 2016).

1.1.3 Highways and messengers involved in the gut-brain axis

The gut is innervated by intrinsic (enteric nervous system) and extrinsic sympathetic, parasympathetic (pelvic nerves and vagus nerves), and sensory (spinal and vagal pathways) neurons (Uesaka et al. 2016). Hence, the central nervous system is involved in the regulation of gastrointestinal function and at the same time the neuronal impulses are signaled from the gut to the brain. Brain to gut communication includes, among other

pathways, the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis (Mayer 2011). The ANS and HPA axis have been suggested to be involved in the emotional influences on the gut, like the alterations in gut motility and inflammation when under stress (Mawdsley, Rampton 2005, Lyte, Vulchanova & Brown 2011). The sympathetic innervation in the gastrointestinal tract causes mainly inhibition and slowing of intestinal transit and secretion, as well as modulation of mucosal immunity (Elenkov et al. 2000, Mayer 2011). Although small in number, enteroendocrine cells are important mediators in the gut-brain axis as their output is involved in digestive functions as well as in brain signaling (Mayer 2011). The parasympathetic innervation influences, among other functions, histamine and serotonin release from the enterochromaffin cells (Mayer 2011). Hence, multiple bidirectional communication systems between gut and brain enable a flexible communication and interaction in the gut-brain axis (Mayer 2011). Signals from the gut can be induced by various stimuli, including but not limited to, stimuli eliciting hunger/satiety, mechanical and chemical stimuli and inflammation (Mayer 2011). Two examples of the gut-brain signaling are the ghrelin-dopamine (hunger and food reward) and cytokine-sickness (inflammation) responses (Watkins, Maier 2000, Cone, McCutcheon & Roitman 2014, Poon et al. 2015). Investigations of the mechanisms and mediators between gut and brain will help us to understand this complex communication better and may guide us towards new therapeutic approaches (Skibicka et al. 2012). Disturbances in gut-brain communication can be seen in symptoms or diseases like nausea and vomiting triggered by drugs or toxins, as well as anxiety, depression and pain in chronic gastrointestinal disorders (Mayer 2011, Moloney et al. 2016).

1.2 The microbiota-gut-brain axis

The human body is colonized by trillions of microbes. These microbes live in special body habitats like the skin, vagina and gut. Due to the different niches the microbial composition of these habitats strongly differs from each other (Human Microbiome Project Consortium 2012). The most heavily colonized organ is the intestinal tract with 10^1 to 10^{12} (increasing from stomach to colon) microbes (Gerritsen et al. 2011). For a long time it was estimated that the number of microbes exceeds the number of human cells by 10 fold, but this was recently revisited and the estimated ratio for microbial to human cells for an average man was strongly reduced to 1.3 : 1 (Abbott 2016). However, this estimation is still uncertain and numbers may vary with each defecation (Abbott 2016, Sender, Fuchs & Milo 2016). Irrespective of the exact number, the gut microbial community was shown to play a role in

the health of the host and further suggested to resemble an entire organ with many functions (Eckburg et al. 2005).

The term microbiota comprises archaea, bacteria, eukaryotes (e.g. fungi) and viruses. Due to established cultivation and sequencing methods, research has mainly focused on the role of the bacteria in this system (although recent research also takes an interest in the virome) (Scarpellini et al. 2015). Nevertheless, this thesis will focus on the role of the bacterial fraction of the gut microbiota. By analyzing the microbiota of the nasal passages, oral cavity, skin, gastrointestinal tract, and urogenital tract of over 242 individuals the Human Microbiome Project was a landmark study in the field of microbiota research (Human Microbiome Project Consortium 2012). The consortium found that the gut microbiota constitutes of dominant phyla (taxonomic rank). The different ratios of these phyla were suggested to describe enterotypes for each person, but these should be considered as a simplification rather than distinct clusters (Arumugam et al. 2011, Arumugam et al. 2014). According to the latest discoveries and investigations in the field of microbiota research, the microbiota is involved in many diseases. The spectrum of these diseases includes mental illnesses (depression, anxiety), neurodevelopmental disorders (autism), inflammatory conditions (allergies) and gastrointestinal disorders (IBD, IBS) (Damman et al. 2012, Collado et al. 2015, Palm, de Zoete & Flavell 2015). Hence, the gut microbiota was suggested to generally play a significant role in the health of the host (Biedermann, Rogler 2015, Collado et al. 2015). The following chapters focus on the indigenous microbiota, its part in the gut-brain axis and the current experimental models used to investigate the intestinal microbiota. Although pathogens often have special abilities (e.g. toxins) to colonize in the host and interact with the immune system, those microbes will not be discussed.

1.2.1 The intestinal microbiota – a complex ecosystem

The gut of an adult harbors hundreds to thousands of microbial species (Donaldson, Lee & Mazmanian 2016). The number of bacteria increases from the small to the large intestine (Gerritsen et al. 2011). Also the composition of bacteria differs in the small intestine compared to the large intestine. The physiology of the gut is suggested to play a role in this phenomenon as there are pH and oxygen gradients from small to large intestine as well as different availability of nutrients (Donaldson, Lee & Mazmanian 2016). Even the shorter transit time in the small intestine was suggested to have an impact on adherence and thereby colonization of the small intestine. As oxygen levels are higher in the small

intestine, the bacteria colonizing this habitat are mainly facultative anaerobes which tolerate lower pH (e.g. Lactobacillaceae) (Gu et al. 2013, Donaldson, Lee & Mazmanian 2016). In contrast, in the colon the availability of simple carbon sources is very low so the bacteria have to be able to digest "resistant" polysaccharides. This promotes the growth of fermentative anaerobes (e.g. Bacteroidaceae) (Donaldson, Lee & Mazmanian 2016).
All parts of the gastrointestinal tract are covered by mucus. The mucus is mainly composed of water, and highly glycosylated gel-forming mucins, which are produced and continuously renewed by goblet cells (Johansson, Larsson & Hansson 2011, Pelaseyed et al. 2014, Kelly et al. 2015). In the colon the mucus layer is much thicker than in the small intestine and composed of two layers (Johansson, Larsson & Hansson 2011) termed as "loosely" (outer) and "firmly" (inner) attached layer (Atuma et al. 2001, Johansson, Larsson & Hansson 2011). The thickness as well as the microbial composition is different between these two layers (Johansson, Larsson & Hansson 2011, Donaldson, Lee & Mazmanian 2016). Also the thickness of these layers is very variable and suggested to be influenced by the bacteria present. Although bacteria seem to be absent from the inner mucus layer, whereas in the outer layer bacterial colonization was observed (Johansson, Larsson & Hansson 2011), a small portion of bacteria near/associated with the epithelium has been described in another study (Swidsinski et al. 2005). Regarding this topic it should be mentioned that the methodology of tissue preparation and visualization impacts on the detection of an association between microbiota and epithelium (Palestrant et al. 2004). As penetration of the mucus is not so easy for bacteria, the microbial community inhabiting the mucus needs to have special colonization abilities (Donaldson, Lee & Mazmanian 2016). The utilization of mucin glycans is one property often represented in the mucosa-associated microbiota (Png et al. 2010).
Besides the different bacterial composition of inner and outer mucus layer, there is high spatial heterogeneity along the colon (Zhang et al. 2014). In addition, the mucosal microbial community differs from the digesta-associated colonic community (Eckburg et al. 2005). Hence, microbial profiling of fecal samples is now viewed more critical (Donaldson, Lee & Mazmanian 2016). This aspect is especially important to consider regarding the influence of the diet on the microbiota (see 1.2.2). While microbial community profiling in fecal samples may provide appropriate conclusions regarding the communities present in the gut lumen, one needs to be aware that the mucosa-associated bacteria could be much less affected by, or need longer periods of, intervention for alterations to become evident (Donaldson, Lee & Mazmanian 2016).

1.2.2 Factors influencing the intestinal microbiota

Many factors have been identified that impact on the gut microbiota. In fact the first critical phase for the human microbiota already occurs in the womb of the mother. Despite former suggestions that the placenta of the mother is germ free, recent research showed that the maternal microbiota already influences the microbiota of the child before birth (Aagaard et al. 2014, Rodriguez et al. 2015, Collado et al. 2015). Hence, deliberate changes of diet and overall life style of the mother have already been suggested as a therapeutic approach for the establishment of a healthy baby microbiota and as an intervention to decrease the risks of certain diseases in the unborn child (Rodriguez et al. 2015, Collado et al. 2015). The way of delivery is the next critical step for the child microbiota. Here studies are quite consistent that caesarian section influences the microbiota in a negative way making the child more susceptible to diseases, including but not limited to asthma, celiac disease and obesity (Thavagnanam et al. 2008, Decker, Hornef & Stockinger 2011, Barros et al. 2012, Collado et al. 2015). Breast milk as a first diet is greatly involved in shaping the infant's gut microbiota (Donaldson, Lee & Mazmanian 2016). This is due to the fact that there are microbes in the milk, as well as milk oligosaccharides, which serve as carbon sources for some microorganisms, and maternal antibodies, which serve as protection and thereby promote homeostasis (Fernandez et al. 2013, Yu, Chen & Newburg 2013, Rogier et al. 2014, Donaldson, Lee & Mazmanian 2016). Although the microbiota is an adaptable system also in later stages of life, especially the window of infancy is important to establish a healthy microbiota (Borre et al. 2014, Rodriguez et al. 2015, Bauer, Huus & Finlay 2016). During the entire life diet, lifestyle, medications, environment and supplements (e.g. pre- and probiotics) impact on the microbiota (summarized in Figure 1) (Gerritsen et al. 2011, Rodriguez et al. 2015). Whereas the aforementioned factors often can be influenced by the host, other factors do not lend themselves to modification, such as sex and age which also shape the intestinal microbiota (Gerritsen et al. 2011, Markle et al. 2013). Currently it is thought that diet has the biggest impact on the microbiota (David et al. 2014, Donaldson, Lee & Mazmanian 2016). Nevertheless, also the immune system (innate and adaptive) influences and puts selective pressure on the microbiota (Donaldson, Lee & Mazmanian 2016).

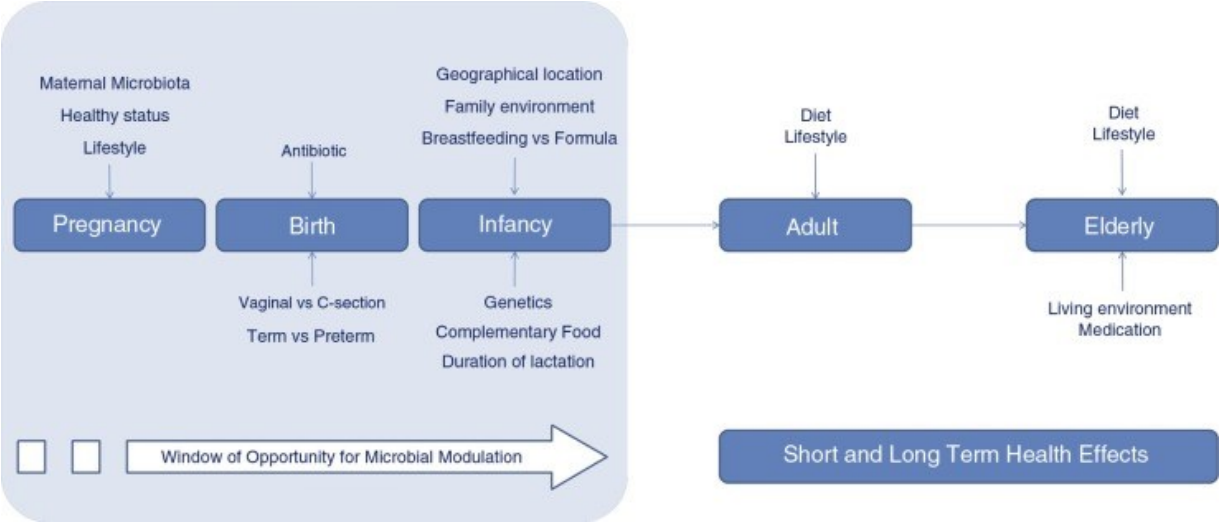

Figure 1 Factors with impact on the gut microbiota of the baby, adult and elderly. The most critical time to improve the health of the child by dietary interventions (microbiota modulation) is the first 3 years of life. Legend adapted from (Rodriguez et al. 2015). Figure taken from (Rodriguez et al. 2015), copyright (2016) under the terms of the Creative Commons Attribution-Noncommercial 3.0 Unported License, https://creativecommons.org/licenses/by-nc-nd/3.0/.

1.2.3 Functions of enteric microbes

The intestinal microbiota fulfills many functions for the host. First, it occupies habitats where otherwise pathogens could colonize (colonization resistance). Besides occupation some bacteria also actively prevent colonization by pathogens through the production of antimicrobial substances (Lawley, Walker 2013). Second, the microbiota supplies nutrients and energy to the host as these bacteria are able to break down otherwise indigestible food components. The production of short-chain fatty acids (SCFAs) and vitamins are of special importance in this context (see 1.2.4). In addition the microbiota also has an impact on fat distribution (Backhed et al. 2004). Third, the microbiota is important for the normal development of the immune system. This observation comes from germ free (GF) mice which display an impaired immunes system (see also 1.2.5) (Maier, Anderson & Roy 2014).

The intestinal microbiota is in close contact with the intestine. Hence, the immune system has the challenging task to maintain, on the one hand, tolerance towards the beneficial microbiota and harmless food components and, on the other hand, to provide protection and immunity against pathogens (Maier, Anderson & Roy 2014). Interestingly, the distribution of immune cells varies between the upper and lower part of the intestine. Given the physiology of the small and large intestine as well as the higher number of bacteria present in the colon, it has been suggested that in the small intestine the immune

response is focused on tolerance towards food antigens and defense against pathogens, whereas in the large intestine the immune response is focused on homeostasis with the commensal microbiota and prevention of an inflammatory response (Mowat, Agace 2014, Maier, Anderson & Roy 2014). Furthermore some bacteria have immunomodulatory abilities, thereby promoting the maintenance of the unique tolerance to the commensal microbiota (see also 1.2.4) (Neish et al. 2000, Round, Mazmanian 2009). The mucosal glial cell network plays a role in maintenance of the intestinal epithelial barrier and the regulation of the intestinal immune response. Just recently it was shown that the microbiota is important for the postnatal development and continuous homeostasis of the glial cell network in the mucosa (Kabouridis et al. 2015). The microbiota itself has also an impact on the mucus renewal process as well as on intestinal barrier function (Yu et al. 2012, Bischoff et al. 2014, Kelly et al. 2015). In view of these observations it seems that the host and the bacteria have both adapted to each other and contribute to this remarkable symbiosis (Maier, Anderson & Roy 2014). Nevertheless, how the immune system distinguishes between pathogens and commensal bacteria is still not completely understood, but some microbial metabolites (see 1.2.4) were suggested to be involved in this intricate maintenance of immune homeostasis (Donaldson, Lee & Mazmanian 2016). Anyhow, recent research indicates that early colonization (critical window) is important to teach the immune system a healthy relationship and maintenance of homeostasis with the intestinal microbiota (Rodriguez et al. 2015, Collado et al. 2015).

As a side note, the microbiota itself is also a highly adaptable and complex ecosystem where many microbes live in a beneficial relationship with each other (Martin, Bermudez-Humaran & Langella 2016). As space is limited and nutrients are sometimes rare, collaborations can make colonization and survival easier. Hence, some bacteria live in biofilms and communicate (quorum sensing) with each other (Rhee, Pothoulakis & Mayer 2009, Li, Tian 2012).

Given that the microbiota was suggested to be a critical node in the gut-brain axis, signals from the brain also influence the gut microbiota and vice versa (Kennedy et al. 2014) (implications of the microbiota in the gut-brain axis summarized in Figure 2). As stress alters gut motility and secretion, this also has a big impact on the microbiota (Zou et al. 2008, Snoek et al. 2010, Collins, Surette & Bercik 2012). The HPA axis reacts to stress and was shown to interact with the microbiota (Cryan, Dinan 2015). Interestingly, the establishment of a healthy microbiota early in life may even have an impact on the stress response (Sudo et al. 2004). Furthermore, enteroendocrine cells are implicated in top-down

signaling by releasing hormones into the gut lumen (Rhee, Pothoulakis & Mayer 2009). Stress and inflammatory mediators in the gut-brain axis were also shown to impact on the interaction between mucosa and microbiota (Gareau, Silva & Perdue 2008, Lyte, Vulchanova & Brown 2011).

As the gut is innervated by many neurons, the involvement of neural pathways is thus unsurprising. Nevertheless, an operant vagus nerve is not always necessary for the transfer of information, so the role of neural pathways in microbiota-gut-brain signaling depends on the specific situation/research question (Luczynski et al. 2016).

In signaling from microbiota to the gut and brain the enteroendocrine cells have an important role. Recently it was shown that the microbiota can influence serotonin biosynthesis from colonic enteroendocrine cells (Yano et al. 2015). Enteroendocrine cells are able to react to the presence of SCFAs via G-protein-coupled receptors (Cani, Everard & Duparc 2013) and recognize microbes via pattern recognition receptors (PRRs) (Abreu 2010, Mayer 2011).

During inflammation, microbiota-gut-brain communication can be altered/disturbed by enhanced gut permeability (Rhee, Pothoulakis & Mayer 2009).

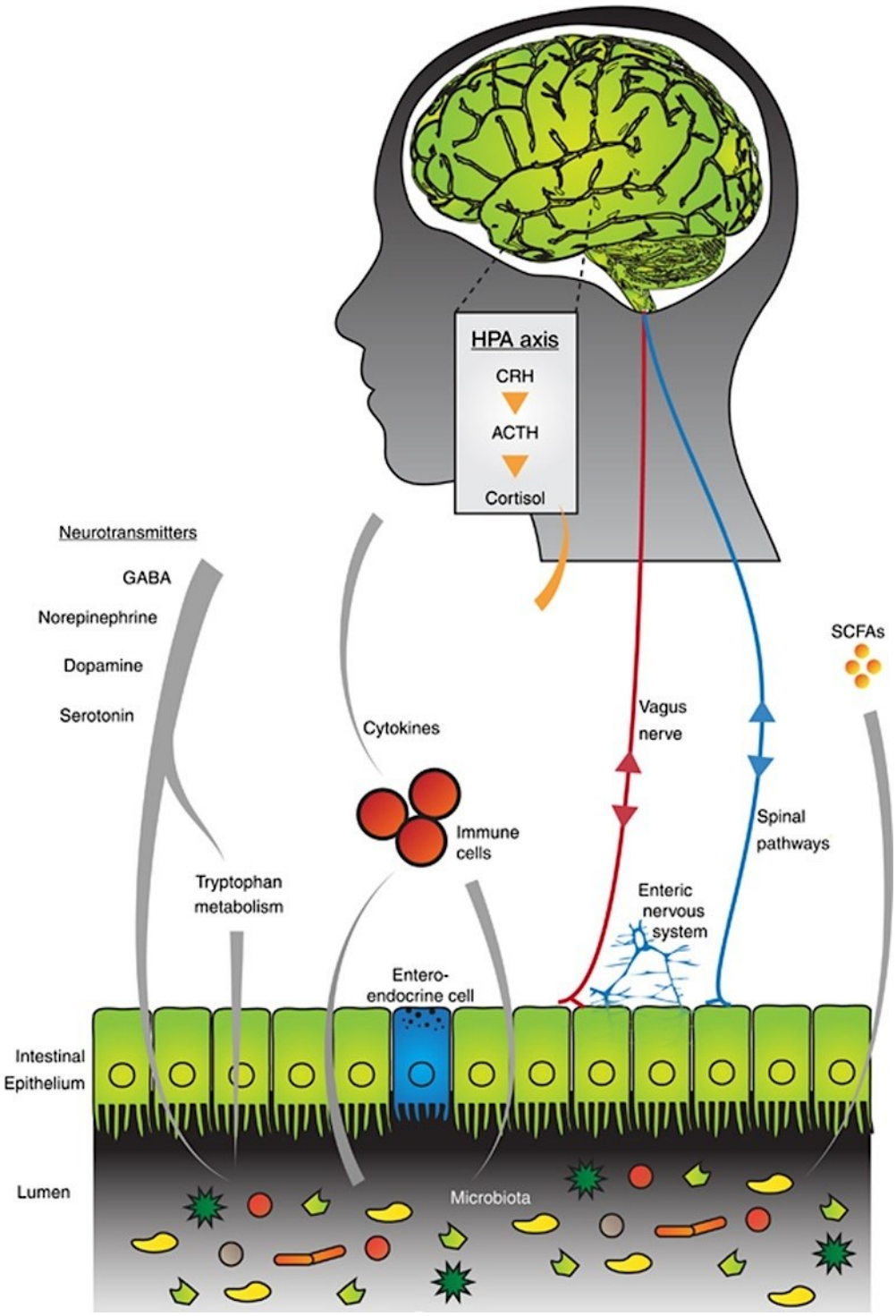

Figure 2 Important pathways in microbiota–gut–brain signaling. Potential pathways through which the intestinal microbiota can influence the gut brain axis include direct and indirect pathways: endocrine (cortisol), immune (cytokines), and neural (vagal, spinal and enteric nervous system) pathways. HPA, hypothalamic–pituitary–adrenal; CRH, corticotropin-releasing hormone; ACTH, adrenocorticotrophic hormone; GABA, gamma-aminobutyric acid; SCFAs, short-chain fatty acids. Reprinted by permission from Macmillan Publishers Ltd: [Neuropharmacology] (Cryan, Dinan 2015), copyright (2016). Legend adapted from (Cryan, Dinan 2015).

1.2.4 Microbial metabolites implicated in the gut-brain axis

Microbial metabolites can either be taken up from the gut into the portal circulation or act directly in the gut (Lyte 2014). SCFAs, like acetate, butyrate and propionate, are produced by starch fermentation in the colon. They are known to impact on intestinal immune responses and influence brain function (Maslowski et al. 2009, Bienenstock, Kunze & Forsythe 2015). Especially butyrate has gained interest as it is the main energy source of colonocytes, promotes and regulates T-regulatory cell homeostasis and promotes glucose and energy homeostasis by activation of intestinal gluconeogenesis (Arpaia et al. 2013, Smith et al. 2013b, De Vadder et al. 2014, Bienenstock, Kunze & Forsythe 2015). Due to these immunomodulatory effects, butyrate has been suggested to play a role in maintaining immune homeostasis and tolerance between the innate immune system and microbiota (Maier, Anderson & Roy 2014). Besides probiotic approaches (see 1.2.6) clinical studies tried to improve IBD symptoms with the help of enemas containing SCFAs or butyrate alone (Scheppach et al. 1992, Senagore et al. 1992, Steinhart, Brzezinski & Baker 1994, Scheppach 1996). Furthermore, SCFAs can bind to receptors on enteroendocrine cells and thereby increase circulating levels of peptide YY, which influences gut motility and appetite (Samuel et al. 2008). Also intraluminal administration of SCFAs was shown to stimulate serotonin release which then impacts on colonic transit (Fukumoto et al. 2003). Moreover, recent research could show that colonic acetate can cross the BBB and alter expression profiles in the hypothalamus (Frost et al. 2014).

Microbes were shown to be able to recognize and produce neurochemicals (Lyte, Primrose Freestone 2010, Lyte, Vulchanova & Brown 2011, Lyte 2014, Halang et al. 2015). This led to the term microbial endocrinology and to the suggestion to use probiotics (see 1.2.6) with the ability to produce neurochemicals (Lyte 1993, Lyte 2011). Gamma-aminobutyric acid (GABA) is one of these neuroactive compounds produced by the intestinal microbiota. GABA can be produced in large milligram quantities by bacteria from the human intestine (Barrett et al. 2012). As a side note, GABA also exerts immunomodulatory effects (Bjurstom et al. 2008). Other neurochemicals produced by microorganisms include acetylcholine, dopamine, histamine and serotonin (Iyer et al. 2004, Asano et al. 2012, Patterson et al. 2014, Lyte 2014). Interestingly it was suggested that the microbial production of these neuroactive substances involves partly the same pathways as those used by the host (Iyer et al. 2004). Whether the microbially produced neuroactive substances are taken up by the host in significant amounts or whether they are simply taken

up by other microbes is still not completely clear (Lyte 2014). One study could show that rats fed with a fermented soymilk enriched with GABA (produced by a *Lactobacillus* strain) exhibit reduced depressive-like behavior (Ko, Lin & Tsai 2013). A further study showed that ingestion of another *Lactobacillus* strain for 4 weeks was sufficient to alter behavior and mRNA expression patterns of GABA receptors in the brain. In this model it was also shown that an intact vagus nerve was necessary to mediate these effects (Bravo et al. 2011).

Nevertheless, at large the mechanisms whereby the intestinal microbiota signals to the brain and which components of the bacteria are truly important to mediate these effects are still not fully elucidated (Foster et al. 2015).

1.2.5 Investigating microbiota implications – germ-free mice and antibiotic-induced dysbiosis

Rodent models offer the possibility of an early and intense intervention in the composition of the gut microbiota. As a model system, they can be used to narrow down the links involved in the microbiota-gut-brain axis (e.g. vagus nerve) (Bravo et al. 2011, Luczynski et al. 2016). Two popular models have evolved to investigate the microbiota-gut-brain axis (Luczynski et al. 2016, Lundberg et al. 2016).

For investigating physiologic and pathologic implications of the microbiota, GF mice have become a powerful and highly popular model (Foster et al. 2015). The GF model was in fact established in the middle of the 19th century (Gustafsson 1959). These mice, which are raised in a germ-free environment, offer the possibility to investigate the physiology of an organism that has never been in contact with bacteria. They also allow for microbiota transplantation studies, including the introduction of human fecal microbiota (humanized mice) (Goodman et al. 2011). In addition, the introduction of only one specific strain makes it possible to investigate specific effects of exactly this strain. Nevertheless, GF mice also have some limitations. Due to their germ-free upbringing these mice exhibit physiological differences compared to their colonized counterparts (Luczynski et al. 2016). Furthermore, their mucosal and systemic immune system is under-developed (Macpherson, Harris 2004). GF mice also display an exaggerated HPA response to restraint stress (Sudo et al. 2004). After that finding the alterations in behavior of GF animals were more closely investigated (Foster et al. 2015). Compared to their conventional counterparts, GF mice display changes in anxiety-like behavior, social preference, self-grooming and recognition memory (Luczynski et al. 2016). In addition, levels of neurochemicals are altered and the

BBB is impaired (Braniste et al. 2014, Luczynski et al. 2016). It should be added that there are contradictory findings with regard to behavior and levels of neurochemicals of GF mice. This is most likely due to the use of different strains, sexes and behavioral tests (Foster et al. 2015, Luczynski et al. 2016). In sum, the pertinent research has revealed that the absence of microbiota in GF mice leads to alterations in immune, digestive, intestinal, metabolic and nervous system function as well as aging (Smith, McCoy & Macpherson 2007, Grenham et al. 2011, Luczynski et al. 2016). Interestingly, although the absence of bacteria makes GF mice more susceptible to infections it also seems to make GF mice resistant to traits associated with multiple sclerosis (Round, Mazmanian 2009, Berer et al. 2011, Lee et al. 2011).

Antibiotic treatment circumvents some of the limitations of GF mice and has been suggested to be potentially more relevant for the elucidation of causal microbiota implications than the use of GF mice (Luczynski et al. 2016). Usually antibiotic-treated mice have been raised in an environment with germs (conventional or specific-pathogen-free housing). Hence, they can be used to induce an acute state of dysbiosis in a healthy and fully microbiota-colonized animal. Interestingly, some of the GF-associated features were also observed in antibiotic-treated mice (Bercik et al. 2011, Desbonnet et al. 2015). The introduction of these two models offers the possibility, on the one hand, to investigate the effects of a lacking microbiota, with the possibility to rescue those effects by introducing specific strains/complex microbiota or simply a microbial product and, on the other hand, to investigate changes due to dysbiosis, and save those by introducing specific strains/complex microbiota or simply a microbial product. By combining the knowledge gained with these two models it may be possible to draw further conclusions on the microbiota-gut-brain axis and to evaluate which changes are actually not based on microbiota alterations but rather due to a reaction to the antibiotics or the abnormalities of GF mice (Luczynski et al. 2016).

1.2.6 Probiotics and prebiotics – caring for the small ones

Given that alterations in the microbiota have an impact on the immune, endocrine and nervous system, a therapeutic approach with beneficial microbes or specific nutrients for the microbiota may be useful in many different clinical applications (Bienenstock, Kunze & Forsythe 2015). Hence, research on the development of probiotics and prebiotics has increased during the last years. Probiotics were defined by the World Health Organisation (WHO) as ‘live microorganisms which, when administered in adequate amounts, confer a

health benefit on the host' (FAO/WHO 2002). Prebiotics are defined as 'non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host' (Roberfroid et al. 2010). As mentioned in 1.2.2 breast milk innately contains not only probiotics but also prebiotics, which makes it a natural synbiotic. Hence, the addition of probiotics and/or probiotics to formulas intended to improve health of infants has been suggested but so far further research on safety and efficacy is still warranted (Braegger et al. 2011). In general, studies with probiotics and prebiotics in rodents look promising but evidence that their neurochemical and behavioral effects can also be induced in humans is still scarce, and more clinical data is needed (Bienenstock, Kunze & Forsythe 2015). In addition, given that there is no universal rationale to define their composition and dosage, studies using probiotics, prebiotics or both (synbiotics) to promote health have yielded rather inconsistent results (Foster et al. 2015). Besides this obvious challenge of standardization, variations in the composition of the microbial community before intervention are a general problem. The use of GF mice circumvents this problem but still has the limitations of this model in general. Although housing conditions are standardized for conventionally housed mice, they do not share an identical microbiota either.

1.2.7 Research on the microbiota – hype and reality

The importance of microbiota homeostasis for the health of the host has long been appreciated. Research on the role of the microbiota increased as it was shown that gut dysbiosis can not only be associated with intestinal (e.g. IBS) but also with extra-intestinal disorders (e.g. autism, atopic and allergic diseases, rheumatoid arthritis) (Gerritsen et al. 2011). The variety of diseases, dysbiosis has been linked with, covers immune, metabolic and neurological disorders (Donaldson, Lee & Mazmanian 2016). As a consequence, treatment strategies in IBD aim at changes of the microbiota with the help of probiotics, antibiotics and diets (Neuman, Nanau 2012). A comorbidity also exists between gastrointestinal complications/diseases and autism spectrum disorder (Kohane et al. 2012, Mayer, Padua & Tillisch 2014). Hence, studies have already used diets and probiotics as therapeutic approaches but clinical success is still limited and further studies are warranted (Li, Zhou 2016).

While the interest and fascination on the gut microbiota is still large, the first boom of excitement vanishes and skepticism is growing. Many factors play a role in this

phenomenon. First, many studies could show correlations between some bacterial species/genera/families or microbial diversity and a disease/symptom but some of these observations were not consistent between studies, difficult to reproduce or even contradictory (Lyte 2014). Second, the way of sampling, processing or analyzing of the microbiota often varies between studies and laboratories and makes comparison difficult (Fraher, O'Toole & Quigley 2012). Third, the initial hype of healthy microbes promoted the use and sale of probiotics and prebiotics, which thereby led to the involvement of non-scientific (marketing) interests in this scientific topic. Fourth, as most of the research in the microbiota-gut-brain axis is performed in animals, the lack of human studies is viewed as a notable drawback (Nguyen et al. 2015), given that mouse-human translation is hampered by distinct differences in the murine and human gut microbiota (Ley et al. 2005). The limitation in the collection of mucus-associated microbiota from the human gut, involving biopsies, is another draw-back in clinical trials. Likewise, in fundamental research the choice of the animal model of the disease under study must consider confounding factors and the results need to be interpreted with due care (Donaldson, Lee & Mazmanian 2016). In summary, the question of cause and consequence is often not clear in the relationship of disease and gut dysbiosis (Frank et al. 2011). There is also increasing awareness that besides the genetic community profiling, the metagenomic and metabolomic properties of the intestinal microbiota need to be analyzed (Gerritsen et al. 2011).
In general, after the big hype in microbiota research and its purported implications in all kinds of physiological functions and diseases, ongoing works starts to view these assertions more critically and strives for the establishment of standardized methods to validate the findings and make them more reproducible and comparable (Fraher, O'Toole & Quigley 2012).

1.3 Pattern recognition receptors (PRRs)

PRRs are critical for the innate immune system. They are expressed by various cell types, most importantly by immune cells, but also by intestinal epithelial cells, and present on the cell surface or intracellularly (Mogensen 2009). These receptors are able to recognize common microbial structures, termed microbe-associated molecular patterns (MAMPs). Some of these patterns can be expressed by commensal as well as pathogenic microorganisms (PAMPs), thus the term pathogen-associated molecular patterns is used as well (Wells, Loonen & Karczewski 2010). Hence, bacteria in the intestinal lumen are recognized by PRRs of the intestinal epithelial cells and immune cells. Toll-like receptors

(TLRs) are one very well described family of PRRs and expressed on a wide range of cell types (Wells, Loonen & Karczewski 2010). The 13 TLRs recognize different microbial structures. After stimulation a downstream signaling cascade is triggered leading to the activation of transcription factor nuclear factor-kappa B (NF-κB). NF-κB then controls the induction of proinflammatory cytokines and chemokines which are crucial in the induction of inflammatory and adaptive immune responses against pathogens (Wells, Loonen & Karczewski 2010). Given that commensal microbes also express MAMPs, there are mechanisms which prevent that the continuous exposure of the gut to MAMPs does not induce a persistent inflammatory state. This challenge seems to be met by the highly regulated expression and distribution of TLRs in the intestine (Maier, Anderson & Roy 2014).

1.3.1 Flagellin

Flagellin is a bacterial protein and agonist of TLR5. Polymerized flagellin monomers form the filaments of the flagellum, which enables the bacterium to directed motility (Song, Yoon 2014). The genes encoding flagellin are part of the chemotactic regulon and can be expressed by gram-negative as well as gram-positive bacteria (Hayashi et al. 2001, Aldridge, Hughes 2002). TLR5 is expressed at the cell surface of several immune cells and also on epithelial cells (Hayashi et al. 2001). Besides pro-inflammatory gene expression, activation of TLR5 by flagellin also mediates antiapoptotic effects in epithelial cells, thereby contributing to the maintenance of the epithelial barrier (Zeng et al. 2006, Fröhlich, Mayerhofer & Holzer 2015). TLR5 expression patterns illustrate how the immune system has adapted to the microbiota challenge. In the colon TLR5 is expressed on the basolateral surface of epithelial cells. In contrast, in the moderately colonized lower airways TLR5 is expressed on the apical surface of epithelial cells (Zhang et al. 2005, Ortega-Cava et al. 2006).

Flagellin has obtained considerable interest in different areas of research, including immunology, vaccine development and cancer (Honko, Mizel 2005, Sfondrini et al. 2006, Honko et al. 2006). Several studies showed that the presence of flagellin can have protective effects against bacterial dissemination by early immune recruitment (Vijay-Kumar et al. 2006, Hawn et al. 2007, Andersen-Nissen et al. 2007, Vijay-Kumar et al. 2008). Furthermore, flagellin was shown to have many advantages as a vaccine and was well-tolerated in clinical trials (Honko et al. 2006, Song et al. 2009, Treanor et al. 2010, Turley et al. 2011, Camacho et al. 2011, Tanomand et al. 2013). The role of flagellin in

cancer is not completely understood as studies showed variable effects of flagellin administration (Sfondrini et al. 2006, Rhee, Im & Pothoulakis 2008, Song et al. 2011). Given that TLR5 is situated on the basolateral surface of colonic epithelial cells (Ortega-Cava et al. 2006), a disruption of the epithelial barrier could cause an elevated TLR5 activation (Pastorelli et al. 2013, McCole 2014). Humans suffering from Crohn's disease, an IBD, were shown to have a high antibody response to flagellin of commensal bacteria (Lodes et al. 2004). Moreover, in patients with diarrhea-predominant IBS the levels of antibodies to flagellin are increased and correlate with the anxiety score (Dlugosz et al. 2015).

1.4 Aims of the project

The concept of the gut-brain axis, the bidirectional communication between gut and brain, has been supported by many experimental and clinical studies. This model has recently been expanded by including the gut microbiota as a critical node in the communication between the gut and brain (Kennedy et al. 2014). Clinical observations that the intestinal microbiota profile is altered in many neurologic and psychiatric disorders and diseases have given rise to the hypothesis that a disordered microbiota is a pathogenetic factor in these pathologies. The spectrum of diseases, that the microbiota has been suggested to play a role in, includes mental illnesses (depression, anxiety), neurodevelopmental disorders (autism), inflammatory conditions (allergies) and gastrointestinal disorders (IBD, IBS) (Damman et al. 2012, Collado et al. 2015, Palm, de Zoete & Flavell 2015).

There is, however, still little evidence for a causal relationship between gut dysbiosis and mental illness. This is a question of particular importance that currently can be addressed only in adequate animal models: GF mice and antibiotic-induced gut dysbiosis. As GF mice were shown to have limitations due to their GF upbringing (Diaz Heijtz et al. 2011, Braniste et al. 2014), antibiotic treatment gained appreciation as an alternative model (Bercik et al. 2011, Farzi, Gorkiewicz & Holzer 2012, Desbonnet et al. 2015). However, so far no standardized model for antibiotic-induced dysbiosis has been established. Moreover, the models of antibiotic-induced dysbiosis that have been published to date have not been characterized in great depth, mostly addressing particular aspects of dysbiosis-induced functional changes.

For this reason, the first part of the current thesis set out to conduct a systematic analysis of the impact of antibiotic-induced dysbiosis on several levels of the gut-brain axis in an attempt to understand the communication between microbiota and brain function.

Antibiotic-induced dysbiosis is not without limitations, inasmuch as it needs to be ensured that physiologic alterations are caused by the dysbiosis and not by the antibiotics themselves. Ideally, the antibiotics used to cause gut dysbiosis should not be absorbed from the gut and be devoid of any direct action on the brain. Of the antibiotics used here (ampicillin, bacitracin, meropenem, neomycin, vancomycin), only ampicillin is known to have some oral bioavailability in humans (Craig, Stitzel 2004, Lafforgue et al. 2008). As ampicillin is used in the clinic routinely, I hypothesized that ampicillin does not reach the murine brain and induce any neuroinflammatory or neurotoxic effects. In order to examine the pharmacokinetics of ampicillin in mice, a fast, sensitive and selective analytical method using solid phase extraction-liquid chromatography-tandem mass spectrometry was developed for the quantitative analysis of ampicillin and vancomycin (as non-absorbable control) in plasma and brain. Neuroinflammatory processes were judged by the expression of the cytokines interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α and interferon (IFN)-γ and chemokine (C-C motif) ligand 2 (CCL2), and a possible impact on reactive oxygen species (ROS) stress was explored by measuring glutathione reductase (GSR) and catalase (CAT) expression. Furthermore, general health was evaluated by measuring food intake, water consumption and body weight of the mice during the 11-day antibiotic treatment.

As the antibiotic cocktail administered in this project was designed to affect a very broad spectrum of bacterial taxa, I hypothesized that the gut microbiota is markedly decreased and antibiotic treatment leads to wide-spread dysbiosis. This was analyzed by 16S rRNA-based microbial community profiling of colon tissue including the luminal contents. Since GF mice are known to have an enlarged cecum and diminished spleen weight (Smith, McCoy & Macpherson 2007), and the weight of these organs is changed by gut dysbiosis (Reikvam et al. 2011), these parameters were also assessed after the 11-day antibiotic treatment.

The gut microbiota produces a huge number of metabolites that may not only act locally but also be absorbed and distributed by the circulation (Wikoff et al. 2009, Antunes et al. 2011, Tremaroli, Backhed 2012, Nicholson et al. 2012, Lu et al. 2014) to distant organs including the brain. Hence, I hypothesized that in antibiotic-treated mice the metabolome is altered compared to control mice. For this reason, the effect of the antibiotic treatment on the metabolic profile in the colon and circulation was investigated. A limited profile of

colonic metabolites was examined by ^{1}H NMR spectrometry-based metabolomics and compared with a broad profile of circulating metabolites examined by LC-MS.

A disturbance of the balance between the gut microbiota and the intestinal immune system by antibiotic-induced dysbiosis may give rise to immune stimulation, cytokine release and inflammation (Wlodarska, Finlay 2010, Dorozynska et al. 2014) and could thereby influence inflammation-related mood changes (Moloney et al. 2014). Dysbiosis and inflammation can, in addition, enhance paracellular permeability of the intestinal mucosa (Hu et al. 2013, Bischoff et al. 2014, Leclercq et al. 2014), which may further disrupt microbiota-immune homeostasis in the intestine. I hypothesized that antibiotic-treated mice do not exhibit markers of overt inflammation or disruption of epithelial barrier. To address these issues, the architecture of the small intestine and colon was investigated by routine histology. Moreover, the expression of the tight junction proteins claudin-1 (CLDN1), occludin (OCLN) and zonulin-1 (TJP1) and the cytokines IL-1β, IL-6, IL-10, TNF-α and IFN-γ were measured in the colon. In addition, the plasma levels of these cytokines were also estimated to address the question whether the antibiotic-induced changes in cytokine expression in the colon translate to changes in circulating cytokine levels and whether immune mediators play a role in the communication between gut dysbiosis and brain.

Since particular changes in emotional-affective and cognitive behavior have been reported to occur in GF mice (Gareau et al. 2011, Diaz Heijtz et al. 2011, Stilling, Dinan & Cryan 2014) and, to some extent, following antibiotic treatment (Bercik et al. 2011, Farzi, Gorkiewicz & Holzer 2012, Desbonnet et al. 2015) I hypothesized that the antibiotic mouse model under study also exhibits changes in these behavioral dimensions. Therefore, anxiety-related, exploratory and locomotor behavior was assessed with the open field test (OFT) and elevated plus maze (EPM) test, depression-like behavior and stress coping with the tail suspension test (TST), novel object memory with the novel object recognition test (NORT) and spatial memory with the Barnes maze (BM) test.

The gut-brain axis communicates via multiple routes including neural and hormonal (Cryan, Dinan 2012). Hence, I hypothesized that these ways are altered in antibiotic-treated mice. Alterations in the expression of bio- and neurochemical markers were analyzed in four microdissected regions of the murine brain: medial prefrontal cortex, hippocampus, amygdala and hypothalamus. Besides behavioral changes, expression of some neurochemical markers is altered in GF mice (Sudo et al. 2004, Neufeld et al. 2011,

Gareau et al. 2011, Clarke et al. 2013). Thus, potential effects of the antibiotic treatment on the expression of neural signaling-related molecules were examined with regard to brain-derived neurotrophic factor (BDNF), glutamate receptor subunit epsilon-2 (glutamate receptor, ionotropic, NMDA2B (epsilon 2), GRIN2B), glucocorticoid receptor (nuclear receptor subfamily 3, group C, member 1, NR3C1), serotonin transporter (solute carrier family 6 (neurotransmitter transporter), member 4, SLC6A4), neuropeptide Y (NPY) and Y1, Y2 and Y5 receptor (NPY1R, NPY2R, NPY5R). Plasma levels of corticosterone were taken as an index of changes in the HPA axis, another feature of GF mice (Sudo et al. 2004). Given that tight junction protein expression in frontal cortex, striatum and hippocampus is diminished in GF mice (Braniste et al. 2014), potential alterations in the blood-brain barrier were probed by analyzing the expression of the tight junction proteins claudin-5 (CLDN5), OCLN and TJP1.

Altogether, the first part of this thesis was therefore undertaken to systematically investigate the effect of antibiotic-induced dysbiosis on murine behavior and brain neurochemistry and to analyze the gut microbiota-brain axis at the level of the microbiota, gut, circulation and brain.

In an independent approach the second part of this thesis investigated the effect of flagellin on anxiety-like and social behavior. Microbial components (MAMPs/PAMPs) can activate the innate immune system via binding of PPRs (Wells, Loonen & Karczewski 2010). Two PAMPs, namely lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid, a synthetic analog of double-stranded RNA which is present in some viruses, have been shown to induce behavioral changes if administered parentally (Sparkman et al. 2006, Frenois et al. 2007, Gibney et al. 2013, Farzi et al. 2015, Leite et al. 2016, Araki et al. 2016). LPS and polyinosinic:polycytidylic acid have both been shown to increase anxiety- and depression-like behavior (Gibney et al. 2013, Sulakhiya et al. 2016) and, in the case of LPS, induce social interaction deficits (Araki et al. 2016).

Flagellin is a PAMP that is recognized by TLR5. Flagellin has been shown to be a potent immune activator (Gewirtz et al. 2001) and to provide protective function against various immune challenges (Vijay-Kumar et al. 2008). Furthermore, in contrast to LPS, flagellin does not show high a potential to generate severe adverse effects (Vijay-Kumar et al. 2008). In patients with diarrhea-predominant IBS the levels of antibodies to flagellin are increased and correlate with the anxiety score (Dlugosz et al. 2015). However, to the best

of my knowledge, flagellin-induced changes in emotional behavior have not been assessed in animal models. I therefore hypothesized that flagellin is also able to induce behavioral changes in mice. To address this issue, anxiety-related, exploratory and locomotor behavior was assessed with the OFT and social behavior with the social interaction test (SIT).

As a TLR5 agonist, flagellin is able to initiate innate immune responses (Hayashi et al. 2001). Moreover, these reactions seem to depend on the type of flagellin (the bacterial species of origin or vector) (Vidhyasekaran 2016). Thus, I further hypothesized that the bacterial species of origin has an additional impact on the behavioral response induced by flagellin. The behavioral response to flagellin from *B. subtilis* and *P. aeruginosa* as well as flagellin from *S. typhimurium* was tested by evaluating short- and long-term anxiety-related, exploratory and locomotor behavior with the OFT and the light-dark (LD) test.

2 Materials and Methods

The description of Materials and Methods is partly reused from Fröhlich et al. (2016).

2.1 Experimental animals

The experiments were carried out with adult male C57BL/6N mice obtained from Charles River Laboratories (Sulzfeld, Germany). Light conditions (12 hour light/dark cycle), temperature (set point 22°C) and relative air humidity (set point 50%) were tightly controlled. Throughout the experiments tap water and standard laboratory chow were provided ad libitum. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.2 Ethics statement

The experimental procedures and number of animals used were approved by the ethical committee at the Federal Ministry of Science, Research and Economy of the Republic of Austria (BMWF-66.010/0026-WF/II/3b/2014) and conducted according to the Directive of the European Parliament and of the Council of 22 September 2010 (2010/63/EU). The experiments were designed in such a way that both the number of animals used and their suffering was minimized. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.3 Reagents

Multiple **antibiotics** were used to deplete a wide range of bacteria in the gut.

Ampicillin sodium salt (catalogue number A9518, Sigma-Aldrich, Vienna, Austria) is a beta-lactam antibiotic with activity against gram-positive and gram-negative bacteria.

Bacitracin from *Bacillus licheniformis* (catalog number 11702, Sigma-Aldrich) is a polypeptide antibiotic active against gram-positive and gram-negative bacteria.

Meropenem (Optinem^{R}, AstraZeneca Österreich GmbH, Vienna, Austria) is a beta-lactam antibiotic with activity against gram-positive and gram-negative bacteria.

Neomycin trisulfate salt hydrate (catalog number N5285, Sigma-Aldrich) is an aminoglycoside antibiotic with activity against gram-negative bacteria and some gram-positive bacteria.

Vancomycin hydrochloride from *Streptomyces orientalis* (cataloge number 4747, Sigma-Aldrich) is a glycopeptide antibiotic with activity against gram-positive bacteria and some species of *Neisseria*.

Flagellin from different organisms was used as TLR5 agonist.

Flagellin isolated from *Salmonella typhimurium* (purified by ultrafiltration and affinity chromatography, catalogue code tlrl-pstfla, InvivoGen, San Diego, CA, USA).

Flagellin isolated from *Pseudomonas aeruginosa* (purified by ultrafiltration and chromatography, catalogue code tlrl-pafla, InvivoGen)

Flagellin isolated from *Bacillus subtilis* (purified by different successive separation techniques, catalogue code tlrl-pbsfla, InvivoGen)

2.4 Behavioral tests

2.4.1 Open field test (OFT)

The open field box consisted of a 50 x 50 x 30 cm (length x width x height) gray plastic box. The central area of the box was defined as the 36 x 36 cm area in the middle of the ground floor of the box and illuminated by 35 lux at floor level (Farzi et al. 2015). Mice were placed individually in the box and their behavior was tracked for 5 min with a video camera positioned above the center of the open field box. The behavior of the mice in the OFT was recorded and evaluated with the VideoMot2 software (TSE Systems, Bad Homburg, Germany). For analysis of anxiety-like behavior the overall time mice spent in the central area of the field and the number of central area entries were calculated. To quantify locomotion, the total distance traveled in the open field box was also measured. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.4.2 Elevated plus maze (EPM) test

The EPM was shaped like a plus sign with two opposing arms surrounded by 15 cm high walls (closed arms) while the other two opposing arms were open (open arms). The four arms (each 30 x 5 cm) were connected by a 5 x 5 cm neutral field, and the EPM was elevated 70 cm above the floor. Illumination of the neutral field, open arms and closed

arms was 20, 30, and 5 lux, respectively (Brunner et al. 2014). Each mouse was placed on the neutral field of the maze facing an open arm, and the behavior on the EPM was tracked for 5 min with a video camera and evaluated with the VideoMot2 software. After 5 min of exploration on the EPM the mice were returned to their home cage. The overall time each mouse spent on the open arms as well as the number of open arm entries were used to assess anxiety-like behavior. The distance traveled on the open and closed arms was used to evaluate locomotion. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.4.3 Tail suspension test (TST)

Mice were suspended by their tail with a 1.9 cm wide strapping tape (Leukotape classic; BSN Medical S.A.S., Le Mans, France) to a lever for 6 min, and their behavior was recorded by a video camera (Hassan et al. 2014). A trained blinded observer analyzed the video recordings with the VideoMot2 software event monitoring module for three types of behavior: swinging, curling and immobility. The mouse was considered swinging when it continuously moved its paws while keeping the body straight and/or moving the body from side to side. The mouse was considered curling when the mouse twisted its trunk (Berrocoso et al. 2013). The time spent swinging, curling and being immobile was calculated. Mice which climbed over their tails were excluded as they had learnt that escape is possible (Cryan, Mombereau & Vassout 2005). This methodological description was also published in an original article (Fröhlich et al. 2016).

2.4.4 Novel object recognition test (NORT)

Mice were habituated to the open field box each day during three consecutive days (days 7-9). On day 10, the mice were placed in the open field apparatus and given 5 min to explore two objects that were placed at adjacent edges of the central area of the field. One hour later the animals were re-exposed to one familiar object (old object) together with a novel object (new object) for 5 min. The exploratory behavior directed at each object was recorded in both sessions. The time of object exploration was measured with the VideoMot2 software and the performance of each mouse was expressed by the memory index (MI) according to the formula: $MI = (t_n - t_o)/(t_n + t_o)$. The time exploring the new object is represented by t_n, whereas t_o represents the time exploring the old object (Redrobe et al. 2004). Mice that explored the objects for less than 5 s in total were excluded from the results. To avoid spatial and object bias, the position of objects was alternated between

trials, and the choice of familiar versus novel object was changed from mouse to mouse. After each test session the objects were cleaned with 70% ethanol. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.4.5 Barnes maze (BM)

The BM test protocol was adapted from that used by Attar et al (Attar et al. 2013), with minor modifications. In short, the BM apparatus consisted of a circular white polyvinyl chloride slab with a diameter of 91 cm elevated 61.5 cm above the floor. At a distance of 2.5 cm from the edge, 20 holes with a diameter of 5 cm were evenly distributed along the perimeter. Below the surface of the maze a small escape cage was affixed underneath one of the 20 holes (target hole). The surface of the BM was illuminated by 35 lux. Four visual cues (colored circles and squares) were mounted around the room for spatial orientation on the slab. The animals were subjected to the BM test on three consecutive days. On day 7 the mice were habituated to the BM, and on days 8 and 9 the mice were trained to quickly find and enter the escape hole. This short training phase consisted of two training trials on day 8 and three training trials on day 9. The probe session during which no escape cage was affixed to the BM apparatus was scheduled on day 10. The movements of the mice on the training and probe days were tracked for 2 min with a video camera and evaluated with the VideoMot2 software. Spatial learning manifested itself in a shortening of the latency to identify the target hole (target latency) during consecutive training trials. Spatial memory was assessed by the time the mice spent in the target area (quadrant of BM with target hole in the center) on the probe day. To avoid spatial or visual cue bias, the location of the escape cage was alternated after every three mice. After each test session the BM and escape cage were cleaned with 70% ethanol. Mice which did not learn the correct position of the target hole, or did not voluntarily enter the escape cage, were excluded from the evaluation. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.4.6 Social interaction test (SIT)

The SIT was adapted from Kaidanovich-Beilin et al. (Kaidanovich-Beilin et al. 2011) with minor modifications. In short, the social interaction apparatus consisted of a rectangular three chambered box made of a transparent Perspex cage and two wire cup-like containers. Each chamber was 20 x 40 x 22 cm. The entrance to the other compartments could be blocked by two sliding doors (5 x 8 cm). First the mice were habituated to the apparatus.

For habituation the two sliding doors were closed and the mice spent 5 min only in the middle chamber. In the consecutive test session a stranger mouse was placed into one wire cup-like container and both containers were each placed in the middle of one outer chamber and the doors were removed. The mice were free to move around in all 3 chambers and interact with the stranger mouse. The movements of the mice were tracked and evaluated with the VideoMot2 software during the 5 min test session. Time spent in the immediate vicinity (5 cm) of the containers (with stranger mouse or empty), time spent in the two compartments with container as well as number of entries into compartments and vicinities were used to assess social behavior. Mice that did not enter all of the three compartments at least once were excluded from the evaluation.

2.4.7 Light-dark (LD) test

The LD box consisted of a cage (37 x 21 x 20.5 cm) which was divided into a light compartment and a dark compartment of equal size. Transparent walls lined the light compartment whereas the dark compartment consisted of black acrylic walls. The areas were connected only through a door (4.5 x 6 cm). Illumination in the light compartment was set to 300-400 lux (Brunner et al. 2014). Each mouse was placed in the light compartment facing the connecting door to the dark compartment. Animals were allowed to explore the box for 10 min. Light-beam frame recording was used and behaviors were recorded using the ActiMot2 system (TSE Systems). For analysis of anxiety-like behavior the overall time mice spent in the light compartment and the number of transitions between the light and dark compartment were calculated. To quantify locomotion, the total distance traveled in both compartments was also measured.

2.5 Experimental protocols

2.5.1 Protocol 1 – Effects of antibiotic treatment on microbiota-gut-brain axis

The aim of protocol 1 was to evaluate the effect of antibiotic-induced dysbiosis on health parameters, behavior, cognition, metabolome, microbiome, expression of cytokines, tight junction proteins and neuronal signaling molecules. Three separate experiments were carried out each with a different cohort of male mice (Figure 3). To minimize environmental stress, mice were transferred to the behavioral test room (12 hour light/dark cycle, set points 22°C and 50% relative air humidity, maximal light intensity 100 lux) 2 days before the start of the antibiotic treatment and maintained in this room until sacrifice.

Mice were kept in groups of 2 to 3 animals per cage. At the age of 8-11 weeks mice were treated with a mix of five antibiotics (pH 6.98-7.14) or vehicle (distilled water) by oral gavage (10 mL/kg) for 11 days (Fig. 1). The antibiotics (108.0 mg bacitracin, 108.0 mg neomycin, 43.2 mg ampicillin, 21.6 mg meropenem, 6.48 mg vancomycin) were dissolved in 4.5 mL distilled water. Dosing of antibiotics was based on studies where ampicillin (Membrez et al. 2008, Khosravi et al. 2014, Desbonnet et al. 2015), bacitracin and neomycin (Bercik et al. 2011), meropenem (Moller et al. 2005, Gadjeva et al. 2010) and vancomycin (Lawley et al. 2012) were added to the drinking water. The antibiotic concentrations used in these studies were converted to equivalent gavage doses, calculated relative to the average daily water intake. Each day the mice were weighed before the first gavage, and the gavage volume was adjusted accordingly. Because of their coprophagic behavior all cage mates received the same treatment. For each gavage session 4.5 mL of the antibiotic mix was prepared and used within 18 h. In all experiments, vehicle- and antibiotic-treated animals were run in parallel. This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.5.1.1 Experiment 1.1

In the first experiment mice were subjected to a behavioral test battery consisting of the OFT on days 7, 8 and 9, EPM test on day 8, TST on day 9, and NORT on day 10. From the first day of treatment (day 0) until day 6 the mice were gavaged twice daily (8:00 am, 5:00 pm). On day 7 the mice were gavaged shortly after the OFT (12:00 am) and before onset of the dark phase (5:00 pm). From day 8 onwards the mice were gavaged once daily at 3:00 pm (after the behavioral testing of the mice scheduled for the respective day had been completed). The mice received the last treatment at 3:00 pm on the day before sacrifice. Mice were sacrificed on day 11. Plasma, colon, luminal colonic contents, and brain tissues were collected for molecular analysis (n = 10-12) (Figure 3). This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.5.1.2 Experiment 1.2

In the second experiment, mice were habituated to the BM on day 7. BM training sessions were performed on days 8 and 9, and the probe session was conducted on day 10. From the first day of treatment (day 0) until day 6 the mice were gavaged twice daily (8:00 am, 5:00 pm). On day 7 the mice were gavaged shortly after habituation to the BM (12:00 am) and before onset of the dark phase (5:00 pm). From day 8 onwards the mice were gavaged once

daily at 3:00 pm (after the behavioral testing of the mice scheduled for the respective day had been completed). The mice received the last treatment at 3:00 pm on the day before sacrifice. Mice were sacrificed on day 11 (n = 9). This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.5.1.3 Experiment 1.3

In the third experiment mice were also treated with vehicle or the antibiotic mix but were not subjected to any behavioral tests; they were sacrificed on day 10 (the time point of cognitive testing) for analysis of antibiotic concentrations in blood and brain, for histological examination of the colon and for measurement of mRNA and protein expression in the colon (n = 6-8) (Figure 3). Treatment was adapted to the first two experiments: from the first day of treatment (day 0) until day 6 the mice were gavaged twice daily (8:00 am, 5:00 pm). On day 7 the mice were gavaged at 12:00 am and before onset of the dark phase (5:00 pm). From day 8 onwards the mice were gavaged once daily at 3:00 pm. The mice received the last treatment at 3:00 pm on the day before sacrifice (day 10). This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

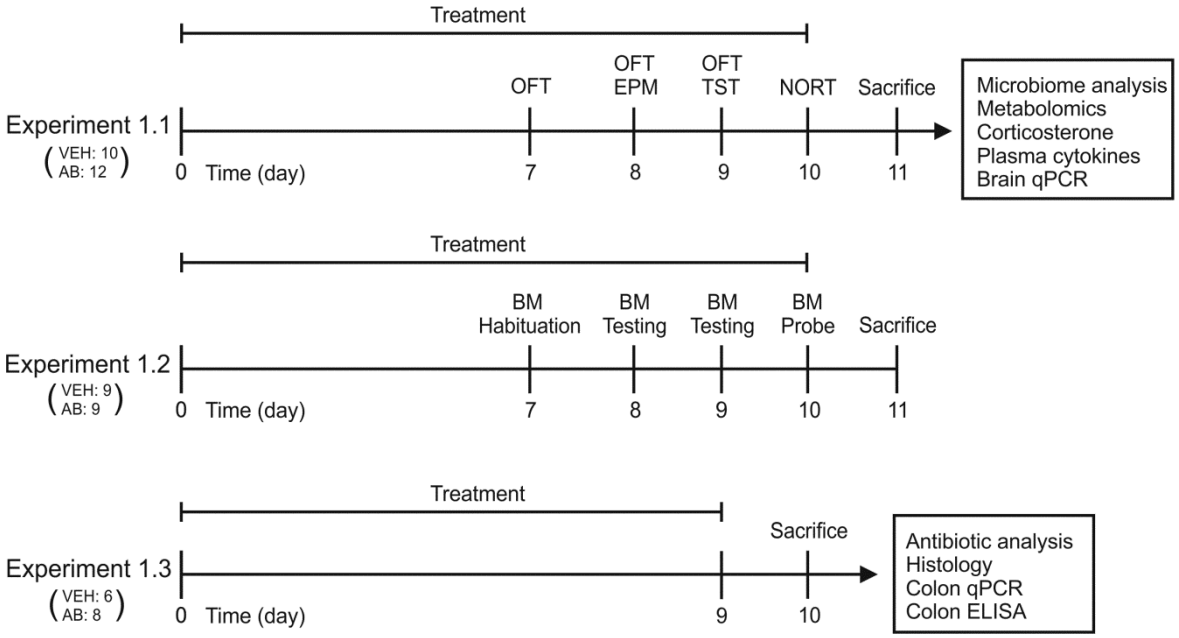

Figure 3 Experimental groups and time lines for protocol 1 - Effects of antibiotic treatment on microbiota-gut-brain axis.

Mice of experiment 1.1 were subjected to a test battery including OFT, EPM test, TST and NORT. After sacrifice, tissues of experiment 1.1 animals were analyzed as indicated. Mice of experiment 1.2 were habituated and trained in the BM. Animals of experiment 1.3 were not subjected to any behavioral tests but euthanized on day 10 (the day of cognitive

evaluation in experiment 1.1 and 1.2) to measure antibiotic concentrations in plasma and brain and investigate the effect of antibiotics alone on colon histology, mRNA and protein expression. In all experiments vehicle (VEH)- and antibiotic (AB)-treated animals were run in parallel, the number of mice in each group being indicated in brackets. OFT, open field test; EPM, elevated plus maze; TST, tail suspension test; NORT, novel object recognition test; BM, Barnes maze; qPCR, real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay. A similar figure has also been published in an original article (Fröhlich et al. 2016).

2.5.2 Protocol 2 – Effects of flagellin on social and anxiety-like behavior

The aim of protocol 2 was to evaluate the short- and long-term effects of flagellin on anxiety-like and social behavior. Two separate experiments were carried out each with a different cohort of male mice (Figure 4). Upon arrival at the animal center mice were habituated for two weeks. Mice were transferred to the behavioral test room (12 hour light/dark cycle, set points 22°C and 50% relative air humidity, maximal light intensity 100 lux) 1 day before treatment. At the age of 10 weeks mice were treated with one intraperitoneal (i.p.) injection of flagellin (200 µg/kg) or vehicle (saline).

2.5.2.1 Experiment 2.1

The first experiment of this protocol was carried out to investigate the effect of flagellin from *S. typhimurium* on anxiety-like and social behavior. Mice were housed in groups of 3 to 4 animals per cage. The OFT was performed approximately 4 h after treatment. The SIT was performed the day after treatment (approximately 24 h after treatment).

2.5.2.2 Experiment 2.2

The second experiment of this protocol was carried out to evaluate whether the origin of flagellin has an impact on anxiety-like behavior. Mice were housed in groups of 3 animals per cage. Mice received either flagellin from *S. typhimurium*, *B. subtilis* or *P. aeruginosa*, or vehicle (saline) via i.p. injection. Mice were subjected to the OFT 3 h after injection. The LD test was performed 24 h after treatment.

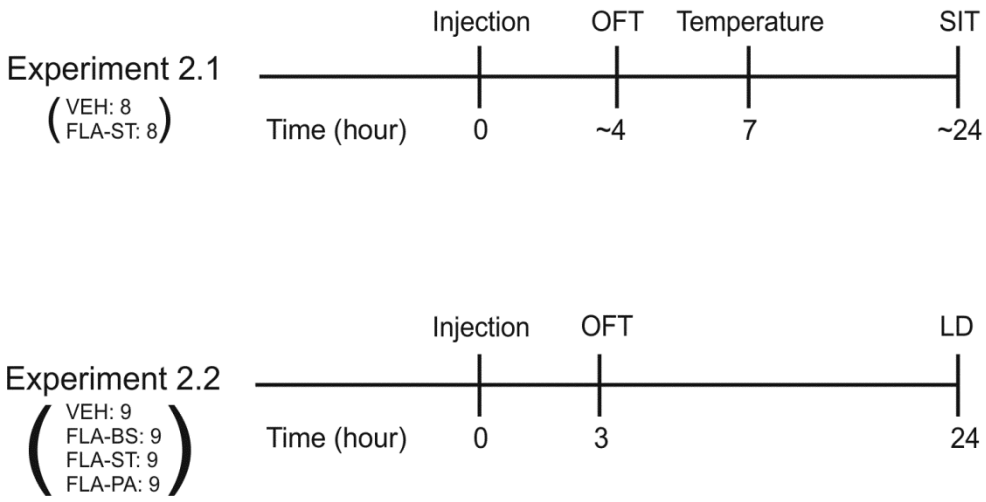

Figure 4 Experimental groups and time lines for protocol 2 - Effects of flagellin on social and anxiety-like behavior.

Mice of experiment 2.1 were subjected to the OFT and the SIT. Mice of experiment 2.2 underwent the OFT and the LD test. In all experiments vehicle (VEH)- and flagellin (FLA)-treated animals were run in parallel, the number of mice in each group being indicated in brackets. FLA-BS, flagellin from *Bacillus subtilis*; FLA-ST, flagellin from *Salmonella typhimurium*; FLA-PA, flagellin from *Pseudomonas aeruginosa*; OFT, open field test; SIT, social interaction test; LD, light-dark.

2.6 Blood and tissue harvesting for biochemical analysis

Animals were anesthetized with pentobarbital (150 mg/kg i.p.). Blood was drawn via cardiac puncture with a syringe that was filled with 100 µL of 3.8% sodium citrate as anticoagulant. The average blood vs sodium citrate ratio was 4:1. After 15 min of centrifugation at 4°C and 7000 rpm, blood plasma was collected and stored at -70°C. Following blood collection, brains were collected and immediately frozen in 2-methylbutane (Sigma-Aldrich) on dry ice for 5 s. Afterwards the brains were wrapped in aluminum foil and kept at -70°C. The spleen and cecum were removed and weighed. For microbiome and metabolome analyses the large intestine including the luminal contents was removed and stored at -70°C. Stool consistency was assessed by rating the fecal boli either as formed or loose. For histology approximately 1 cm segments of the jejunum, ileum and colon were excised, washed with tap water and placed in 10% buffered formalin (Roti-Histofix, Carl Roth, Karlsruhe, Germany). For RNA extraction approximately 1 cm of the colon was collected, cleaned with tap water, shock frozen in liquid nitrogen and stored at -70°C. This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.7 Microbiome analysis

Frozen colon tissue including the luminal contents was homogenized on a MagNA Lyser Instrument with MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). Bacterial DNA was extracted with the Power Lyzer® Power Soil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, the DNA concentration was determined, and bacterial 16S rRNA was amplified by PCR with the Rotor-Gene SYBR Green PCR Kit (Qiagen, Hilden, Germany) using 20 ng DNA as template (see Appendix 6.1). To this end, the 16S primers F27—AGAGTTTGATCCTGGCTCAG and R357—CTGCTGCCTYCCGTA were used as fusion primers containing Ion Torrent sequencing adapters. In order to assess contaminations imported during the microbiome analysis workflow, a sample devoid of any tissue and colon content (blank) was included in the PCR run. Afterwards PCR products were gel-purified and the amplicon DNA concentration was determined. Sequencing of pooled amplicons was performed with the Ion PGM Sequencer and an Ion Sequencing 400 Kit (both from Life Technologies, Carlsbad, CA, USA). Sequencing was performed by Andrea Thüringer at the Institute of Pathology of the Medical University of Graz. Contaminating non-bacterial sequences were removed and Acacia error correction was applied on all reads using standard parameters (Bragg et al. 2012). Chimeras were identified by Usearch algorithm and removed. The resulting bam file was introduced into QIIME (v1.8.0) 16S workflow (www.qiime.org) (Caporaso et al. 2010). Differences in microbial communities between groups were investigated using the phylogeny-based weighted UniFrac distance metric. Alpha diversity and ADONIS calculations were performed with the respective QIIME scripts (alpha_diversity.py and compare_categories.py). Sequence analysis was performed by Karl Kashofer at the Institute of Pathology of the Medical University of Graz and the methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016). Furthermore, real-time PCR (qPCR) was performed to evaluate the number of bacteria in the individual samples. Bacterial 16S rRNA was amplified by qPCR with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, California, USA) using 20 ng DNA as template (see Appendix 6.1). The 16S primers F515—GATTGCCAGCAGCCGCGGTAA and R806— GGACTACCAGGGTATCTAAT were used as primers. Results from qPCR were analyzed with the Bio-Rad CFX Manager software (Bio-Rad).

2.8 Hematoxylin and eosin staining

Segments of the small and large intestine were excised and kept in 10 % Roti-Histofix (Carl Roth) for 48 h and then rehydrated in running water for 1 h. After 3.5 h dehydration in 50% ethanol, tissues were stored in 70% ethanol in the fridge. Tissues were embedded using automated embedding (Tissue TeK VIP, Sakura Finetek, Vienna, Austria) with the embedding console system (Tissue TeK -TEC, Sakura) according to a standard protocol. Paraffin sections were cut into 5 µm slices using a Microm HM360 (Histocom, Vienna, Austria) rotary microtome. Sections were mounted on superfrost ultra plus slides (Thermo Scientific, Waltham, MA, USA). After overnight drying, the slides were deparaffinized with Roticlear (Carl Roth) followed by a descending series of ethanol (100%, 96%, 80%, 50%) and distilled water. Then the slides were stained with hematoxylin solution (Carl Roth) for 8 min, rinsed with tap water followed by staining with eosin Y (Sigma-Aldrich) for 1 min. After being 10 min in tap water, slides were dehydrated with an ascending series of ethanol (70%, 80%, 96%,100%), Roticlear/ethanol (50%) solution and Roticlear. Subsequently the slides were mounted using Entellan (VWR, Vienna, Austria). Images were taken at an upright brightfield microscope (Olympus BX51, Olympus, Vienna, Austria) equipped with a DP71 camera. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.9 Brain microdissection

The working area and dissection instruments were cleaned with RNeasy AWAY (Carl Roth). The microdissection was performed by a trained researcher on a cold plate (Weinkauf Medizintechnik, Forchheim, Germany) set at -20°C. Brain areas (medial prefrontal cortex (Bregma, +3.20 to −0.22), hypothalamus (Bregma, +0.26 to −2.92), amygdala (Bregma, −0.58 to −2.54), hippocampus (Bregma, −0.94 to −4.04)) were dissected as previously described (Brunner et al. 2014). The dissected brain tissues were collected in micro packaging vials filled with some Precellys beads (Peqlab, Erlangen, Germany) and stored at -70°C until RNA extraction. This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.10 Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR (qPCR)

Brain tissues were homogenized with the Precellys 24 homogenizer (Peqlab). Colon tissues were disrupted with an ULTRA TURRAX T8 (IKA-Werke, Staufen, Germany). RNA

extraction was performed according to the manufacturer's instructions using the RNeasy lipid tissue mini kit (Qiagen) and RNeasy mini kit (Qiagen) for brain and colon tissues, respectively. The RNA concentration was measured and 2 µg of RNA was reverse-transcribed in the Mastercycler Gradient (Eppendorf, Hamburg, Germany), using the high capacity cDNA reverse transcription kit (Life Technologies) according to the manufacturer's instructions (see Appendix 6.2). A control without reverse transcriptase for each group and area was always included. For relative quantification of mRNA levels, qPCR was performed on a LightCycler®480 System with TaqMan inventoried gene expression assays for BDNF (Mm04230607_s1), CAT (Mm00437992_m1), CCL2 (Mm00441242_m1), CLDN1 (Mm00516701_m1), CLDN5 (Mm00727012_s1), GRIN2B (Mm00433820_m1), GSR (Mm00439154_m1), IFN-γ (Mm01168134_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm01288386_m1), NPY (Mm03048253_m1), NPY1R (Mm00650798_g1), NPY2R (Mm01218209_m1), NPY5R (Mm00443855_m1), NR3C1 (Mm00433832_m1), OCLN (Mm00500912_m1), SLC6A4 (Mm00439391_m1), TJP1 (Mm00493699_m1), TNF-α (Mm00443258_m1), ACTB (Mm00607939_s1), GAPDH (Mm99999915_g1), PPIL3 (Mm00510343_m1) and the TaqMan gene expression master mix (Life Technologies). All samples were measured as triplicates. ACTB, GAPDH and PPIL3 were used as reference (endogenous housekeeping) genes for quantification of target gene expression in the brain, while ACTB and PPIL3 were used as reference (endogenous housekeeping) genes for expression quantification in the colon. Quantitative measurements of target gene levels relative to controls were performed with the 2-ΔΔCt method using the mean value of the VEH-treated group as the calibrator (Livak, Schmittgen 2001). Group differences were expressed as fold changes. This methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.11 Colonic content metabolomics

Metabolites in the colonic content of mice were analyzed by untargeted ^{1}H NMR analysis. Colonic content extracts were prepared by mixing 20 mg of frozen luminal colonic content material with 1 mL of saline phosphate buffer that consisted of 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl and 1 mM sodium 3-(trimethylsilyl)-propionate-D_4 (TSP) in D_2O. After mixing thoroughly, samples were centrifuged at 17,000 x g for 5 min. Each supernatant was filtered through a 0.2 µm membrane filter, and 300 µL filtrate was transferred to a 3 mm NMR tube for analysis. High resolution ^{1}H NMR spectra were

recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). The sample temperature was controlled at 298K. Each spectrum consisted of 128 scans, and the *noesypr 1d* pre-saturation sequence was used to suppress the water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8=0.15 s). A 90^{o} pulse length of 8.2 µs was set for all samples. ^{1}H NMR spectra were Fourier transformed (LB = 0.3 Hz) and zero filling, manually phased and base line corrected using TOPSPIN 2.0 software. The AMIX software (Bruker) was used to reduce the ^{1}H NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed, and the spectral data were reduced to include regions of equal width (0.04 ppm) equivalent to the region of δ 10.00–0.40. The signals of identified metabolites were integrated manually using MestReNova (v. 9.0.1) and normalized to the intensity of the internal standard (TSP) (Khoyratty et al. 2015). This measurement was performed by Young Hae Choi and Hye Kyong Kim at the Institute of Biology of the Leiden University and the methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.12 Plasma metabolomics

Metabolites in the plasma of mice were analyzed by targeted LC-HRMS metabolomics. The plasma samples were processed according to Yuan et al. (Yuan et al. 2012). In short, 800 µl of precooled (-80°C) methanol was added to 200 µl sample, mixed and incubated overnight at -80°C. Protein precipitates were removed by 10 min centrifugation at 13,000 g, supernatants dried under nitrogen flow and finally reconstituted in 200 µl 30% methanol for analysis. Samples were analyzed by chromatographic separation on a Luna NH2 column (2×150 mm; 3 µm; Phenomenex, Torrance, USA) according to Bajad et al. (Bajad et al. 2006) by hydrophilic interaction liquid chromatography (HILIC) with an Ultimate 3000 UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a high resolution mass spectrometer Q-Exactive (Thermo Fisher Scientific, Bremen, Germany). Full scan spectra were recorded in positive and in negative electrospray from m/z 70–1050 with a resolution of 140,000 (at m/z 200) using data dependent fragmentation.

Samples were measured randomized in four sample blocks with one blank and one QC (pool of equal parts from all sample extracts) in-between and two blank/QC pairs at the beginning and end of the measurement series, yielding in total nine QC runs.

Raw data were converted into mzXML by msConvert (ProteoWizard Toolkit v3.0.5) (Chambers et al. 2012), and metabolites were targeted-searched by the in-house developed tool PeakScout, with a reference list containing accurate mass and retention times in agreement to standards outlined by Sumner et al. (Sumner et al. 2007). Molecular masses for all substances were taken from the literature and available online databases (HMDB, KEGG, Metlin) (Kanehisa, Goto 2000, Kanehisa et al. 2014, Smith et al. 2005, Wishart et al. 2007, Wishart et al. 2009, Wishart et al. 2013). Additionally, pure substances of all analytes, except fatty acid and lipids, were run on the same system to obtain reference retention times and fragmentation spectra. PeakScout excised chromatograms with mz-slices of ±50 ppm of targeted masses according to the reference list. Chromatographic peaks of every substance in every sample were confirmed manually and integrated (area under the curve, AUC). In case of ambiguities, fragmentation spectra were manually rechecked to ensure correct metabolite identification (identification error rate estimated to be below 2% for metabolites, and below 15% for lipids). Metabolites were categorized according to analytical quality as suitable for multivariate analysis (MVA) + univariate analysis (UVA) or suitable for UVA only, while all other metabolites found were excluded. Filters were (i) median of ppm difference to accurate mass for MVA+UVA <15 ppm, for UVA <20 ppm; (ii) range of ppm difference to accurate mass for MVA+UVA <25 ppm, for UVA <50 ppm; (iii) relative standard deviation of peak retention times in samples MVA+UVA <15%, UVA <30%; (iv) relative standard deviation of peak retention times in QC MVA+UVA <10%, UVA <30%; (v) percentage of median blank AUC from median QC AUC for MVA+UVA <10%, for UVA <75%; (vi) relative standard deviation of QC AUC for MVA+UVA <30%, for UVA <75%, and (vii) percentage of missing data in samples for MVA+UVA <30%, for UVA <50%.

To correct for dilution differences resulting from different blood volumes (100-560 µL) median normalization was applied as follows. Each metabolite was scaled 0 to 1 as $AUC_{0\text{-}1scaled} = (AUC\text{-}minimum(AUC))/(range(AUC))$, for each sample the $sample_{median}$ was calculated as the *median of all* $AUC_{0\text{-}1scaled}$ in the sample, and AUC values were normalized as *median normalized AUC* = $AUC/sample_{median}$. Finally, *median normalized AUC* values were log_{10} transformed to achieve sufficiently normal distribution and homoscedasticity in the data set.

The results for samples of mouse 2 and 6 were excluded from analysis because contamination with bile during blood collection impacted too strongly on the metabolite

profile. The results for samples of mouse 1 and 22 were also excluded from analysis because blood volumes of 100 µl resulted in a very high 1:1 dilution with citrate, which impacted on metabolic profiles so strongly that median normalization was not able to correct the dilution. This measurement was performed by a collaborator (Natalie Bordag and team, HEALTH Institute for Biomedicine and Health Sciences, JOANNEUM RESEARCH Forschungsgesellschaft mbH) and the methodological description was also published in a similar fashion in an original article (Fröhlich et al. 2016).

2.13 Antibiotic analysis

A sensitive and selective analytical method for the quantitative analysis of antibiotics in plasma and brain was developed. Plasma samples were thawed to room temperature. A 50 µL aliquot was diluted with 100 µL of 0.1% acetic acid (Sigma-Aldrich), spiked with 10 ng amoxicillin (Sigma-Aldrich) as internal standard and mixed. A 30 mg strata-x solid phase extraction cartridge (30 mg; 1 cc; Phenomenex, Aschaffenburg, Germany) was activated (1 mL methanol (Sigma-Aldrich) followed by 1 mL 0.1% acetic acid), after which the diluted sample was loaded on the cartridge and washed with 1 mL of 0.1% acetic acid. Analytes were eluted with 1 mL methanol. The elution solvent was evaporated to dryness and the residual sample was resolved in 100 µL of 0.1% formic acid (Sigma-Aldrich).

For brain analysis, half a brain was placed into 2 mL ZR Bashing Bead Lysis Tubes (VWR, Vienna, Austria), spiked with 10 ng amoxicillin (internal standard) and 1 mL water. After homogenization the vial was centrifuged at 10,000 rpm for 10 min and the supernatant was used for solid phase extraction. The supernatant was diluted with 1 mL of 0.1% acetic acid and mixed. A 100 mg strata-x solid phase extraction cartridge (100 mg, 3 cc; Phenomenex) was activated (3 mL methanol followed by 3 mL 0.1% acetic acid), and the diluted sample was loaded on the cartridge and washed with 3 mL of 0.1% acetic acid. Analytes were eluted with 3 mL methanol. The elution solvent was evaporated to dryness and the residual sample was resolved in 200 µL of 0.1% formic acid.

Analysis was performed with an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray ionization source coupled to a 1290 binary UHPLC system (Agilent, Waldbronn, Germany). For chromatographic separation a Phenomenex Kinetex C18 (100x2.1mm; 2.6 µm) column (Phenomenex) was used.

Gradient elution with a binary mobile phase system of channel A (0.1% formic acid in water) and channel B (0.1% formic acid in acetonitrile (Sigma-Aldrich)) was performed at 30°C column temperature and a 0.4 mL /min flow rate. The gradient profile was 3% channel B for 0.5 min, linearly increased to 24% channel B over 1.5 min and ramped to 90% channel B over 0.1 min. The run time was set to 3.5 min following a post time of 1.5 min. The autosampler was maintained at 4°C and the injection volume was set at 5 µL. Mass spectrometry detection was conducted in positive mode, using dynamic multiple reaction monitoring. The cycle time was set at 250 ms. Two precursor/product ion transitions were used as quantifier and qualifier for each analyte, respectively. Quantitation was performed using the ion transitions *m/z* 366.1 → *m/z* 349;114 (amoxicillin; internal standard), *m/z* 350.1 → *m/z* 114;106 (ampicillin) and *m/z* 724.8 → *m/z* 144;100.1 (vancomycin). According to bioanalytical validation guidelines the method was tested for the following parameters: selectivity, linearity, lower limit of quantification (LLOQ), accuracy and precision, extraction efficiency, matrix effect, and autosampler stability. Calibration curves were constructed using linear regression with 1/x2 weighting based on a minimum of 8 calibrator peak area ratios, excluding the blank. Calibration levels were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 50, 100 and 200 ng/mL for ampicillin and 1.0, 2.0, 5.0, 10, 20, 50, 100 and 200 ng/mL for vancomycin. Calibrators and triplicate quality control samples at low, medium and high concentrations were analyzed in each set of specimens. Samples in which no antibiotic was detected were labeled with the respective LLOQ value for numerical evaluation and statistical comparison. This measurement was performed by a collaborator (Bernhard Wagner, Institute of Biomedical Science, FH JOANNEUM University of Applied Sciences) and the methodological description was also published in an original article (Fröhlich et al. 2016).

2.14 Circulating corticosterone analysis

Plasma corticosterone levels were determined with an enzyme immunoassay kit (Assay Designs, Ann Arbor, Michigan, USA) as described previously (Farzi et al. 2015). According to the manufacturer's specifications, the sensitivity of the assay was 27 pg/mL, and the intra- and inter-assay coefficient of variation amounted to 7.7 % and 9.7 %, respectively. Two plasma samples (from mouse 2 and 6) were contaminated with bile during blood collection and excluded from all analyses. In two other plasma samples (from mouse 1 and 22) the plasma vs. sodium citrate ratio was 1:1. Since this high dilution of plasma is likely to give false (low) values of plasma concentrations, those two samples

were excluded from further analysis as well. This methodological description was also published in an original article (Fröhlich et al. 2016).

2.15 Circulating cytokine analysis

Plasma concentrations of IL-1β, IL-6, IL-10, TNF-α and IFN-γ were simultaneously quantified with the ProcartaPlex™ immunoassay (eBioscience, San Diego, CA, USA). According to the manufacturer's instructions the cytokines were determined using analyte-specific magnetic beads coated with target-specific antibodies. Target-specific fluorescent signals were measured with the Bio-Plex 200 multiplex suspension array system employing Luminex xMAP-technology in combination with the Bio-Plex 5.0 Software (Bio-Rad). Standard curves for each analyte were generated by using the reference analyte concentration supplied, and concentrations were calculated with a five-parameter logistic curve-fitting method (Reichmann et al. 2015). According to the manufacturer, the sensitivity for the respective cytokines was: IL-1β: 0.14 pg/mL, IL-6: 0.21 pg/mL, IL-10: 0.69 pg/mL, TNF-α: 0.39 pg/mL, IFN-γ: 0.09 pg/mL. In all plasma samples examined, the concentrations of IL-1β were too low to render detectable values. Levels of IL-10 were also excluded from analysis as the majority of samples were below the detection limit (no group preference being observed). This methodological description was also published in an original article (Fröhlich et al. 2016).

2.16 Cytokine expression in colonic tissue

Protein levels of IL-1β, IL-6, IL-10, IFN-γ and TNF-α in colonic tissue were each determined with an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Houston, Texas, USA). Protein extraction was performed according to the manufacturer's instructions (see Appendix 6.3). According to the manufacturer's specifications, the sensitivity for the respective cytokine was: IL-1β: 7.8 pg/mL, IL-6: 7.8 pg/mL, IL-10: 1.56 pg/mL, TNF-α: 15.6 pg/mL, IFN-γ: 15.6 pg/mL.

2.17 Statistics

Results were statistically evaluated either with SPSS 22 (SPSS Inc., Chicago, IL, USA) or with R (R Development Core Team 2011) (v3.2.1, packages stats, missMDA, nlme) using Tibco® Spotfire® (v7.0.0). Principal component analysis (PCA) was performed centered and scaled to unit variance (R function *prcomp*). Missing values of NMR metabolites were imputed by zero, missing values of LC-MS metabolites by a regularized expectation-maximization (function *imputePCA* and *estim_ncpPCA*). For all data except LC-MS

metabolomics differences between two independent groups were analyzed with the independent samples *t*-test in SPSS. The homogeneity of variances was assessed with the Levene test. In case of a non-parametric distribution of data the Mann–Whitney U test was used. For repeated measurements repeated measures analysis of variance (ANOVA) was performed. For multiple comparisons, *p*-values were adjusted with the false discovery rate. Probability values of $p \leq 0.05$ were regarded as statistically significant. Pearson's correlation coefficient was used to determine correlations between variables.

For microbiome analysis the ADONIS test of weighted UniFrac distances was conducted with the QIIME compare_categories script. For LC-MS metabolomics the normal data distribution was found to be sufficient according to Shapiro-Wilk (91% normally distributed) and Kolmogorov-Smirnov (99% normally distributed) after log_{10} transformation of *median normalized AUC*. Scedasticity was found to be sufficient according to Bartlett (86% homoscedastic) and Levene (81% homoscedastic) after log_{10} transformation of *median normalized AUC*. Differences between two independent groups were analyzed by a simple ANOVA (R function *aov*) model with the categorically fixed factor treatment. *p*-Values were adjusted according to Benjamini-Hochberg (R function *p.adjust*). Statistical analysis of microbiome data was performed by Karl Kashofer at the Institute of Pathology of the Medical University of Graz. Statistical analysis of metabolome data was performed by Natalie Bordag at the HEALTH Institute for Biomedicine and Health Sciences, JOANNEUM RESEARCH Forschungsgesellschaft mbH. This methodological description was also published in an original article (Fröhlich et al. 2016).

3 Results – Findings

3.1 Antibiotic treatment induces microbial community disruption, depletion of bacteria-derived metabolites in the colon, and alterations of lipid species and converted microbe-derived molecules in the plasma

3.1.1 At the time point of cognitive testing levels of ampicillin and vancomycin are below the lower limit of quantification in the brain

Ampicillin is known to have some oral bioavailability in humans (Lafforgue et al. 2008). In order to examine the pharmacokinetics of antibiotics in mice, a fast, sensitive and selective analytical method using solid phase extraction-liquid chromatography-tandem mass spectrometry was developed for the quantitative analysis of antibiotics in plasma and brain. Plasma and brains were extracted on day 10, the day on which cognitive performance was assessed. The levels of ampicillin and vancomycin, a non-absorbable antibiotic (Craig, Stitzel 2004), were compared. Concentrations of ampicillin in plasma were around 2 ng/mL in antibiotic-treated mice (Figure 5A) while the plasma levels of vancomycin were, except for two samples, below the LLOQ of 1 ng/mL (Figure 5B). Both ampicillin (Figure 5A) and vancomycin (Figure 5B) levels were below the respective LLOQ in the brain. These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

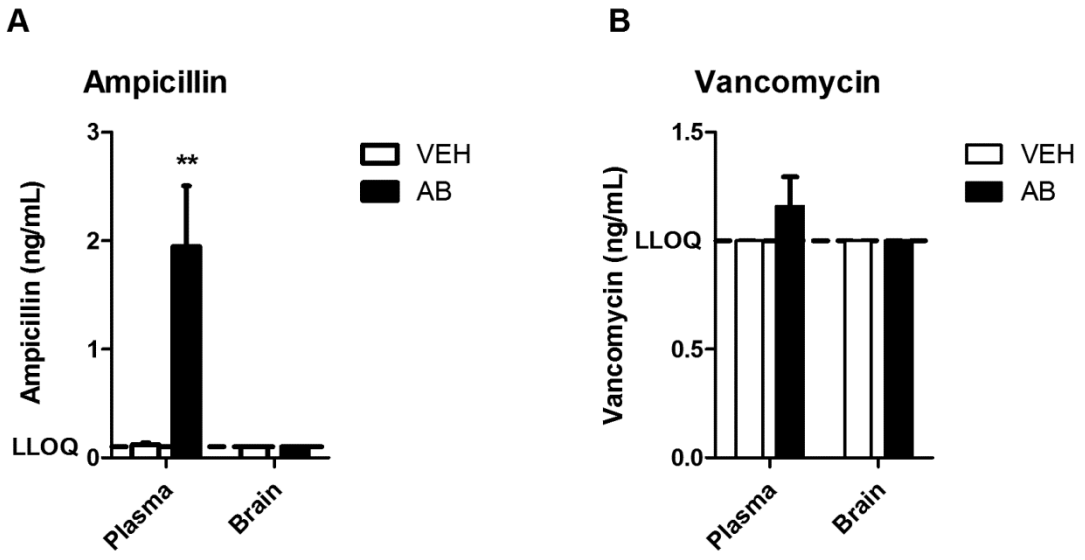

Figure 5 Brain concentrations of ampicillin and vancomycin are below the respective lower limit of quantification. The graphs depict concentrations of ampicillin (A) and vancomycin (B) in plasma and brain after 10 days of antibiotic (AB) treatment. The bars represent means + SEM, n = 6–8; $^{**}p \leq 0.01$ compared to vehicle (VEH)-treated mice, Mann-Whitney U test. These findings have been published in an original article (Fröhlich et al. 2016).

3.1.2 Antibiotic treatment does not change ingestion and body weight but alters cecum and spleen weight

Given that the gut microbiota has an impact on body weight (Rosenbaum, Knight & Leibel 2015), food intake, water consumption and body weight of the mice were measured during the 11-day antibiotic treatment. Repeated measures ANOVA revealed that food intake increased over the treatment period ($F_{(1.572, 6.288)} = 8.908$; $p \leq 0.05$) to the same extent in vehicle- and antibiotic-treated animals (Fig. 4A). Drinking behavior was influenced by the treatment period ($F_{(1.296, 5.186)} = 11.689$; $p \leq 0.05$) but not by the treatment itself. Noteworthy, the antibiotic-treated mice drank nominally more water at all time points than the vehicle-treated animals, but this difference did not reach statistical significance (Figure 6B). Repeated measures ANOVA disclosed that neither the treatment period nor the type of treatment had a significant effect on body weight (Figure 6C). It is worth mentioning that the consistency of the colonic contents and feces did not differ between vehicle- and antibiotic-treated mice and did not change during the experiment.

Since GF mice are known to have an enlarged cecum and diminished spleen weight (Smith, McCoy & Macpherson 2007), and the weight of these organs is changed by gut

dysbiosis (Reikvam et al. 2011), these parameters were assessed on day 11 when the mice were sacrificed after the 11-day antibiotic treatment. The antibiotic-treated mice presented with a significantly increased cecum weight (Figure 6D) whereas the weight of the spleen was significantly reduced in antibiotic-treated animals (Figure 6E).

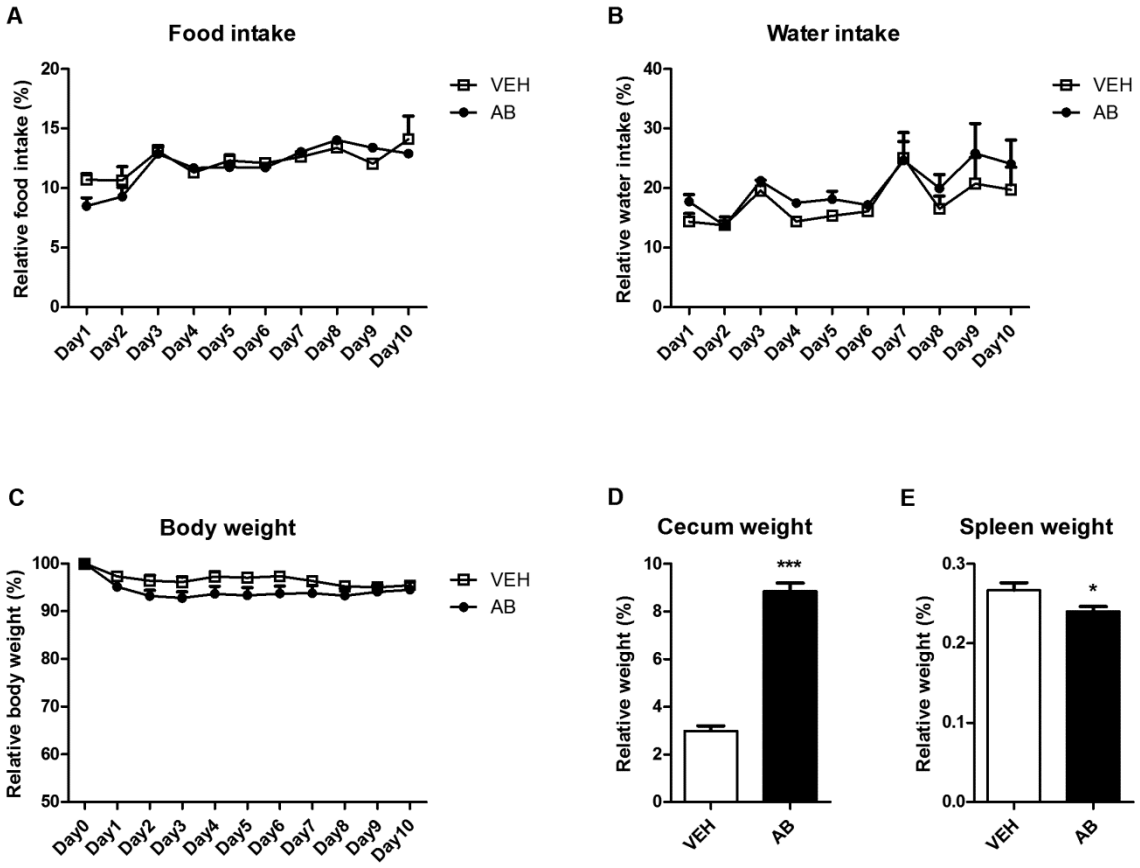

Figure 6 Antibiotic treatment does not change ingestion and body weight but alters cecum and spleen weight. Mice were treated with antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) or vehicle (VEH) by gavage for up to 11 days. (A) Daily food intake expressed as percentage of body weight. (B) Daily water intake expressed as percentage of body weight. (C) Relative body weight expressed as percentage of body weight at day 0 (measured immediately before treatment start). (D) Relative cecum weight and (E) relative spleen weight measured on day 11 and expressed as percentage of body weight. Values represent means + SEM, n=20-21 (C,D,E), n=15-17 (A,B); $^{*}p \leq 0.05$, $^{***}p \leq 0.001$ compared to VEH-treated mice, *t*-test (E) or Mann-Whitney U test (D). These findings have been published in an original article (Fröhlich et al. 2016).

3.1.3 Colonic microbial composition is strongly disrupted by antibiotic treatment

The ability of the antibiotic mix to disrupt gut microbial composition was confirmed by microbiome analysis of colon tissue including the luminal contents. Composition of the microbiome was vigorously changed by antibiotic treatment marked by a reduction in

bacterial load (Figure 7A,C) and diversity of bacteria (Figure 7B). Principal coordinate analysis (PCoA) revealed that antibiotic-treated mice had a significantly different (p = 0.001 by ADONIS test) microbial community than vehicle-treated mice (Figure 7A). Moreover, the red dots representing the profile of the microbial composition of antibiotic-treated mice clustered more closely to the blank (grey) than to the profile of vehicle-treated mice (blue), indicating that most of the commensal bacteria were erased by antibiotic treatment (Figure 7A). The residual operational taxonomic units (OTU) in antibiotic-treated mice comprise mainly common contaminant bacterial species originating from DNA extraction and PCR reagents and solutions (Salter et al. 2014) (see Appendix 6.4). As common contaminants have an impact on the overall calculation of bacterial load, the decrease in bacterial number was also shown with qPCR (Figure 7C). These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

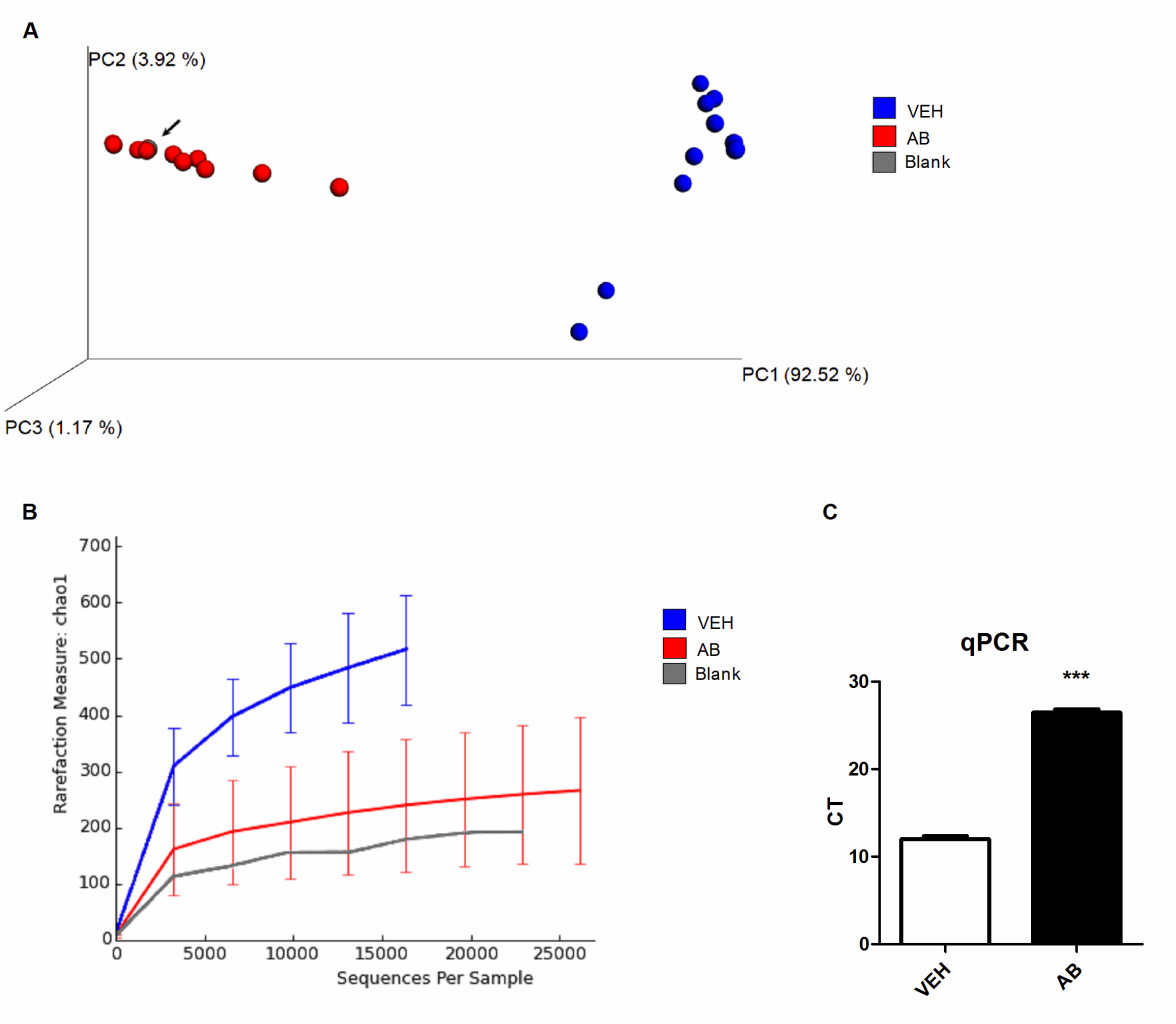

Figure 7 Antibiotic treatment strongly disrupts and diminishes the microbial community in the colon.

Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. (A) Principal coordinate analysis (PCoA) plot based on weighted UniFrac distance between samples. The microbiome analysis blank is depicted as a dot highlighted by an arrow. Two individual dots of antibiotic-treated mice are not visible because they are overlaid by other dots. (B) Alpha-rarefaction curves using the chao1 index. (C) Real-time PCR (qPCR) using the cycle threshold (CT). Values in B represent medians ± SD, n = 10–12. The bars in C represent means + SEM, n = 10-12; $^{***}p \leq 0.001$ compared to VEH-treated mice, *t*-test. A similar figure has also been published in an original article (Fröhlich et al. 2016).

3.1.4 In the colonic luminal contents microbial metabolite levels are strongly diminished by antibiotic treatment

Based on the knowledge that the gut microbiota contributes to digestion (RW.ERROR - Unable to find reference:261), and that the profile of the microbial community in antibiotic-treated mice is altered, antibiotic treatment could have an impact on the colonic metabolite profile. This was assessed by untargeted ^{1}H NMR analysis. Chemometric analysis showed a separation of the treatment groups, indicating a considerable difference in colonic metabolic composition (Figure 8A, B). For the most part metabolites known to be produced by the colonic microbiota were diminished in the luminal contents of antibiotic-treated mice. Particularly the levels of microbial fermentation products such as the SCFAs acetate, butyrate and propionate as well as the levels of trimethylamine, adenine and uracil were significantly decreased by antibiotic treatment (Figure 8C). The antibiotics themselves gave a strong background signal in the region of δ 5.5-3.3. These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

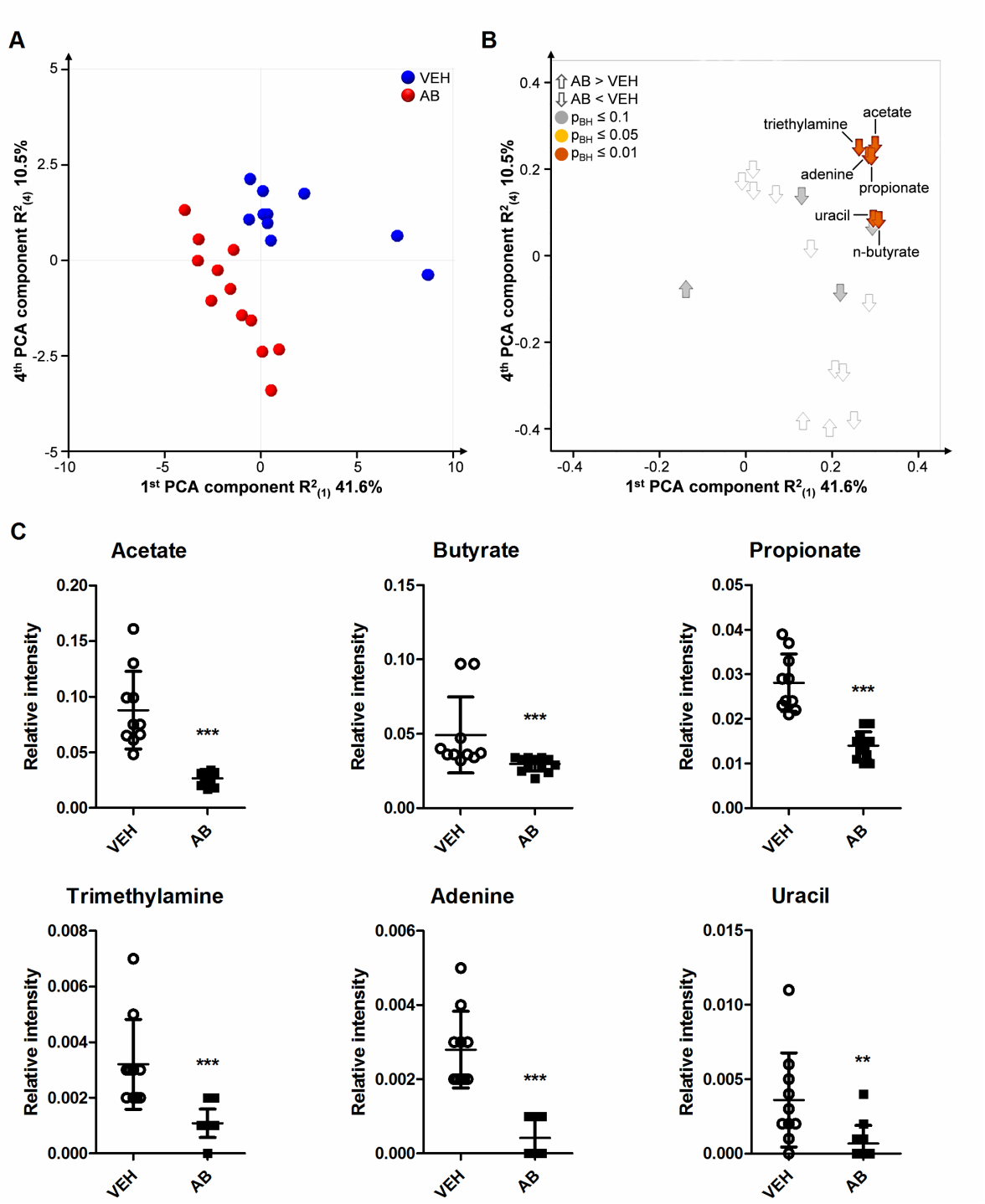

Figure 8 Distinct microbial metabolite levels in the colonic contents are markedly decreased by antibiotic treatment.

Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. (A) Scores plot of the principal component analysis (PCA) with 21 identified colonic metabolites analyzed by ^{1}H NMR, showing group separation in the first and fourth components. (B) Corresponding loadings plot, showing the contribution of metabolites to group separation. The metabolites are colored according to *p*-values calculated with the Mann-Whitney U test. (C) Graphs of significantly different metabolites. Vertical bars represent the group mean, whiskers SD, n = 10–12; $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, Mann-Whitney U test. These findings have been published in an original article (Fröhlich et al. 2016).

3.1.5 The profile of circulating metabolites is intensely altered by antibiotic treatment

Given that microbial colonization influences circulating metabolites (Wikoff et al. 2009), the plasma metabolic profile was analyzed with targeted LC-MS metabolomics. Metabolomic analysis was able to identify 170 metabolites of which 142 were suitable for multiple analyses. PCA revealed that the treatment groups separated to a significant extent, indicating a considerable difference in overall plasma metabolic composition (Figure 9A, B). More precisely, 11 metabolites were present in significantly different (Benjamini Hochberg adjusted p-value ≤ 0.05) amounts in antibiotic-treated mice compared to vehicle-treated animals (Figure 9B). In antibiotic-treated mice the levels of corticosterone, sphingomyelin 34:1, phosphatidylinositol 38:5, phosphatidylcholine (PC) 36:2, PC 38:5, PC 40:5 and PC 40:8 were significantly raised. In contrast, the levels of lysophosphatidylcholine (LPC) 20:3 and *p*-cresyl sulfate were significantly decreased (Figure 9C). Corticosterone levels measured by metabolomic analysis corresponded well with those quantified immunochemically (see 3.2.3). Furthermore, the antibiotic-induced decrease of circulating trimethylamine-*N*-oxide levels (Figure 9C) corresponded with the decrease of this metabolite in the colonic contents (Figure 8C). Due to isomerism deoxycholic acid and chenodeoxycholic acid could not be distinguished by LC-MS. While in vehicle-treated mice deoxycholic acid and chenodeoxycholic acid were clearly present, levels of these two metabolites were below the limit of detection in antibiotic-treated mice (Figure 9C). These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

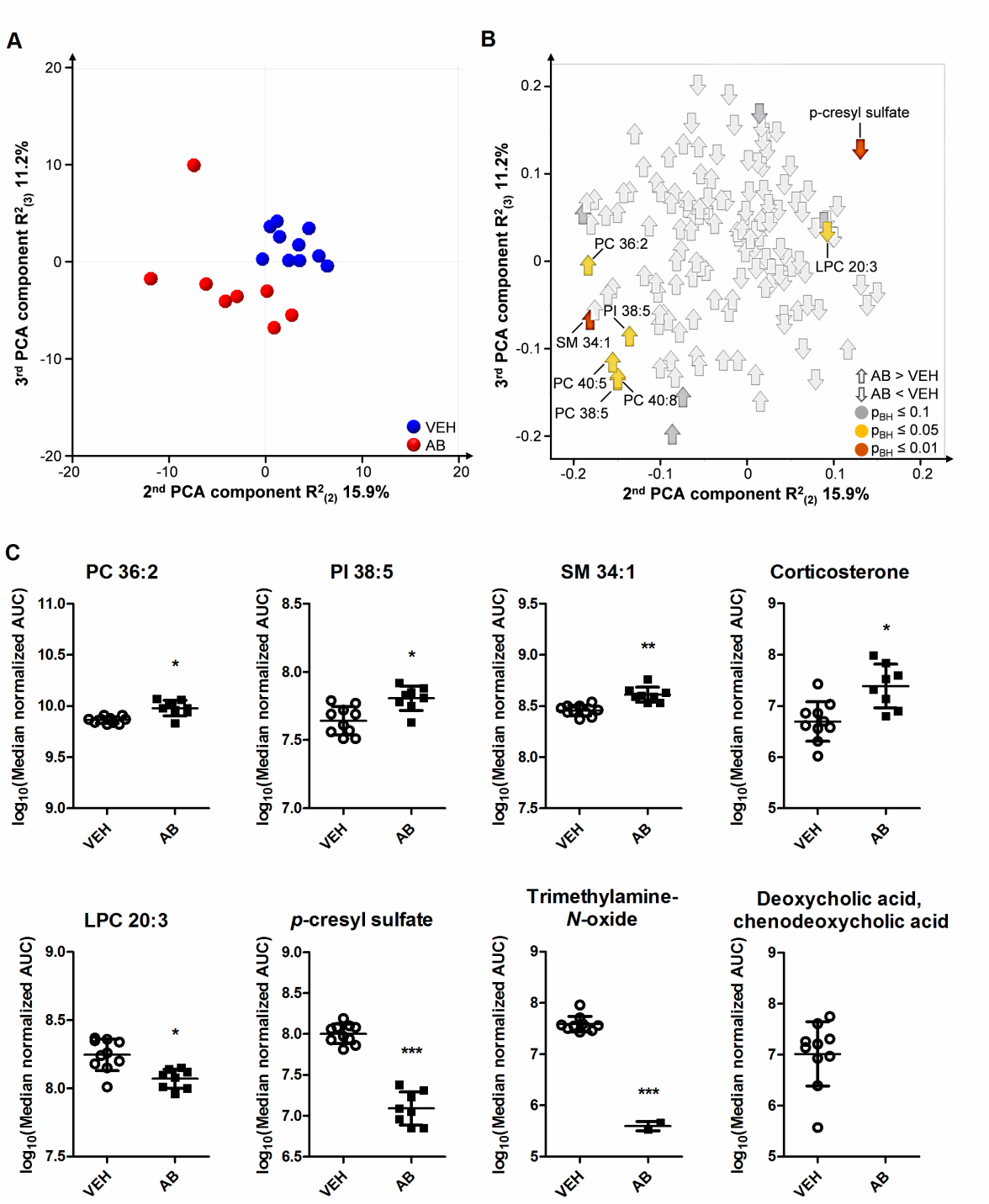

Figure 9 Circulating metabolite levels are markedly altered by antibiotic treatment. Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. (A) Scores plot of the principal component analysis (PCA) with 148 identified circulating metabolites analyzed by LC-MS, showing group separation in the second and third components. (B) Corresponding loadings plot, showing the contribution of metabolites to group separation. Metabolites are colored according to Benjamini-Hochberg adjusted *p*-values. (C) Graphs of selected metabolites that were significantly different. Corticosterone and trimethylamine-*N*-oxide are not shown in the loadings plot because they were only suitable for univariate analysis (UVA). Levels of deoxycholic acid/chenodeoxycholic acid were below the limit of detection in AB-treated mice. Vertical bars represent the group mean, whiskers SD, n = 8–10; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, Benjamini-Hochberg adjusted *p*-values. AUC, area under the curve;

PC, phosphatidylcholine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; SM, sphingomyelin. These findings have been published in an original article (Fröhlich et al. 2016).

3.2 Antibiotic-induced dysbiosis enhances cytokine expression in the colon, but not in the circulation

3.2.1 Antibiotic treatment does not alter gross histology of the small and large intestine

The potential ability of the antibiotic treatment, relative to vehicle, to cause damage to the intestine was evaluated by hematoxylin and eosin staining of the small intestine and colon. There were no gross differences in histology between the two groups, and no pathologic alterations such as inflammation, epithelial or mucosal alterations, hyperplasia or villous blunting were observed (Figure 10). Stool consistency was normal (formed fecal boli) in all mice in both groups.

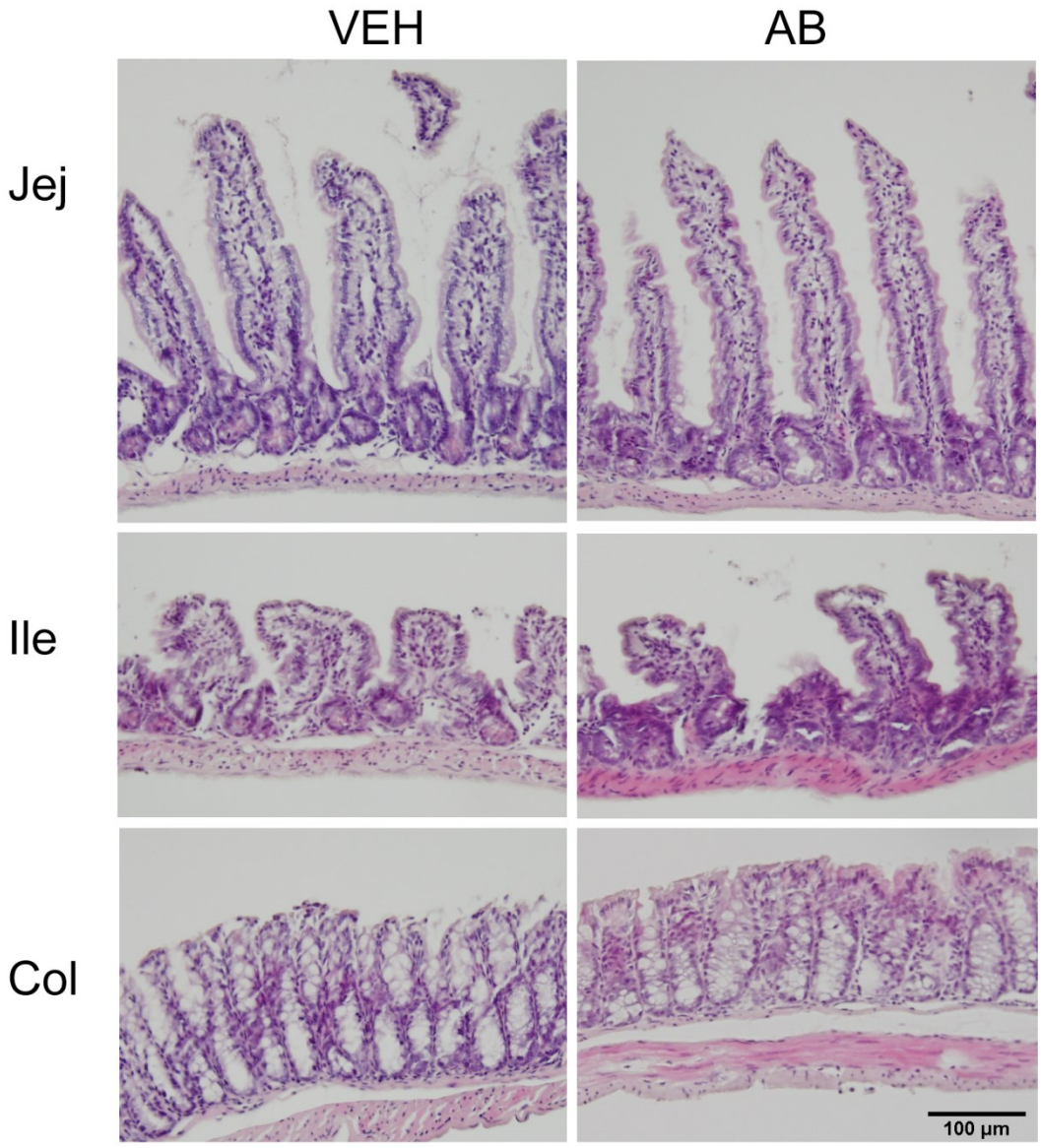

Figure 10 Antibiotic treatment does not alter gross histology of the small and large intestine.
Mice were treated with antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) or vehicle (VEH) by gavage for 10 days. The panels show representative histological micrographs of hematoxylin and eosin staining of jejunum (Jej), ileum (Ile) and colon (Col). The left column panels depict images from VEH-treated mice, the right column panels images from AB-treated mice. Scale bar: 100µm. These findings have been published in an original article (Fröhlich et al. 2016).

3.2.2 Cytokine, but not tight junction protein, mRNA expression patterns in the colon are changed by antibiotic treatment

In keeping with the ability of dysbiosis to alter intestinal immune responses (Round, Mazmanian 2009), I found that antibiotic treatment changed cytokine mRNA expression in the colonic tissue. While the expression of IL-10 and IFN-γ mRNA was significantly enhanced in antibiotic-treated mice, the expression of TNF-α mRNA was reduced by half compared to vehicle-treated mice (Figure 11A). The expression of IL-1β and IL-6 mRNA was similar in both treatment groups. These strong changes were not observed on the protein level; only the protein expression of IFN-γ was significantly increased in antibiotic-treated mice Figure 11B).

The potential ability of the antibiotic treatment to change mucosal permeability and immune function in the colon was addressed by measuring mRNA expression of tight junction proteins and cytokines. As shown in Figure 11C, there was a trend ($p = 0.059$) towards increased expression of CLDN1 in antibiotic-treated mice. In contrast, TJP1, also known as zonulin-1, and OCLN mRNA were expressed in similar amounts in the two treatment groups (Figure 11C).

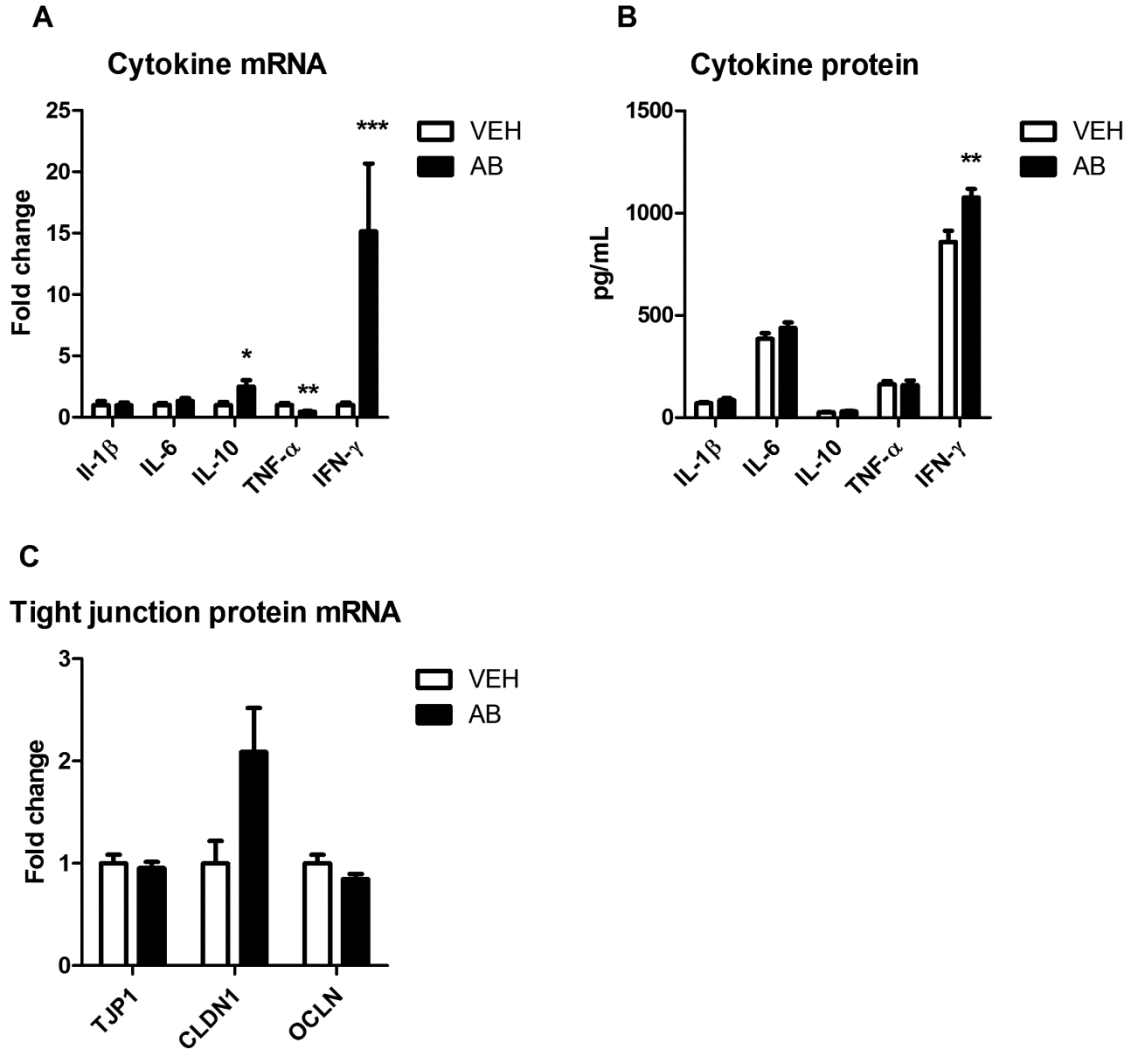

Figure 11 Colonic expression patterns of cytokine mRNA and protein are altered by antibiotic treatment.
Mice were treated with an antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) by gavage for 11 days. (A) Expression of interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α and interferon (IFN)-γ mRNA depicted as fold change relative to VEH-treated mice. (B) Protein levels of IL-1β, IL-6, IL-10, TNF-α and IFN-γ. (C) Expression of tight junction protein-1 (TJP1), claudin-1 (CLDN1) and occludin (OCLN) mRNA depicted as fold change relative to vehicle (VEH)-treated mice. Values represent means + SEM, n=6-8; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, Mann-Whitney U test.

3.2.3 Antibiotic treatment enhances plasma corticosterone, but not cytokine, levels

In addressing the question whether the antibiotic-induced changes in cytokine mRNA expression in the colon translate to changes in circulating cytokine levels, I did not observe any differences in the cytokine concentrations in the plasma of vehicle- and antibiotic-treated mice (Figure 12A). In all plasma samples examined, the concentrations of IL-1β were too low to render detectable values. Levels of IL-10 were also excluded from analysis

as the majority of samples were below the detection limit (no group preference being observed).

Circulating corticosterone levels reflect HPA axis activity and are influenced by gut colonization (Sudo et al. 2004). When measured by an immunoassay, the plasma levels of corticosterone were significantly enhanced after 11 days of antibiotic treatment (Figure 12B), which confirmed the measurements taken by plasma metabolomics (Figure 9C).

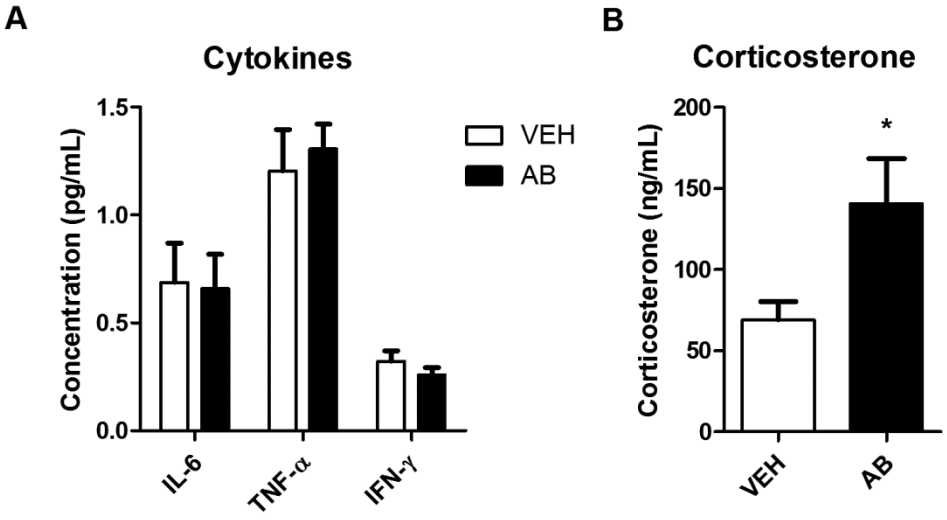

Figure 12 Cytokine concentrations remain unaltered in antibiotic-treated mice whereas plasma levels of corticosterone are elevated.

Mice were treated with antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) or vehicle (VEH) by gavage for 11 days. The graphs show concentrations of cytokines (A) and corticosterone (B) in plasma. Values represent means + SEM, n=6-12; $^{*}p \leq 0.05$ compared to VEH-treated mice, *t*-test. These findings have been published in an original article (Fröhlich et al. 2016).

3.3 Antibiotic treatment induces cognitive impairment that is associated with brain region-specific changes in the expression of neuronal signaling molecules

3.3.1 Antibiotic treatment has no impact on anxiety- and depression-like behavior

Since GF mice are known to be less anxious than their conventionalized counterpart (Neufeld et al. 2011) I used two tests to assess anxiety-like behavior in antibiotic-treated mice. On day 7 of the antibiotic treatment, locomotor and anxiety-like behavior were examined with the OFT. In this test, the time spent in the central area of the open field and

the number of central area visits were evaluated as parameters inversely correlated to anxiety, while locomotor behavior was deduced from the total traveling distance. The results of the OFT show that neither anxiety-like behavior nor locomotor behavior was significantly changed by antibiotic treatment (Figure 13A-C).

On day 8 of the antibiotic treatment the animals were placed on the EPM, which is another test to evaluate anxiety and locomotion. In this test, the time spent on the open arms and the number of entries into the open arms are inversely correlated to anxiety-like behavior, while the total distance traveled is an index of locomotor behavior. The results obtained with the EPM test confirmed the observations made with the OFT. Antibiotic-treated mice spent a similar duration of time on the open arms of the EPM and entered the open arms as often as the vehicle-treated mice (Figure 13D, E). Locomotion was not altered either since the total traveling distance was not significantly different between the two groups (Figure 13F).

Given that there is a comorbidity of gastrointestinal disorders with psychiatric disorders such as depression (Graff, Walker & Bernstein 2009), the TST was used to assess depression-like behavior on day 9 of the antibiotic treatment. In this test, the time of immobility, swinging and curling was recorded, the time of immobility primarily reflecting the degree of depression-like behavior. Antibiotic-treated animals did not significantly differ in any of the parameters of the TST from vehicle-treated animals (Figure 13G-I), which indicates that depression-like behavior remained unaltered.

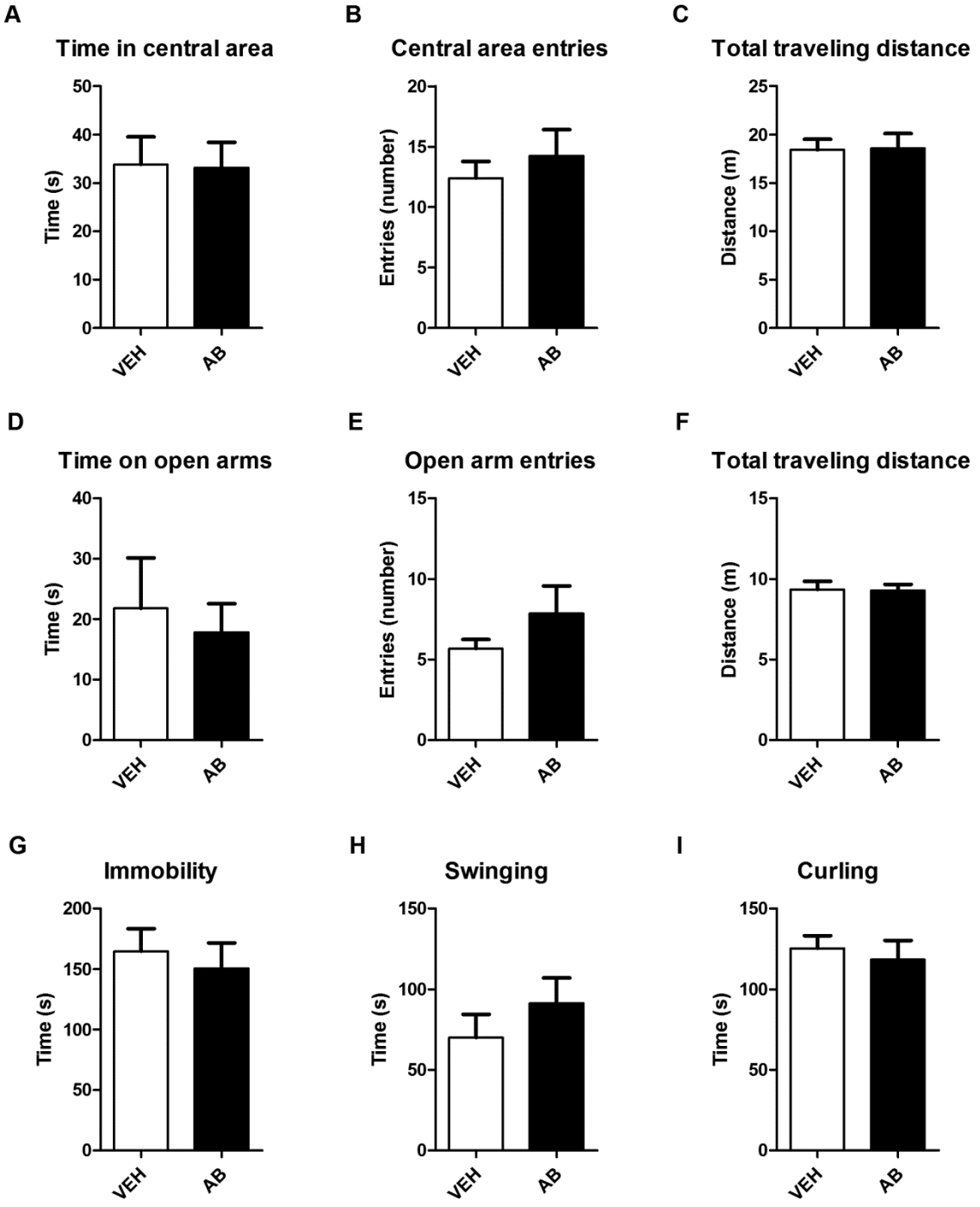

Figure 13 Antibiotic treatment does not affect locomotor, anxiety- and depression-like behavior.
Mice were treated with antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) or vehicle (VEH) by gavage for 11 days. The open field test (A-C) was conducted on day 7, the elevated plus maze test (D-F) on day 8 and the tail suspension test (G-I) on day 9 of the antibiotic treatment. Values represent means + SEM, n=10-12 (A-C), n=9-12 (D-F), n=8-9 (G-I). These findings have been published in an original article (Fröhlich et al. 2016).

3.3.2 Antibiotic treatment impairs novel object recognition memory but has no impact on spatial learning and memory

Given that memory is impaired in GF mice (Gareau et al. 2011), I assessed non-spatial memory with the NORT on day 10 of the antibiotic treatment. Furthermore, I evaluated spatial learning and memory in a separate group of animals, with the BM test on days 7-10 of the antibiotic treatment (Figure 14A-C). The NORT tested the ability of the animals to discriminate a familiar object from a novel one. Antibiotic-treated mice displayed a disrupted novel object recognition memory reflected by a significantly lower memory index than vehicle-treated mice (Figure 14A). In the BM test animals were trained to identify the location of a specific hole which leads to an escape cage (target hole) among a choice of 20 holes. This test showed that spatial learning (Figure 14B) and memory (Figure 14C) were not altered by the antibiotic treatment. Repeated measures ANOVA showed that only the number of trials significantly influenced target latency ($F_{(4, 52)} = 7.561$; $p \leq 0.001$). These results indicate that antibiotic treatment altered particular aspects of memory rather than generally impairing cognition. These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

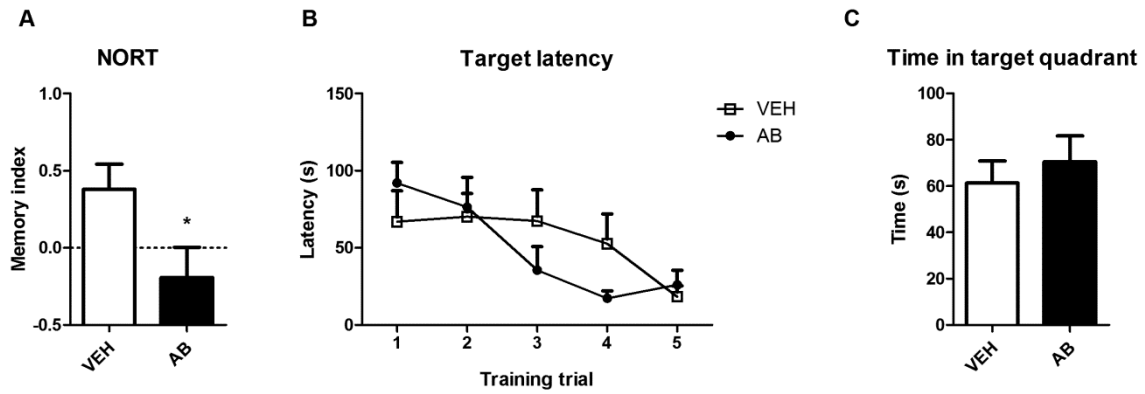

Figure 14 Object recognition memory is impaired in antibiotic-treated mice whereas spatial learning and memory remain intact.

Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. In the NORT (A) a positive memory index (MI) indicates that the mice spent more time exploring the novel object than the known object.

In the BM test (B and C) panel B shows the decrease in target latency with consecutive training trials, and panel C shows memorization of the target hole in the target quadrant on the probe day. Values represent means + SEM, n = 6–8 (A), n = 7–8 (B), n = 6–7 (C); $^{*}p \leq 0.05$ compared to VEH-treated mice, *t*-test. These findings have been published in an original article (Fröhlich et al. 2016).

3.3.3 mRNA of tight junction proteins are differentially expressed in the amygdala and hippocampus

Given that tight junction protein expression in frontal cortex, striatum and hippocampus is diminished in GF mice (Braniste et al. 2014), I analyzed mRNA expression of three tight junction proteins, namely CLDN5, TJP1 and OCLN, in four brain regions of antibiotic- and vehicle-treated mice (Figure 15A-D). Brain regions were selected based on their importance in cognition and memory (amygdala, medial prefrontal cortex, hippocampus), and the established influence of the gut microbiota on the HPA axis (hypothalamus) (Sudo et al. 2004). In the medial prefrontal cortex (Figure 15A) and hypothalamus (Figure 15D) the expression of the tight junction proteins CLDN5, TJP1 and OCLN was comparable in vehicle- and antibiotic-treated animals. In contrast, in the hippocampus antibiotic treatment significantly decreased the expression of CLDN5 and OCLN mRNA (Figure 15B). The expression of TJP1 and OCLN mRNA in the amygdala was enhanced in antibiotic-treated mice relative to vehicle controls (Figure 15C). These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

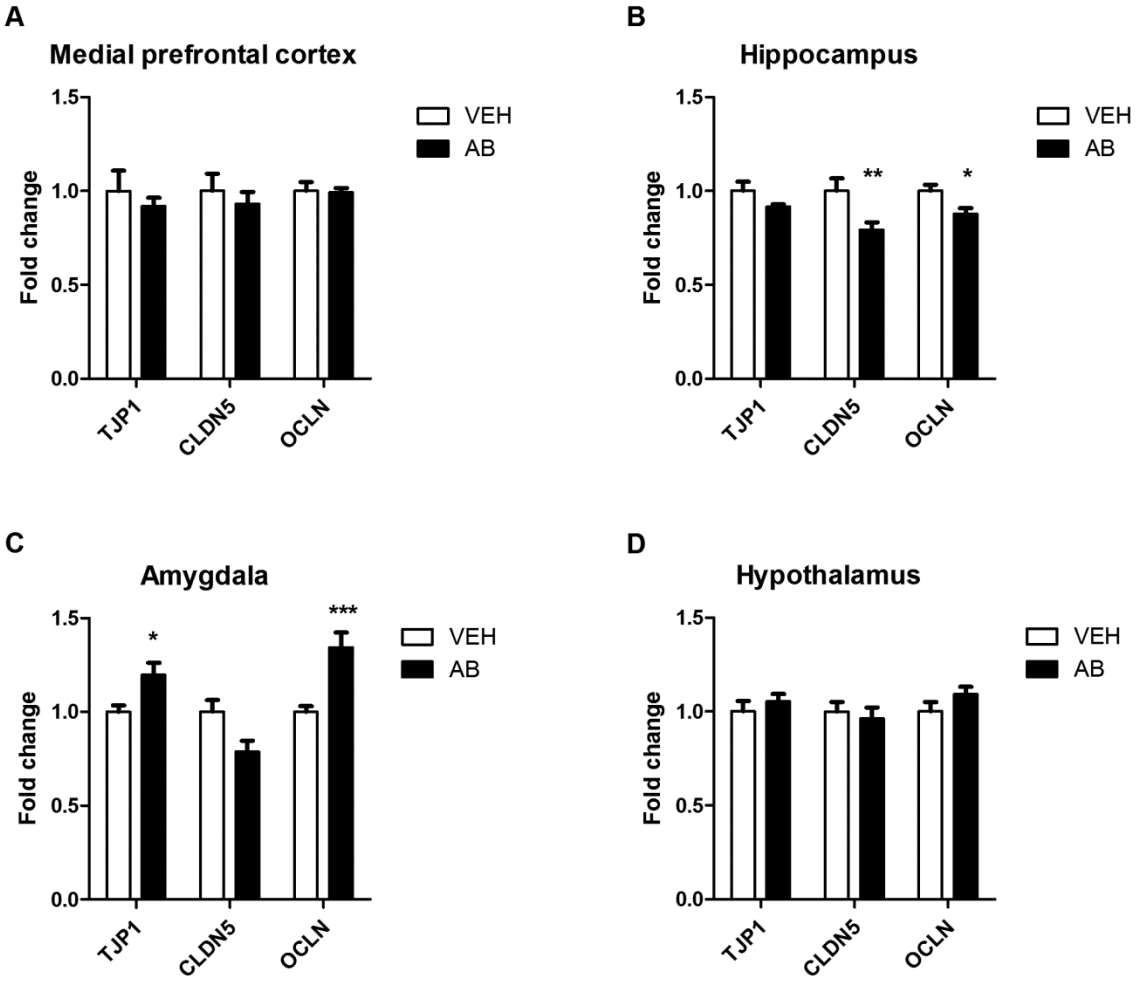

Figure 15 Antibiotic treatment alters tight junction protein mRNA expression in amygdala and hippocampus. Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. The panels show the expression of tight junction protein 1 (TJP1), claudin 5 (CLDN5) and occludin (OCLN) mRNA in the medial prefrontal cortex (A), hippocampus (B), amygdala (C), and hypothalamus (D). mRNA expression is expressed as fold change relative to VEH-treated mice. Values represent means + SEM, n = 10–12; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, *t*-test. These findings have been published in an original article (Fröhlich et al. 2016).

3.3.4 Antibiotic treatment blunts expression of IL-1β in the hippocampus and hypothalamus but does not alter expression of other cytokines in the brain

Given that antibiotic treatment altered cytokine expression in the colon, its effect on the expression of cytokine and chemokine (C-C motif) ligand 2 (CCL2) mRNA in four brain regions (Figure 16A-D) was measured. In the medial prefrontal cortex (Figure 16A) and amygdala (Figure 16C) no significant changes in cytokine (IL-1β, IL-6, TNF-α, IFN-γ) and CCL2 mRNA expression were found following antibiotic treatment. In contrast, the expression of IL-1β mRNA in the hippocampus (Figure 16B) and hypothalamus (Figure

16D) was significantly reduced in antibiotic-treated mice, whereas the expression of the other cytokines and of CCL2 remained unaltered. In all brain samples examined, the expression of IL-10 mRNA was too low to render detectable values.

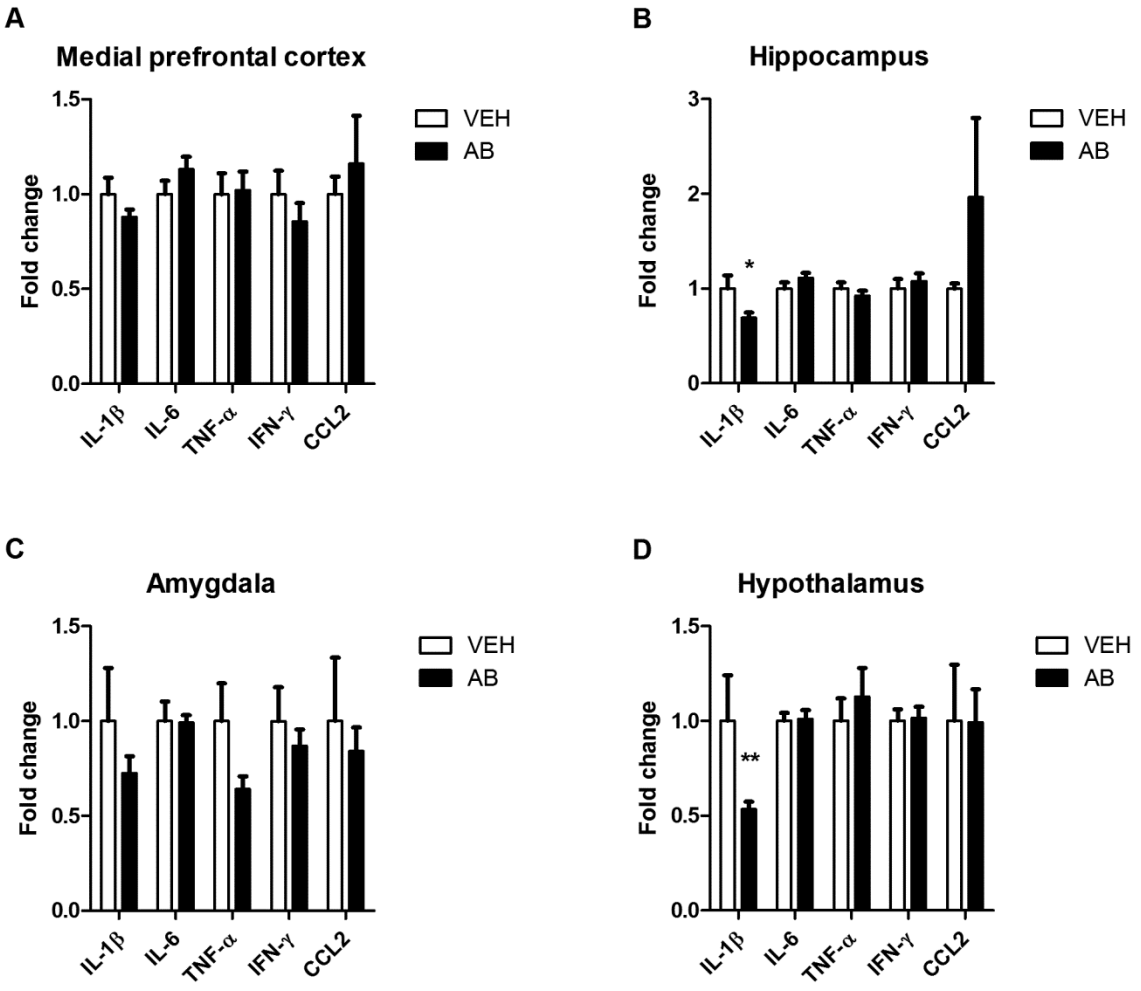

Figure 16 Antibiotic treatment decreases the expression of IL-1β mRNA in the hippocampus and hypothalamus. Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. The panels show the expression of cytokine and chemokine (C-C motif) ligand 2 (CCL2) mRNA in the medial prefrontal cortex (A), hippocampus (B), amygdala (C), and hypothalamus (D). mRNA expression is expressed as fold change relative to VEH-treated mice. Values represent means + SEM, n=10-12; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared to VEH-treated mice, Mann-Whitney U test. These findings have been published in an original article (Fröhlich et al. 2016).

3.3.5 Antibiotic treatment increases GSR but not CAT expression in the amygdala

Since ampicillin was claimed to increase reactive oxygen species stress and thereby to exert a neurotoxic effect (El-Ansary et al. 2015), I measured CAT and GSR mRNA expression in the brain to test if antibiotic treatment causes reactive oxygen species stress (Figure 17A-D). GSR mRNA in the amygdala was significantly increased by antibiotic

treatment (Figure 17C) whereas in the medial prefrontal cortex (Figure 17A), hippocampus (Figure 17B) and hypothalamus (Figure 17D) neither GSR nor CAT mRNA expression was altered in antibiotic-treated animals.

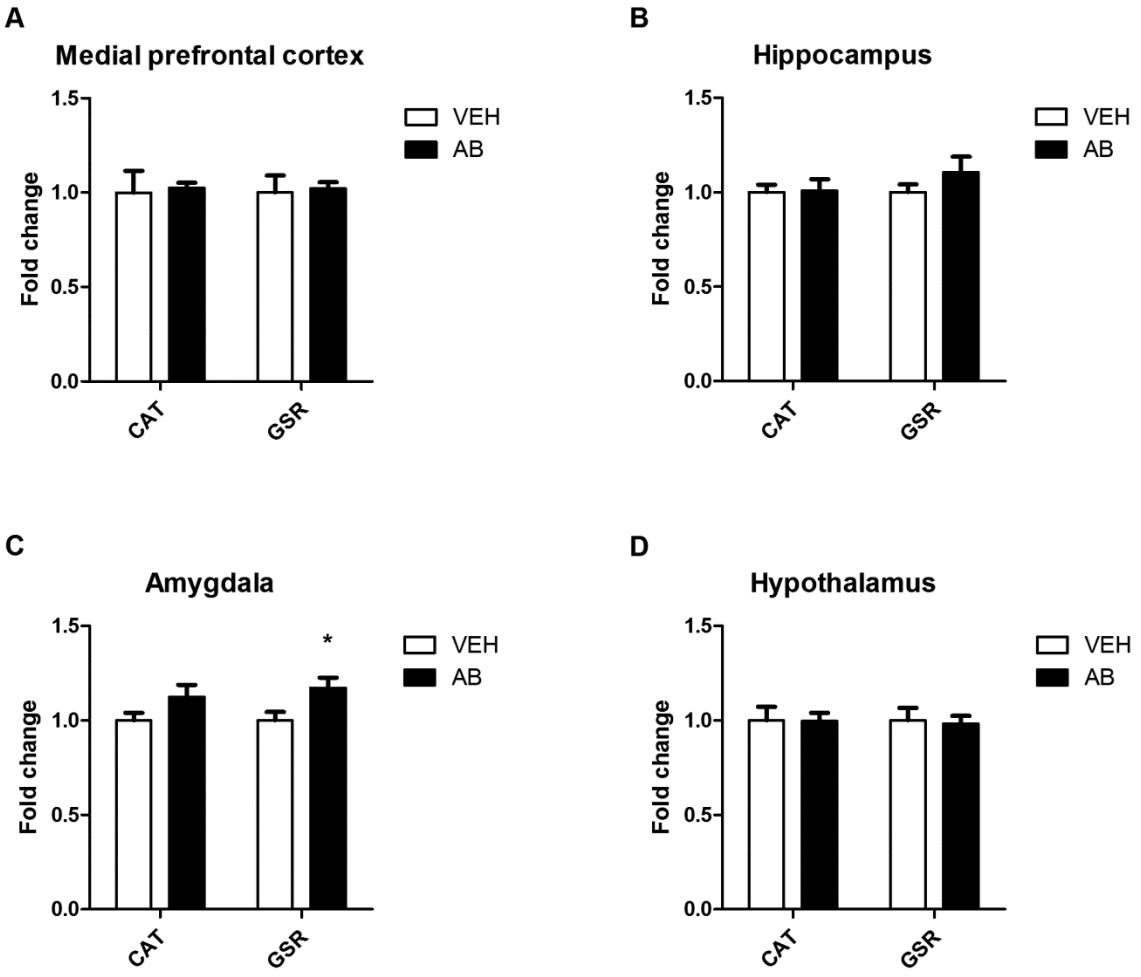

Figure 17 Antibiotic treatment increases glutathione reductase (GSR) but not catalase (CAT) expression in the amygdala.

Mice were treated with an antibiotic (AB) mix (ampicillin, bacitracin, meropenem, neomycin, vancomycin) by gavage for 11 days. The panels show the expression of CAT and GSR mRNA in the medial prefrontal cortex (A), hippocampus (B), amygdala (C), and hypothalamus (D). mRNA expression is expressed as fold change relative to vehicle (VEH)-treated mice. Values represent means + SEM, n=10-12; $^{*}p \leq 0.05$ compared to VEH-treated mice, *t*-test.

3.3.6 Expression patterns of neuronal signaling molecules are altered by antibiotic treatment

Since cognitive performance is affected in antibiotic-treated mice, I investigated the cerebral expression of neuronal signaling-related molecules that are related to learning and memory. The first focus was put on BDNF, N-methyl-D-aspartate receptor subunit

GRIN2B, the nuclear glucocorticoid receptor NR3C1 and serotonin transporter SLC6A4. Those four were chosen due to their altered expression as well as their involvement in the cognitive deficit of GF mice (Sudo et al. 2004, Neufeld et al. 2011, Gareau et al. 2011, Clarke et al. 2013). In the medial prefrontal cortex (Figure 18A), hippocampus (Figure 18B) and hypothalamus (Figure 18D) BDNF mRNA expression was significantly decreased in antibiotic-treated mice. The expression of GRIN2B, NR3C1 and SLC6A4, however, was left unaltered by antibiotic treatment in these three brain regions. In contrast, in the amygdala the expression of GRIN2B and SLC6A4 mRNA was significantly increased while that of BDNF and NR3C1 mRNA was not impacted (Figure 18C). These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

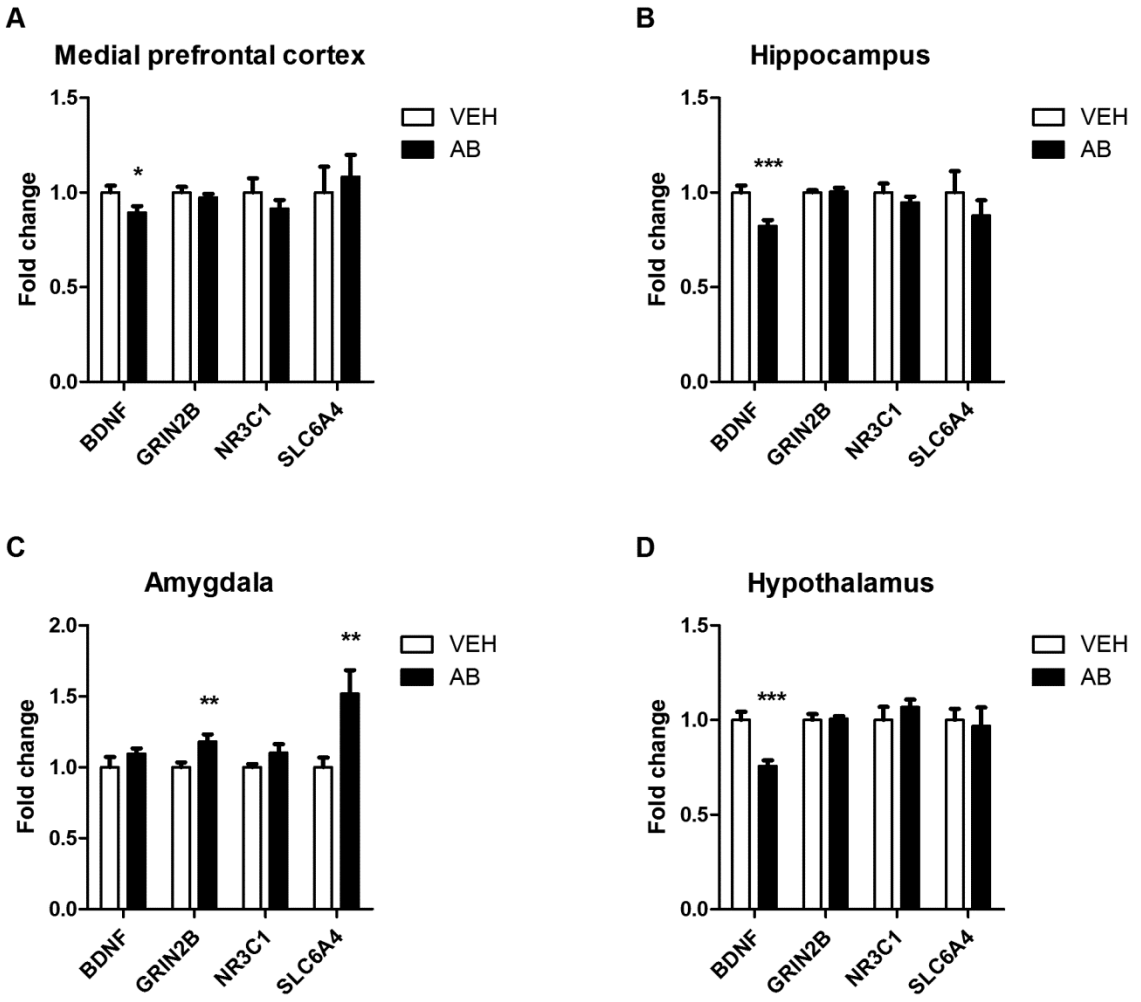

Figure 18 Antibiotic treatment alters the expression of neural signaling-related molecules in a brain region-specific manner.

Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. The panels show the expression of brain-derived neurotrophic factor (BDNF), N-methyl-D-aspartate receptor subunit 2B (GRIN2B), glucocorticoid receptor

(NR3C1) and serotonin transporter (SLC6A4) mRNA in the medial prefrontal cortex (A), hippocampus (B), amygdala (C), and hypothalamus (D). mRNA expression is expressed as fold change relative to VEH-treated mice. Values represent means + SEM, n = 10–12; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, *t*-test. These findings have been published in an original article (Fröhlich et al. 2016).

Given that NPY is involved in the regulation of cognitive processes (Lach, de Lima 2013, Reichmann, Holzer 2015, Tasan et al. 2015) and its mRNA expression is elevated in GF mice (Schele et al. 2013), I examined the expression of NPY and three of its receptors (Y1, NPY1R; Y2, NPY2R; Y5, NPY5R) in four brain regions of antibiotic- and vehicle-treated mice (Figure 19A-D). In the medial prefrontal cortex the expression of NPY, NPY1R, NPY2R and NPY5R mRNA remained unaltered (Figure 19A). Conversely, antibiotic treatment led to a significant reduction of NPY1R and NPY2R mRNA expression in the hippocampus (Figure 19B), but no difference was observed in NPY and NPY5R expression. In contrast, in the amygdala of antibiotic-treated mice NPY mRNA expression was significantly enhanced and NPY5R expression diminished whereas NPY1R and NPY2R mRNA expression remained unaltered (Figure 19C). In the hypothalamus antibiotic treatment also led to a prominent rise in NPY mRNA expression but had no impact on the expression of any NPY receptor under study (Figure 19D). These findings have been published in a similar fashion in an original article (Fröhlich et al. 2016).

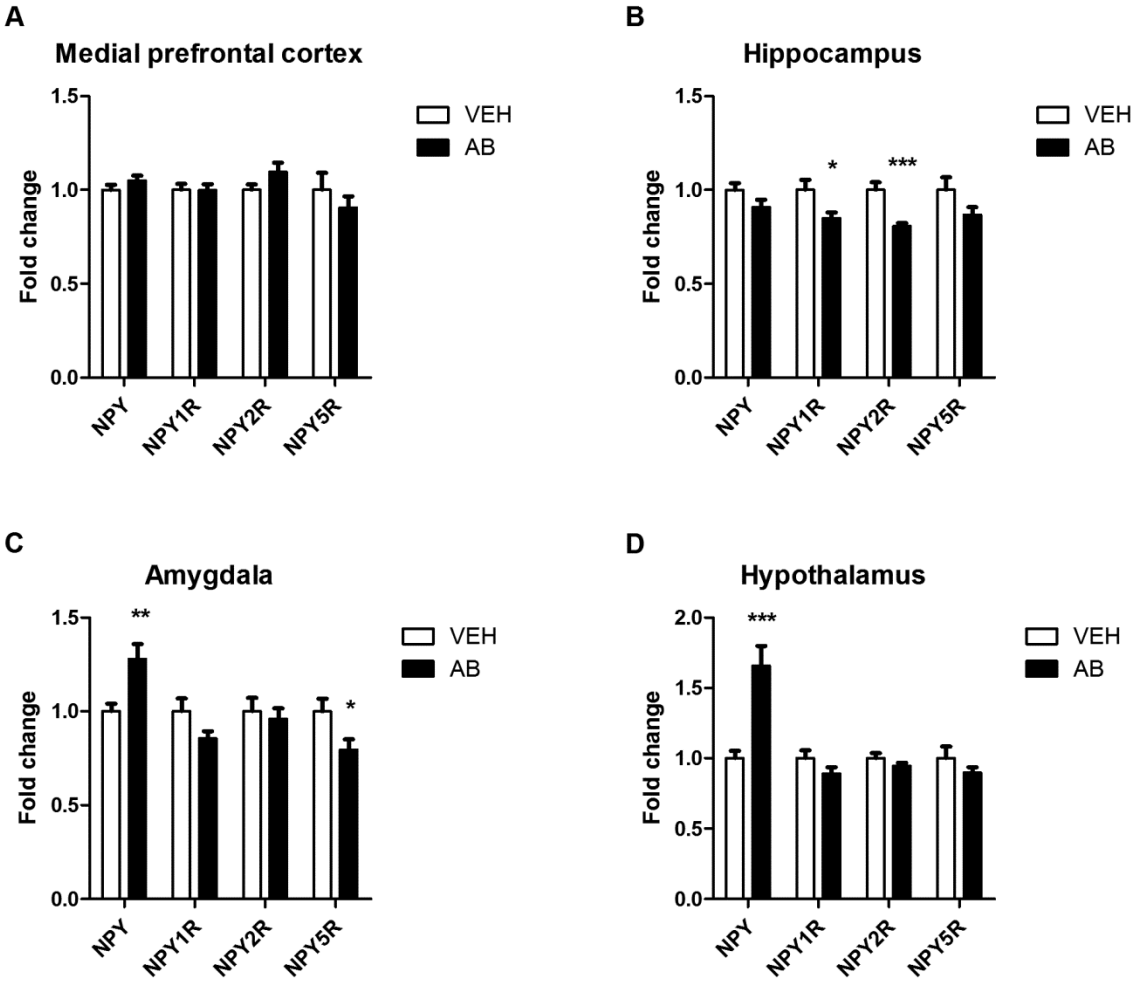

Figure 19 Antibiotic treatment alters the expression of neuropeptide Y and its receptors in a region-specific manner.

Mice were treated with antibiotic (AB) mix or vehicle (VEH) by gavage for 11 days. The panels show the expression of neuropeptide Y (NPY) and the NPY receptors NPY1R, NPY2R and NPY5R in the medial prefrontal cortex (A), hippocampus (B), amygdala (C), and hypothalamus (D). mRNA expression is expressed as fold change relative to VEH-treated mice. Values represent means + SEM, n = 10–12; $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ compared to VEH-treated mice, *t*-test. These findings have been published in an original article (Fröhlich et al. 2016).

3.4 Flagellin (200 µg/kg) does not exert any short- or long-term effects on anxiety-like and social behavior

3.4.1 Flagellin does not exert a consistent effect on anxiety-like behavior

Since the administration of LPS was shown to be anxiogenic (Farzi et al. 2015) I first used the OFT to assess anxiety-like behavior in mice treated with flagellin from *S. typhimurium*. Flagellin-treated mice spent significantly less time in the central area of the field compared to vehicle-treated mice (Figure 20A) indicating an increased level of anxiety following flagellin injection. Noteworthy, there was no significant difference in the total traveling distance, indicating that increased anxiety did not result from sickness induced reduction of locomotion (Figure 20C).

To evaluate long-term effects of flagellin treatment I used the SIT to assess social behavior. Both groups spent significantly more time in the compartment with the mouse indicating normal social behavior (Figure 20D).

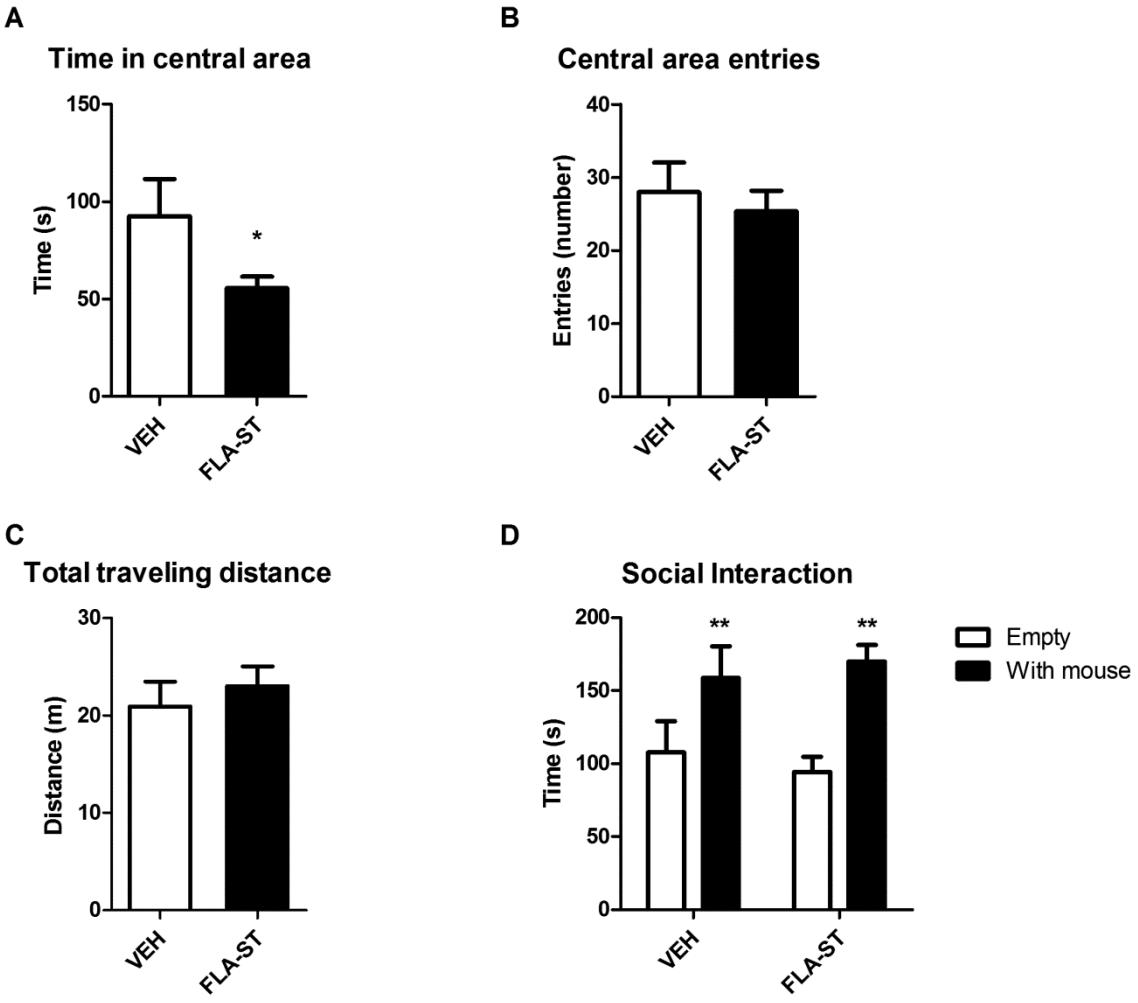

Figure 20 Flagellin from *S. typhimurium* induces a short-term anxiogenic-like effect.

Mice were treated i.p. with 200 µg/kg flagellin from *S. typhimurium* (FLA-ST) or saline (VEH). The open field test (A-C) was conducted approximately 4 h and the social interaction test (D) approximately 24 h after treatment. Values represent means + SEM, n=7-8 (A-C), n=5-7 (D); $^{*}p \leq 0.05$ compared to VEH-treated mice, *t*-test (A-C); $^{**}p \leq 0.01$ paired sample *t*-test (D).

To evaluate the impact of the bacterial species of origin, flagellin from *B. subtilis* and *P. aeruginosa* as well as flagellin from *S. typhimurium* were tested. An OFT was performed 3 h after treatment (Figure 21A-C). Contrary to the pilot study, none of the flagellins was able to induce anxiety-like behavior in this experiment. Sickness was ruled out as a contributing factor because the total traveling distance was comparable in the vehicle- and flagellin-treated groups (Figure 21C). To ensure that any late effects of flagellin were not missed, an LD test was performed 24 h after treatment, but none of the flagellins induced significant behavioral changes at this time point (Figure 21D-F).

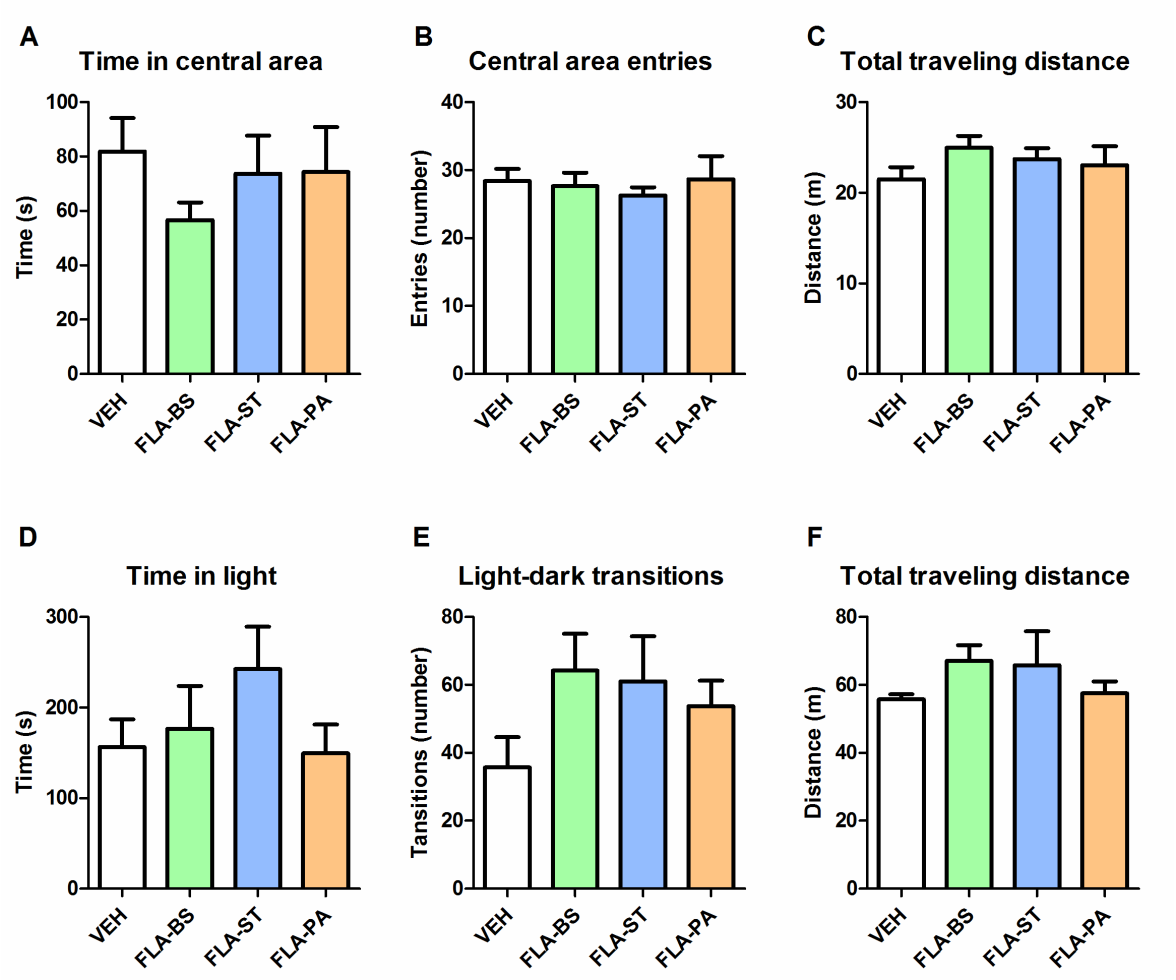

Figure 21 Flagellin from three different organisms does not exert short- or long-term effects on anxiety-like behavior.

Mice were treated i.p. with 200 µg/kg flagellin from *B. subtilis* (FLA-BS), *S. typhimurium* (FLA-ST) or *P. aeruginosa* (FLA-PS), or with saline (VEH). The open field test (A-C) was conducted 3 h and the light-dark test (D-F) 24 h after treatment. Values represent means + SEM, n=7-9 (A-C), n=9 (D-F).

4 Discussion

4.1 Rationale of the project

The concept of the gut-brain axis, the bidirectional communication between gut and brain, has been widely acknowledged in the analysis of pathophysiologic disturbances of the gastrointestinal tract and the central nervous system. Recently the intestinal microbiota has also been suggested to serve as a critical node in the gut-brain axis (Kennedy et al. 2014). According to the latest discoveries and investigations in the field of microbiota research, the microbiota may be involved in many diseases. The spectrum of disorders that the microbiota has been suggested to play a role in includes mental illnesses (depression, anxiety), neurodevelopmental disorders (autism), inflammatory conditions (allergies) and gastrointestinal disorders (IBD, IBS) (Damman et al. 2012, Collado et al. 2015, Palm, de Zoete & Flavell 2015).

Hence, researchers from different scientific areas are now increasingly interested in the microbiota, its role in health and disease, and in experimental approaches to dissect the pathophysiologic implications of gut dysbiosis. As GF mice were shown to have limitations due to their GF upbringing (Diaz Heijtz et al. 2011, Braniste et al. 2014), antibiotic treatment gained appreciation as a model for studying dysbiosis (Bercik et al. 2011, Farzi, Gorkiewicz & Holzer 2012, Desbonnet et al. 2015) and causality in microbiota-host interactions. However, so far no standardized model for antibiotic-induced dysbiosis has been established. Moreover, the different models have not been characterized in great depth, mostly concentrating on single aspects of changes in microbial composition. Therefore, this thesis aimed at establishing a model of antibiotic-induced dysbiosis that is broadly characterized and allows to shed more light on the complex system of the microbiota-gut-brain axis. Furthermore, it is of particular interest to identify bacterial components that are relevant to microbiota-host interactions and, in particular, to alterations in brain function.

Two PAMPs, namely LPS and polyinosinic:polycytidylic acid, a synthetic analog of double-stranded RNA, have been shown to induce behavioral changes if administered parentally (Sparkman et al. 2006, Frenois et al. 2007, Gibney et al. 2013, Farzi et al. 2015, Leite et al. 2016, Araki et al. 2016). To the best of my knowledge flagellin-induced changes in emotional behavior have not been assessed in animal models. In view of the literature on LPS and polyinosinic:polycytidylic acid I hypothesized that flagellin is also

able to induce behavioral changes. Hence, in an independent approach the second part of this thesis investigated the effect of flagellin on anxiety-like and social behavior.

4.2 Effects of antibiotic-induced dysbiosis on overall health

Previous research has shown that administration of antibiotics via the drinking water can lead to a significant reduction of body weight (Farzi, Gorkiewicz & Holzer 2012). It is believed that this change results from the aversive taste of some antibiotics. As a considerable loss of body weight is probably stressful for mice, it was one of this project's objectives to establish an antibiotic-induced dysbiosis model that has no impact on overall health parameters of mice. Administration of antibiotics did not induce significant changes in body weight, water and food intake. Thus, although the microbiota is important for digestion and energy uptake, the antibiotic-treated mice were still able to gain enough energy from the food without increasing their food intake. To minimize stress for the animals, an approach administering the antibiotics via the drinking water would be desirable. As the antibiotics might themselves influence the amount of water consumed and thus alter the quantity of antibiotics administered, the antibiotics were given by gastric gavage to secure consistent dosing.

As the microbiota harbors hundreds to thousands of different bacterial species, the choice of antibiotics is crucial with regard to the question which sector of the bacterial community is abandoned and for this reason has a big impact on which effects are observed in antibiotic-induced dysbiosis (Donaldson, Lee & Mazmanian 2016). Additionally, it should not be neglected that some antibiotics themselves can exert systemic effects when present in toxicologically relevant concentrations in the brain. In the clinic high concentrations of antibiotics in the plasma are required for the treatment of systemic infections, lung infections or infections of the brain (e.g. meningitis), in which case the antibiotic has to be given parenterally. Depending on the antibiotic in use and the functionality of the BBB, excess entry of an antibiotic into the brain could negatively affect brain function (e.g. seizures) (Nau, Sorgel & Eiffert 2010). The antibiotic mouse model to be used in this thesis was designed to cause a thorough depletion of microbes from the gut without any systemic effects induced by the antibiotics themselves. Hence, five antibiotics with minimal oral bioavailability were chosen to ablate a wide range of bacteria yet microbial depletion is limited to the intestine (Craig, Stitzel 2004, Lafforgue et al. 2008). When given orally, non-absorbable antibiotics will only act in the intestine as is the case for bacitracin, meropenem, neomycin and vancomycin. As one of the antibiotics, ampicillin, has low oral

bioavailability in humans (Lafforgue et al. 2008), concentrations of this antibiotic were measured in the plasma and brain at a time point when cognitive performance was assessed. Analysis revealed that ampicillin levels in the plasma were measurable whereas in the brain levels were below the LLOQ. The use of ampicillin in our study was modeled along other studies with this penicillin (Membrez et al. 2008). Ampicillin, like other penicillins, is one of the most specific antibiotics available, given that the molecular target of this beta-lactam compound is missing in eukaryotic cells. The mouse has been reported to tolerate doses of 5 g/kg per day given parenterally (Stewart 1964). Neurotoxic reactions, primarily seizures, are seen only when very high doses of ampicillin are given parenterally, the blood-brain barrier is leaky (as is the case in babies and small children), and consequently the penicillin concentrations in the cerebrospinal fluid are elevated (Browning, Tune 1983, Grill, Maganti 2011). In the present study, ampicillin was given orally and very unlikely to have any direct effect on the brain, since no consistent change in blood-brain barrier permeability was observed, as deduced from the expression of tight junction proteins, and consequently no measurable levels of ampicillin were detected in the mouse brain.

In this context the dose of ampicillin administered to the mouse is worth considering in relation to its dosage in humans. The daily oral dose of ampicillin given to the mice was approximately 192 mg/kg. If this concentration is translated to human doses, including the body surface area normalization method (Reagan-Shaw, Nihal & Ahmad 2008), it is equivalent to a dose of around 16 mg/kg. Although this calculation does not take account of any species differences in pharmacokinetics (Blanchard, Smoliga 2015), it is relevant to state that in children a dose of 200-300 mg/kg/day ampicillin given parenterally is recommended for diseases like uncomplicated community-acquired pneumonia (Newman et al. 2012). Despite the confounding factors in these equivalence considerations it is obvious that the dose of ampicillin administered to the mouse is way smaller than the dose used in human therapy and, hence, by all means is devoid of adverse effects on the eukaryotic cells of the host organism.

In this context particular note should also be taken of the study of Desbonnet et al. (2015). These investigators administered an antibiotic mix including ampicillin to weaning mice for a much longer period than in the current study. Assuming that the brain of young mice with a BBB not yet fully developed may be more susceptible to any deleterious effect of ampicillin than adult animals, one would expect adverse effects to occur more likely than in adult animals. However, the animals showed normal body weight gain and drinking

behavior (Desbonnet et al. 2015). Hence, the pertinent literature together with the analysis of ampicillin in plasma and brain indicates that changes of behavior and brain function observed in antibiotic-treated adult mice were not the result of a direct toxic effect of ampicillin on the brain.

4.3 Dysbiosis induced by antibiotic treatment

Microbiome analysis showed that the multiple antibiotic treatment studied here was indeed capable of greatly reducing bacterial load and diversity. Microbial community profiling was conducted not only to confirm the efficacy of antibiotic treatment but also to obtain a picture of the microbial composition in the gut of control and antibiotic-treated animals. Given the large reduction of bacterial load in antibiotic-treated mice, it is likely that rare species are not seen in the overall picture as their number is too low (Hamady, Knight 2009) and the residual OTUs already account for one hundred percent of bacterial load. With the method of analysis used here the composition of the microbiota in antibiotic-treated mice is thus difficult to assess, as common contaminants have an impact on the overall calculation of bacterial load (Weiss et al. 2014). Hence, the presence of contaminants will be blown out of proportion as there are only low numbers of inherent OTUs left (samples of low biomass) (Salter et al. 2014, Weiss et al. 2014). As a result, contaminant OTUs contribute greatly to the overall bacterial load in antibiotic-treated mice. As can be seen in Appendix section 6.4, Table 2, antibiotic-treated mice often harbor similar amounts of OTUs as the blank (water). In the vehicle-treated mice these OTUs are very low in number or seemingly non-existent. In the case of the vehicle-treated mice, it is likely that the contaminant OTUs are “rare species”, thus, in the overall calculation they are extremely low. This makes interpretation of the presence of OTUs in antibiotic-treated mice very difficult and comparison with the OTUs of vehicle-treated mice inaccurate. Nevertheless, as bacterial load is so low that contaminant OTUs constitute the main families in the antibiotic-treated mice, a definite reduction in bacterial load and diversity has been achieved by antibiotic treatment. Furthermore, the decrease in bacterial load was supported by the qPCR results. As the reduction is so vast, yet no gross changes in histology and tight junction protein expression are visible, the antibiotic-induced dysbiosis model described here is characterized by a massive impoverishment of the bacterial community without impairment of its habitat.

4.4 Further local effects of antibiotic-induced dysbiosis

Oral consumption of antibiotics can lead to diarrhea (antibiotic-associated diarrhea), intestinal morphologic alterations and malabsorption (Högenauer et al. 1998, Wlodarska et al. 2011). The antibiotic mix administered in this project consisted of 5 antibiotics which were dosed rather high, hence local effects of the antibiotic mix on the intestine were examined. None of the mice showed signs of diarrhea or loose stool, and gross stool consistency remained normal throughout the experiment. Histology of the jejunum, ileum and colon of antibiotic-treated mice also failed to reveal gross alterations. In contrast, the weight of the cecum was almost tripled by antibiotic treatment, which is in line with observations made in GF mice (Smith, McCoy & Macpherson 2007). It should not go unnoticed in this context that oral administration of enroflaxin has been reported to cause histologic damage in the intestine (Romick-Rosendale et al. 2014) and changes in colonic mucus thickness of mice (Wlodarska et al. 2011), which can make the animals more susceptible to infections (Wlodarska et al. 2011, Romick-Rosendale et al. 2014). In order to develop a selective gut dysbiosis model, the antibiotics in use need to be devoid of any adverse influence on the gastrointestinal mucosa besides bacterial clearance. This criterion implies that the habitat of the gut microbiota stays as normal as possible and is not accompanied by colonization of pathogenic microbes. In the present study, the similarity of intestinal histology in control and antibiotic-treated mice attests to the absence of any adverse influence on the microbial habitat, which can be viewed as an advantage of the model under study. Nevertheless, it should be noted that the outer mucus layer in the colon, which is colonized by many bacteria (Johansson, Larsson & Hansson 2011), was not evaluated in the current study, as it is usually lost during preparation for H&E staining (Palestrant et al. 2004). Of further note, the microbial habitat may also be influenced by the ability of the microbiota to affect colonic motility and gut transit.

Several mechanisms have been proposed to be implicated in the enlargement of the cecum in GF animals (Gordon, Pesti 1971, Heneghan 1973). It has been suggested that a reduced muscle tone is the main cause for the enlargement (Gordon, Pesti 1971). Moreover, the presence and transport of ions is altered in the cecum of GF mice (Asano 1969), thereby possibly attracting more water into the cecal fluid (Heneghan 1973). Besides other alterations in GF animals, oxygen uptake and cardiac output are reduced in GF rats. These alterations can be restored when the cecum is removed (Wostmann, Bruckner-Kardoss & Knight 1968), indicating that the enlarged cecum has an impact on metabolic deviations

observed in GF animals (Heneghan 1973). An enlargement of the cecum is observed in GF mice as well as in mice treated with antibiotics. When antibiotic treatment is terminated, the cecal weight returns to normal levels (Savage, Dubos 1968). This indicates that this physiologic abnormality in GF animals is inducible and reversible (Reikvam et al. 2011). According to the study of Savage and Dubos (1968), the histology of the cecum of antibiotic-treated mice does not differ from that of control animals, and the excess of water is located primarily in the lumen rather than in the cecal wall (Savage, Dubos 1968). Taken these observations together, an integrated approach addressing muscle tone as well as ion and water secretion/absorption will be required to analyze the mechanisms underlying the cecal enlargement in GF as well as antibiotic-treated mice.

Immunologically, antibiotic-induced dysbiosis led to a significant rise of colonic IFN-γ and IL-10 mRNA expression whereas TNF-α mRNA expression was blunted. This pattern may mirror a reciprocal interaction between stimulant and inhibitory immune processes, preventing the development of overt inflammation. On the other hand, except for IFN-γ, the mRNA expression changes did not translate to the protein level. Also IFN-γ protein levels in antibiotic-treated mice were only 25% higher than in the controls, whereas at the mRNA level IFN-γ was 15 fold higher in antibiotic-treated mice compared to controls. On the other hand, gut dysbiosis did not translate into gross histological alterations in the gut, which negates the presence of overt inflammation. In addition, the absence of significant changes in the colonic expression of tight junction proteins could imply that paracellular permeability in the mucosa did not increase to a relevant extent. Antibiotic analysis supports this assumption as the non-absorbable antibiotic vancomycin (Craig, Stitzel 2004) did not enter the blood stream to a significant extent. Nevertheless, additional permeability assays would be necessary to establish and prove the state of gut permeability in this model.

The metabolome analysis of the colonic content of antibiotic-treated mice supports the findings in microbiome analysis as especially bacteria-derived metabolites were reduced. The SCFAs acetate, n-butyrate and propionate are products of microbial fermentation of dietary fiber. Depletion of these SCFAs is in accordance with other antibiotic studies (Yap et al. 2008, Romick-Rosendale et al. 2009, Swann et al. 2011a). The loss of SCFAs might also explain why antibiotic treatment tended to increase water consumption in an attempt to compensate for the physiologic action of SCFAs to stimulate water absorption in the intestine (Whelan et al. 2004). SCFAs have been linked to several health benefits (Macfarlane, Macfarlane 2011, Subaric et al. 2012). Moreover, they have even been

proposed as a treatment/prevention for different diseases (Harig et al. 1989, Vernia et al. 1995, Scheppach 1996, Di Sabatino et al. 2005, Gao et al. 2009, Scharlau et al. 2009, Hu et al. 2010, Blouin et al. 2011, Tang et al. 2011, Patterson et al. 2014). Oral administration of sodium butyrate was shown to decrease BBB permeability in adult GF mice (Braniste et al. 2014). Colonic acetate was recently shown to be able to cross the BBB and be taken up by the brain (Frost et al. 2014). Furthermore, acetate was suggested to suppress appetite by a direct effect on hypothalamic circuits (Frost et al. 2014). In the present study, however, there was no change in food intake in antibiotic-treated mice. SCFAs can signal via activation of G-protein-coupled receptors and inhibition of histone deacetylases (HDAC) (Tan et al. 2014, Galland 2014). Interestingly, HDAC inhibitors have been proposed as cognitive enhancers and treatment for anxiety and trauma (Graff, Tsai 2013, Whittle, Singewald 2014). Hence, the strong depletion of SCFAs in antibiotic-treated mice might have partly contributed to the deficits observed in recognition memory. Given that SCFAs were also shown to have immunomodulatory properties (Maslowski et al. 2009, Smith et al. 2013b), SCFA depletion might also have played a role in the observed increase in IFN-γ in the colon. As the potential implications of the SCFAs in general health are widespread (Galland 2014), systematic studies are required to pin down the effects and mechanisms operated by SCFAs at the concentrations that are present in the gut and circulation. Trimethylamine is a product of bacterial enzymes processing dietary choline (Zeisel, DaCosta & Fox 1985) and L-carnitine (Koeth et al. 2013), and the reduction of the trimethylamine concentration in the luminal contents very likely results from a lack of bacterial enzymes in the gut. Excess excretion of unmetabolized trimethylamine in the urine (and also breath, sweat and reproductive fluids) is the hallmark of trimethylaminuria (Mackay et al. 2011). This disease is usually lacking physical symptoms but is characterized by a fishy odor (Phillips, Shephard 1993). As affected individuals appear healthy (Phillips, Shephard 1993), it seems as if the increased levels of unmetabolized trimethylamine do not have a negative impact on the host. However, it should be mentioned that mood disorders have been observed frequently in these patients, but it is not clear whether this originates from social isolation (due to the odor) or a biochemical mechanism (Mackay et al. 2011). On the other hand, oral treatment for 16 weeks with a structural analog of choline that inhibits microbial trimethylamine formation did not cause any signs of toxicity or changes in liver and kidney function (Wang et al. 2015b). Trimethylamine is known to be a gap junction opener (Perez-Velazquez, Valiante & Carlen 1994). Gap junctions are connections between adjacent cells and are important for a

particular type of cell-to-cell communication (Dere, Zlomuzica 2012). They have been shown to play a role in epilepsy, and trimethylamine has been found to have a proepileptic effect in rat models when administered into certain brain areas (Gajda et al. 2006, Medina-Ceja, Ventura-Mejia 2010). So far only three cases of epilepsy in trimethylaminuria have been described (McConnell et al. 1997, Pellicciari et al. 2011). Thus the role of trimethylamine in the occurrence of seizures and behavioral problems is not fully elucidated and further research on this topic is warranted (Pellicciari et al. 2011). Of note in this context, trimethylamine is involved in species-specific social communication in mice (Li et al. 2013), and trimethylamine production/secretion seems to be dependent on sex and sexual hormones (Carnicelli et al. 2010, Li et al. 2013). How this metabolite may influence human (social) behavior has not been elucidated so far (Wallrabenstein et al. 2013). On a molecular basis, trimethylamine has been shown to activate the ortholog receptor of murine trace amine-associated receptor 5 expressed in the human olfactory epithelium (Carnicelli et al. 2010, Wallrabenstein et al. 2013).

As there is also a mammalian pathway that processes dietary choline to glycine (Zeisel, DaCosta & Fox 1985), it awaits to be examined if the levels of glycine are increased in antibiotic-treated mice. In this setup the measurement of glycine was not possible with ^{1}H NMR as the antibiotics gave a strong background signal in the area of the glycine peak (δ 3.56-3.57). The reduction of adenine and uracil levels in the luminal colonic content is in accordance with data from GF mice (Matsumoto et al. 2012). This decrease is likely due to the reduced bacterial number in antibiotic-treated mice.

4.5 Effects of antibiotic-induced dysbiosis on circulating cytokines and metabolites

Unlike in the colon, I did not find any alterations in the cytokine profile in the plasma in which cytokine levels were largely below the LLOQ. Circulating cytokines are thus unlikely to mediate between antibiotic-induced gut dysbiosis and disturbances of brain function. The lack of a change in circulating cytokine levels also negates the involvement of systemic inflammation in the behavioral perturbations under study (Moloney et al. 2014) and affirms that dysbiosis-induced inflammatory processes were largely confined to the intestine. Immunologic messages, however, appear to be transmitted to the spleen, the weight of which was significantly diminished by antibiotic treatment, as also seen in related studies with GF mice (Smith, McCoy & Macpherson 2007). The decrease in spleen weight has also been observed in other antibiotic treatment models (Sullivan 1960,

Reikvam et al. 2011, Desbonnet et al. 2015). Apart from the weight/size reduction, the spleens of GF mice contain a skewed lymphocyte profile (Mazmanian et al. 2005), which is also true for other lymphoid tissues of GF mice (Hrncir et al. 2008). The number of lymphocyte subpopulations can be partially corrected by treatment with live bacteria (Savidge et al. 1991, Mazmanian et al. 2005) or bacterial components (Mazmanian et al. 2005, Hrncir et al. 2008). However, the results regarding an improvement of the weight of the spleen are contradictory (Savidge et al. 1991, Hrncir et al. 2008), probably due to different experimental setups. As both reduction in spleen weight and enlargement of the cecum are observed in both GF mice and antibiotic-treated animals, it has been suggested that these changes represent reversible effects typical of an under-stimulation by commensals (Reikvam et al. 2011). This supports the conclusion that the antibiotic treatment diminished not only bacterial colonization, but also decreased stimulation of lymphatic tissues.

In contrast to circulating cytokines, the plasma metabolome of antibiotic-treated mice showed significant alterations compared to vehicle-treated mice. More precisely, lipid species and converted bacteria-derived metabolites were affected by antibiotic-induced dysbiosis. A change in the levels of lipid species has also been observed in fasted GF mice (Velagapudi et al. 2010). Nevertheless, the pattern of up- and downregulated circulating lipid species in the antibiotic-treated mice examined here is not completely consistent with the data obtained from GF mice. While the increase in PC 36:2 is in accordance with results from fasted GF mice, the levels of PC 40:5, PC 40:8 and sphingomyelin 34:1 are not decreased as in the present model but left unaltered in fasted GF mice (Velagapudi et al. 2010). Interestingly, in fasted GF mice there is a trend towards decreased PC, cholesteryl ester and sphingomyelin levels and increased triglyceride concentrations (Velagapudi et al. 2010). PC 36:2, PC 36:5 and PC 38:7 were the only PCs increased in fasted GF mice (Velagapudi et al. 2010). In contrast to the present findings in antibiotic-treated mice, the levels of LPC 20:3 were unaffected in fasted GF mice (Velagapudi et al. 2010). As the metabolism of PC and LPC is interconnected (Richmond, Smith 2011) a decrease in PC lipolysis could be an explanation for the increased PC and concomitant decrease in LPC levels. In fasted GF mice, there was rather a small trend towards increased LPC levels, with one of four LPCs, namely LPC 20:0, reaching significance (Velagapudi et al. 2010). Given that many PCs are decreased in fasted GF mice this would also fit to the proposed change in PC lipolysis. Unlike the LPC levels, the plasma levels of phosphatidylinositol 38:5 were increased in antibiotic-treated mice, which may be

indicative of an anti-inflammatory process, given that exogenous phosphatidylinositol ameliorates inflammation (Davies et al. 2014). Taken together, these findings could mean that there is a specific up- and downregulation of lipid species in GF mice. In antibiotic-treated mice only the increment of PC 36:2 levels and the unchanged levels of PC 32:2, PC 36:3, PC 36:4, PC 40:4, PC 40:7 and LPC 18:2 were in accordance with the lipid profile of fasted GF mice. Nevertheless, also in the present model a specific up- and downregulation of lipid species was observed, indicating that the microbial community has an impact on the lipid species profile of the host. Given the distinct functions of these different lipid species, including biological membrane structure and membrane-mediated cell signaling (Exton 1990) as well as inflammation (Davies et al. 2014) and cell signaling (Schmitz, Ruebsaamen 2010), changes in plasma levels could have an influence on many organs, including the brain. The ways in which the gut microbiota alters hepatic biosynthesis and dietary absorption of these lipid species remain to be investigated (Velagapudi et al. 2010). Due to the importance of corticosterone in the HPA axis, the impact of increased circulating corticosterone levels is discussed in section 4.7.

Besides lipid species, also converted bacteria-derived metabolites were altered in antibiotic-treated mice. While in the plasma of GF mice *p*-cresyl sulfate was totally absent (Wikoff et al. 2009), in the antibiotic-dysbiosis model studied here *p*-cresyl sulfate levels were distinctly decreased but not completely absent. *p*-Cresyl sulfate is generated by bacterial fermentation of dietary tyrosine to *p*-cresol (Bone, Tamm & Hill 1976, Smith, Macfarlane 1996, Meijers, Evenepoel 2011), which is then absorbed and further metabolized by the host to *p*-cresyl sulfate (Meijers, Evenepoel 2011). Given the metabolism of *p*-cresyl sulfate, the reduced *p*-cresyl sulfate levels in antibiotic-treated mice are most likely a reflection of the lack of gut bacterial enzymes. *p*-Cresyl sulfate has been associated with cardiovascular and chronic kidney disease (Meijers, Evenepoel 2011). Hence, a reduction in bacterial metabolism and absorption has been proposed as a therapeutic target in chronic kidney disease (Meijers, Evenepoel 2011), and prebiotic intervention has already been shown to reduce serum *p*-cresyl sulfate levels in hemodialysis patients (Meijers et al. 2010). In patients with chronic kidney disease *p*-cresyl sulfate is thought to be an uremic toxin (Vanholder et al. 2011, Vanholder et al. 2014). Uremic toxins are taken up via specific transporters which are present not only in the kidney but also in the BBB (Enomoto, Niwa 2007, Miyamoto et al. 2011, Hosoya, Tachikawa 2011, Nigam et al. 2015). Thus, in chronic renal failure a possible

accumulation of uremic toxins in the CNS has been proposed (Enomoto, Niwa 2007). As concentrations of *p*-cresyl sulfate are much lower in healthy subjects (Duranton et al. 2012) and the mechanisms of uremic neurotoxicity are still only partially understood (Zgoda-Pols et al. 2011), the overall relevance of *p*-cresyl sulfate in health and disease awaits further analysis and calls for an explanation of discrepant observations. While a recent study failed to observe any association between cognitive impairment and *p*-cresyl sulfate in early-stage chronic kidney disease (Yeh et al. 2016), urinary *p*-cresol and *p*-cresyl sulfate have recently been associated with autism spectrum disorder in young children and proposed as a biomarker in autism (Altieri et al. 2011, Persico, Napolioni 2013, Gabriele et al. 2014).

Circulating trimethylamine-*N*-oxide is an oxidation product of trimethylamine (Rebouche, Chenard 1991), and the decrease of the trimethylamine-*N*-oxide concentrations in the plasma of antibiotic-treated mice are very likely related to the trimethylamine depletion in the colonic contents. The results of Koeth et al. (2013) support this assumption. Furthermore, this group showed that formation of trimethylamine-*N*-oxide is strongly influenced by long-term dietary habits (Koeth et al. 2013). Trimethylamine-*N*-oxide has been associated with a risk of atherosclerosis, cardiovascular and kidney diseases (Koeth et al. 2013, Tang et al. 2013, Tang et al. 2015). Hence, a reduction in trimethylamine-*N*-oxide plasma levels may contribute to cardiovascular health (Koeth et al. 2013). Recently, a natural compound that inhibits microbial trimethylamine formation was suggested as a potential treatment for atherosclerosis (Wang et al. 2015b, Jonsson, Backhed 2015). Interestingly, the pathogen *Vibrio cholerae* was observed to produce more cholera toxin during anaerobic trimethylamine-*N*-oxide respiration, a growth mode that was suggested to play a role in the human intestine (Lee et al. 2012). On the other hand, trimethylamine-*N*-oxide has been shown to prevent protein misfolding (Brown, Hong-Brown & Welch 1997, Bennion, DeMarco & Daggett 2004) and stabilize protein structure (Jaravine, Rathgeb-Szabo & Alexandrescu 2000, Rajan et al. 2011). Thus, trimethylamine-*N*-oxide was suggested as a possible therapeutic approach in protein misfolding diseases (e.g. Alzheimer's disease) (Chaudhuri, Paul 2006). A small study in rats found that a diet supplemented with high amounts of trimethylamine-*N*-oxide leads to disruption of the BBB (Liu, Huang 2015). Another study in mice revealed that oral treatment with sorafenib, a chemotherapeutic drug, leads, besides altered expression of several other metabolites, to a downregulation of trimethylamine-*N*-oxide in the hippocampus (Du et al. 2015). As the number of studies on the role of trimethylamine-*N*-oxide in the brain is limited, it is not yet

fully understood what impact this metabolite has on brain function.

Deoxycholic acid is an unconjugated secondary bile acid, produced by intestinal bacteria (Ridlon, Kang & Hylemon 2006) while chenodeoxycholic acid is a primary bile acid produced by the liver (Ridlon, Kang & Hylemon 2006). Due to isomerism the LC-MS was not able to distinguish between those two bile acids. In antibiotic-treated mice deoxycholic acid and chenodeoxycholic acid were completely depleted from the plasma. This is in accordance with data from GF mice in which the levels of unconjugated bile acids are markedly decreased in the plasma and other tissues (Swann et al. 2011b). Hence the microbial community seems to have a strong impact on bile acid metabolism and profile. When secreted from the liver, chenodeoxycholic acid is in a conjugated form (glycine or taurine), and in the intestine it is again deconjugated by bacterial enzymes (Midtvedt 1974, Swann et al. 2011b). Thus the lack of bacterial enzymes is very likely the reason why deoxycholic acid and chenodeoxycholic acid are completely absent in antibiotic-treated mice. On a physiologic level bile acids play a role in fat digestion as well as in energy, glucose and lipid homeostasis (Watanabe et al. 2004, Stayrook et al. 2005, Watanabe et al. 2006, Swann et al. 2011b). In this capacity, bile acids have been shown to prevent obesity and insulin resistance by increasing energy expenditure in brown adipose tissue in mice (Watanabe et al. 2006). Bile acid manipulation has thus been suggested as a possible approach for diabetes type 2 therapy (Prawitt, Caron & Staels 2011), plasma bile acids being positively correlated with body mass index (Prinz et al. 2015). A negative association in obese patients between plasma bile acids and cognitive restraint of eating was also observed, but this interaction needs to be further investigated (Prinz et al. 2015). Bile acids can activate the farnesoid X receptor which plays a role in intestinal innate immunity (Vavassori et al. 2009) and, in the fed state, in autophagy in the liver (Seok et al. 2014, Lee et al. 2014). This relationship between intestinal microbiota and autophagy has recently been proposed to be of therapeutic benefit in disorders caused by autophagy disturbances, like neurodegenerative diseases (Nie, Hu & Yan 2015).
With regard to microbiota-brain interaction it is important to note that bile acids can directly and indirectly affect the intestinal microbiota (Begley, Gahan & Hill 2005, Nie, Hu & Yan 2015). In advanced cirrhosis both dysbiosis and a decrease in bile acid levels in the intestine have been found (Ridlon et al. 2015). Changes in the microbiota, which were accompanied by changes in bile acid profile (Kakiyama et al. 2013), were shown to improve cognitive function in hepatic encephalopathy (Bajaj et al. 2013). Further research

on this topic is warranted (Ridlon et al. 2013) in order to analyze the mechanisms behind this relationship. Of note, during cholestasis bile acids have been shown to permeabilize the BBB in rats (Quinn et al. 2014). Likewise, injections of chenodeoxycholic acid or deoxycholic acid cause a permeabilization of the BBB in normal rats (Quinn et al. 2014). Furthermore, during acute liver failure (McMillin et al. 2016) and cholestatic liver injury (McMillin et al. 2015) bile acids have been reported to gain entry into the brain. In the murine model of acute liver failure bile acids appear to contribute to the neurological decline associated with hepatic encephalopathy, and feeding with deoxycholic acid worsened the neurological decline (McMillin et al. 2016). Hence, bile acid modulation and reduction of serum bile acids have been proposed as potential (supplemental) therapeutic option for hepatic encephalopathy due to acute liver failure (McMillin et al. 2016). Whereas some bile acids have been implicated in the suppression of the HPA axis during cholestatic liver injury in rats, chenodeoxycholic acid and deoxycholic acid did not induce such effects (McMillin et al. 2015). In a different manner, excess bile acids in the colon seem to play a role in the symptoms of diarrhea-predominant IBS (Camilleri 2012). Furthermore, bile acids have been suggested to be involved in hepatic and intestinal carcinogenesis (Baptissart et al. 2013).

4.6 Effects of antibiotic-induced dysbiosis on behavior and cognition

As reported in a preliminary communication (Farzi, Gorkiewicz & Holzer 2012), short-term antibiotic treatment abolished novel object recognition. This finding was confirmed in the present study which, in addition, disclosed that spatial learning and memory as studied in the Barnes maze test were not affected by antibiotic treatment. A reduction of object recognition memory has also been observed following long-term treatment with another antibiotic regimen (amphotericin B, ampicillin, metronidazole, neomycin, vancomycin) from the weaning period into adulthood (Desbonnet et al. 2015). In the model of the study at hand the specificity of cognitive deficit (object recognition memory but not spatial memory) in antibiotic-treated mice is an important finding which indicates that the antibiotic mix did not induce any global brain damage. There is a general consensus that the hippocampus is implicated in spatial memory (Warburton et al. 2000, Forwood, Winters & Bussey 2005, Vann, Albasser 2011, Warburton, Brown 2015). It is also generally acknowledged that the perirhinal cortex plays a critical role in recognition memory (Ennaceur, Neave & Aggleton 1996, Warburton et al. 2003, Winters et al. 2004, Balderas, Rodriguez-Ortiz & Bermudez-Rattoni 2015). In contrast, in spatial memory the

perirhinal cortex is thought to be less important than the hippocampus, as it depends importantly on the processing of object-related information (Barker, Warburton 2011, Warburton, Brown 2015). However, the contribution of the hippocampus to recognition memory is still an ongoing debate (Warburton, Brown 2015, Kinnavane, Albasser & Aggleton 2015). Studies often use lesion models to test the importance of specific brain areas on a particular type of memory. Lesions in the hippocampus or hippocampal inactivation have been shown to impair (Clark, Zola & Squire 2000, Broadbent et al. 2009, Cohen et al. 2013) or to have no impact on recognition memory (Aggleton, Hunt & Rawlins 1986, Winters et al. 2004, Forwood, Winters & Bussey 2005). These contradictory results could be influenced by several factors including strain/species differences, different methods to assess recognition memory and differences in lesion models. The results obtained in the current study rather points towards a role of the hippocampus in recognition memory (see section 4.7). However, there are complex interactive networks between brain areas involved in cognition, including the perirhinal cortex and hippocampus. Therefore, it cannot be ruled out that the antibiotic-induced impairment of recognition memory derives from major changes in the perirhinal cortex which projects to the hippocampus directly and indirectly via the entorhinal cortex and vice versa (Albasser, Poirier & Aggleton 2010, Kinnavane, Albasser & Aggleton 2015). The insular cortex (Bermudez-Rattoni et al. 2005, Roozendaal et al. 2010, Balderas, Rodriguez-Ortiz & Bermudez-Rattoni 2015), amygdala (Rossato et al. 2013, Beldjoud, Barsegyan & Roozendaal 2015) and prefrontal cortex (Rossato et al. 2013, Balderas, Rodriguez-Ortiz & Bermudez-Rattoni 2015) were also shown to play a role in recognition memory, although contradictory findings on the latter two brain areas have also been published (Barker et al. 2007, Balderas et al. 2008). Hence, the brain circuits underlying recognition memory have not yet been fully elucidated.

While GF mice and antibiotic-treated mice have also been reported to display enhanced activity and exploratory behavior as well as reduced anxiety-like behavior (Bercik et al. 2011, Diaz Heijtz et al. 2011, Desbonnet et al. 2015), no changes in anxiety- and depression-like behavior were observed under the current experimental conditions. The lack of effect on anxiety- and depression-like behavior of antibiotic treatment was rather intriguing and is another example that the composition of the microbiota is a sensitive factor with a particular impact on host health. Furthermore the lack of alterations in anxiety- and depression-like behavior offers the opportunity to relate the bio- and

neurochemical alterations in the brain specifically to the disruption of novel object recognition memory.

4.7 Effects of antibiotic-induced dysbiosis on the brain

To further investigate the specific cognitive deficits in antibiotic-treated mice I analyzed the neurochemical alterations in four different brain areas. The medial prefrontal cortex, hippocampus and amygdala were chosen due to their distinct contributions to object recognition (Moses et al. 2005, Balderas et al. 2008, Broadbent et al. 2009, Barker, Warburton 2011, Antunes, Biala 2012, Beldjoud, Barsegyan & Roozendaal 2015). Given that the gut microbiota has an impact on HPA axis activity (Sudo et al. 2004), and that corticosteroids can influence cognitive function (McEwen 2007) the hypothalamus was included as well. Due to the dynamic distribution of signaling molecules between neuronal somata, dendrites and axons and the homeostatic balance between translation, storage capacity, axonal transport and neuronal release in peptide or protein levels, mRNA expression was chosen as the most sensitive parameter for molecular analysis (Larsen et al. 1993, Ji et al. 1994).

The expression of tight junction proteins is important for an intact BBB and normal BBB permeability (Tietz, Engelhardt 2015). A decrease of tight junction protein expression can easily lead to an increase in BBB permeability giving harmful molecules easier access to the brain (Tietz, Engelhardt 2015). The expression of tight junction proteins at the BBB is another entity regulated by the gut microbiota (Braniste et al. 2014). In GF mice the protein levels of OCLN and CLDN5, but not TJP1, are decreased in the frontal cortex, hippocampus and striatum (Braniste et al. 2014). The present data show that OCLN and CLDN5 were likewise downregulated in the hippocampus of antibiotic-treated mice whereas no change was seen in the medial prefrontal cortex and hypothalamus. In the amygdala, however, OCLN and TJP1 were upregulated. A leaky BBB in the hippocampus could imply that this region is exposed to enhanced oxidative stress, yet expression of CAT and GSR remained unchanged in this region whereas GSR expression was increased in the amygdala in which tight junction protein expression was amplified. However, the altered expression pattern of cerebral tight junction proteins did not translate into neuroinflammatory processes since cerebral cytokine expression was not increased. The mechanisms underlying the distinct changes in tight junction protein expression are not yet understood. Nevertheless, the present findings add to the contention that the gut microbiota

has an impact on the BBB, thereby further influencing the information transfer between the gut and the brain and vice versa.

BDNF is known to be important for synaptic plasticity and cognition (Rattiner, Davis & Ressler 2005, Lu et al. 2014). Antibiotic-treated mice in the present study exhibited decreased expression levels of BDNF mRNA in the medial prefrontal cortex, hippocampus and hypothalamus but unaltered levels in the amygdala. In addition, there was a positive correlation between hippocampal BDNF mRNA expression and the NORT memory index in antibiotic-treated mice (Pearson's correlation; $r = 0.775$, $p = 0.024$). This further supports a role of BNDF in learning and memory measured by the NORT and emphasizes the role of the hippocampus in novel object recognition memory. BDNF expression is also altered in GF mice (Sudo et al. 2004, Diaz Heijtz et al. 2011, Clarke et al. 2013, Schele et al. 2013) and other models of antibiotic-induced dysbiosis (Bercik et al. 2011, Desbonnet et al. 2015). Specifically, BDNF expression in the cortex, hippocampus and amygdala is decreased in GF mice (Sudo et al. 2004, Diaz Heijtz et al. 2011, Clarke et al. 2013), and in the hippocampus of antibiotic-treated mice (Desbonnet et al. 2015), whereas that in the hypothalamus is upregulated (Schele et al. 2013) or remains unchanged (Sudo et al. 2004). The differential impact of gut dysbiosis on BDNF expression in different brain areas may be related to other bio- and neurochemical alterations that show regional differences. For instance, the attenuation of hippocampal and hypothalamic BDNF expression seen in the present study was paralleled by a decrease in IL-1β mRNA expression. Given that IL-1β can impair BDNF-induced signal transduction (Tong et al. 2008), it could be argued that downregulation of IL-1β expression is a mechanism to enhance BDNF signaling despite reduced BDNF levels. However, the effect of IL-1β on cognition depends on experimental conditions and type of memory tested (Yirmiya, Winocur & Goshen 2002, Goshen et al. 2007, Cunningham, Sanderson 2008, Majumder et al. 2012), as intracerebroventricular administration of 1 ng IL-1β to mice has been reported to improve contextual fear memory, whereas blockade of IL-1 signaling or administration of 10 ng IL-1β results in impaired memory (Goshen et al. 2007).

The N-methyl-D-aspartate (NMDA) receptor has been shown to play a role in learning and memory (Rezvani 2006). Specifically, enhanced expression of the subunit encoded by the gene GRIN2B has been associated with improved performance in the NORT (Tang et al. 1999, Niimi, Takahashi & Itakura 2008). In the antibiotic-dysbiosis model at hand the expression of GRIN2B mRNA was increased in the amygdala but remained unaltered in the medial prefrontal cortex, hippocampus and hypothalamus. This is contrary to the

findings in GF mice, where GRIN2B is decreased in the central amygdala (Neufeld et al. 2011). Given the role of the amygdala in anxiety (Allsop et al. 2014, Calhoon, Tye 2015) the divergent expression of GRIN2B in this region may provide an explanation for the lacking change in anxiety-like behavior in antibiotic-treated mice, in contrast to GF mice (Diaz Heijtz et al. 2011) and other antibiotic-induced dysbiosis models (Bercik et al. 2011, Desbonnet et al. 2015).

Kynurenine and serotonin are tryptophan-derived mediators known to play an important role in depression (Oxenkrug 2010). The gut microbiota has already been shown to influence the metabolism and activity of those molecules (Diaz Heijtz et al. 2011, Desbonnet et al. 2015, O'Mahony et al. 2015b, Yano et al. 2015). SLC6A4 encodes the serotonin transporter, which is responsible for the reuptake of serotonin from the synaptic cleft into the presynaptic nerve terminals. The transport of serotonin back into the presynaptic nerve is a crucial step in serotonin-mediated neurotransmission and a common target for antidepressants (Owens, Nemeroff 1994). Fluoxetine belongs to the group of selective serotonin reuptake inhibitors (Sommi, Crismon & Bowden 1987). In stressed mice, treatment with fluoxetine has been found to improve novel object recognition memory (El Hage et al. 2004, Urani et al. 2011) whereas in non-stressed mice the same substance impairs this type of memory (Carlini et al. 2012). In the present study with antibiotic-treated mice the expression of SLC6A4 mRNA in the amygdala was significantly enhanced whereas it remained unaltered in medial prefrontal cortex, hippocampus and hypothalamus. This region-specific increase of SLC6A4 expression was negatively correlated with the novel object recognition memory index (Pearson's correlation; $r = -0.711$, $p = 0.048$) and hippocampal BDNF expression (Pearson's correlation; $r = -0.679$, $p = 0.015$). In the present model this indicates that not only decreased BDNF levels in the hippocampus but also enhanced levels of SLC6A4 in the amygdala may be involved in the observed novel object recognition deficit in antibiotic-treated mice. These findings further support the interplay between the gut microbiota and the serotonin pathway (Clarke et al. 2013), and their involvement in memory (O'Mahony et al. 2015a, Jenkins et al. 2016).

The neuropeptide NPY is involved in numerous physiologic functions, including but not limited to digestion, energy homeostasis, anxiety, depression and cognition (Farzi, Reichmann & Holzer 2015, Reichmann, Holzer 2015). Antibiotic-treated mice in the present study showed a specific increment of NPY expression in the amygdala and hypothalamus. This is in accordance with data from GF mice (Schele et al. 2013). Given

that NPY is an orexigenic substance, an increase in food intake would have been expected to result from the increased NPY expression in the hypothalamus but no change in food intake was observed. It could be argued, however, that hypothalamic upregulation of NPY was counterbalancing a decrease in food intake caused by antibiotic-induced gut dysbiosis. Another functional implication of increased NPY expression in the amygdala and hypothalamus may be connected to the increased circulating levels of corticosterone (Lee et al. 2009, Gelfo et al. 2012, Farzi, Reichmann & Holzer 2015). Increased corticosterone levels are associated with enhanced anxiety- and depression-like behavior (Lee et al. 2009, Kutiyanawalla, Terry & Pillai 2011, Baldock et al. 2014, Farzi, Reichmann & Holzer 2015). Since NPY is known to suppress anxiety- and depression-related behavior (Sajdyk, Vandergriff & Gehlert 1999, Stogner, Holmes 2000, Thorsell et al. 2000, Bannon et al. 2000, Redrobe et al. 2002, Painsipp et al. 2011), the enhanced expression of NPY mRNA in the hypothalamus and amygdala of antibiotic-treated mice may reflect a counterregulatory response of the NPY system, preventing any increase in anxiety- and depression-like behavior caused by the increased corticosterone levels. In these implications, NPY is likely to interact with other neuronal signaling molecules in the brain. For instance, the decreased hypothalamic BDNF expression may have also been affected by the enhanced NPY expression (Gelfo et al. 2012).

In concordance with the enhanced expression of NPY in the amygdala the expression of NPY5R decreased in this brain area. The role of NPY5R in cognition is still not fully elucidated (Nichol et al. 1999, Bari et al. 2015) hence, it remains to be investigated what kind of functional relevance this finding has. Interestingly, there was no change of NPY expression in the hippocampus but a specific downregulation of NPY1R and NPY2R. Given their preferential localization to post- and presynaptic sites, respectively, it has frequently been observed that those two receptors mediate opposing effects of NPY on behavior and neurotransmission (Farzi, Reichmann & Holzer 2015). NPY1R, which is preferentially located postsynaptically plays an important role in the maintenance of emotional-affective homeostasis and stress resilience (Farzi, Reichmann & Holzer 2015). In contrast, NPY2R is preferentially located on presynaptic nerve terminals and plays a role in cognition, emotional-affective behavior and schizophrenia (Farzi, Reichmann & Holzer 2015). In this context it is of particular interest that a similar deficit in object recognition memory, as observed in antibiotic-treated mice in the present study, has been observed in NPY2R knockout mice (Redrobe et al. 2004, Painsipp et al. 2008). Hence, in addition to decreased hippocampal BDNF expression, reduced hippocampal NPY2R

expression could be a contributing factor in the cognitive deficit observed in antibiotic-treated mice.

The plasma concentration of glucocorticoids is increased after HPA axis activation, and increased levels of glucocorticoids are commonly associated with stress responses (Matousek, Dobkin & Pruessner 2010). The enhanced concentration of circulating corticosterone in antibiotic-treated mice could therefore be construed as a stress response to gut dysbiosis. A relationship between the gut microbiota and HPA axis activity has previously been envisaged from the exaggerated corticosterone response of GF mice to stress (Sudo et al. 2004). When C57BL/6N mice are chronically treated with corticosterone, anxiety-like and depression-related behavior are enforced (Sturm et al. 2015). In the antibiotic-treated mice investigated here, however, no changes in anxiety- and depression-like behavior, locomotion and spatial memory were observed. In addition, the expression of the NR3C1 gene, which encodes the nuclear glucocorticoid receptor, remained unaltered in all of the four brain regions examined. It would thus appear that the duration and/or magnitude of the increase in plasma corticosterone was insufficient to alter glucocorticoid receptor expression and emotional-affective behavior. In contrast, the increase of plasma corticosterone in antibiotic-treated mice may have, in analogy to other findings (Dwivedi, Rizavi & Pandey 2006, Naert et al. 2015), contributed to the decrease of BDNF in the hypothalamus.

4.8 Effects of flagellin on behavior

Independently of the first part of this thesis, the second part investigated the role of the PAMP flagellin and its effect on emotional behavior. Microbial components (MAMPs/PAMPs) can activate the innate immune system via binding of PPRs (Wells, Loonen & Karczewski 2010). As the gut is highly colonized, the presence of PAMPs in the intestine is rather high. This can lead to complications when gut permeability is increased (Bischoff et al. 2014, Wang et al. 2015a). The intestinal barrier absorbs nutrients and prevents translocation of intraluminal bacteria (Wang et al. 2015a). Gut permeability can be influenced by many factors including, but not limited, to microbiota, diet (Cani et al. 2008, Lam et al. 2012, Bischoff et al. 2014), toxins (Sonoda et al. 1999, Fasano, Nataro 2004, Bischoff et al. 2014) and inflammatory mediators (Nusrat, Turner & Madara 2000, Bruewer et al. 2003). Increased intestinal permeability fails to effectively prevent translocation, which can result in (chronic) intestinal inflammation (Bischoff et al. 2014) or translocation of bacteria/PAMPs to distant organs (Wang et al. 2015a).

Flagellin is a PAMP that is recognized by TLR5. In the colon, TLR5 expression is restricted to the basolateral surface of the epithelial cells (Ortega-Cava et al. 2006). Flagellin has been shown to be a potent immune activator (Gewirtz et al. 2001) and to provide protective function against various immune challenges (Vijay-Kumar et al. 2008). Furthermore, in contrast to LPS, flagellin does not show high a potential to generate severe adverse effects (Vijay-Kumar et al. 2008). In addition, flagellin has been shown to have many advantages as a vaccine and is well-tolerated in clinical trials (Honko et al. 2006, Song et al. 2009, Treanor et al. 2010, Turley et al. 2011, Camacho et al. 2011, Tanomand et al. 2013). Other PAMPs, namely LPS and polyinosinic:polycytidylic acid, a synthetic analog of double-stranded RNA which is present in some viruses, have been shown to induce behavioral changes if administered parentally (Sparkman et al. 2006, Frenois et al. 2007, Gibney et al. 2013, Farzi et al. 2015, Leite et al. 2016, Araki et al. 2016). In patients with diarrhea-predominant IBS the levels of antibodies to flagellin are increased and correlate with anxiety in IBS patients (Dlugosz et al. 2015). However, to the best of my knowledge flagellin-induced changes in emotional behavior have not been assessed in animal models. I hypothesized that flagellin is also able to induce behavioral changes in mice, hence, the effect of flagellin on anxiety-like and social behavior was assessed. Regardless of the bacterial species of origin, administration of flagellin did not induce any changes in anxiety-like and social behavior. To the best of my knowledge, this is the first report that flagellin is devoid of any impact on anxiety-like and social behavior. This is in contrast to other TLR agonists like LPS and polyinosinic:polycytidylic acid which have both been shown to increase anxiety- and depression-like behavior (Gibney et al. 2013, Sulakhiya et al. 2016) and, in the case of LPS, induce social interaction deficits (Araki et al. 2016). As a TLR5 agonist flagellin is able to initiate innate immune responses (Hayashi et al. 2001) which seem to depend on the type of flagellin (the bacterial species of origin or vector) (Vidhyasekaran 2016). The concentration of flagellin was chosen such that it should elicit a widespread immune response (Rolli et al. 2010, Sanders et al. 2006). Nevertheless, in my study the potential immune response to flagellin did not lead to any short- or long-term changes in behavior. Higher concentrations of flagellin were not used because any contaminations with, e.g., LPS would increase the risk of skewed behavioral responses due to LPS-induced sickness behavior. The TLR4 agonist LPS has been shown to induce a multitude of behavioral changes including increased anxiety-like behavior (Farzi et al. 2015). Even in large scale production the purification of PAMPs without

concomitant contamination with LPS can be rather challenging. Hence, a possible explanation for the increased anxiety-like behavior seen after administration of flagellin from *S. typhimurium* in the first experiment of this study is that this flagellin batch was contaminated with LPS. Very small concentrations of only 0.1 mg/kg LPS are already sufficient to induce an anxiety-like response (Farzi et al. 2015). In addition, LPS is difficult to remove from biological preparations (Magalhaes et al. 2007). As in cell culture experiments no synergistic effect of TLR4 and TLR5 activation has been observed (Makela et al. 2009), I speculate that it was LPS contamination alone rather than a synergistic effect that induced behavioral changes in the first experiment.

4.9 Conclusions

In conclusion, this study systematically characterized a mouse model of antibiotic-induced gut dysbiosis in its impact on the intestinal and circulating metabolite profile as well as brain function and behavior. Thus, the communication between the gut microbiota and the brain was analyzed at several levels of the gut-brain axis, using distinct molecular, biochemical and neurobiological methods. This analysis was complemented by the study of bacterial components that activate the innate immune system, such as flagellin, and their potential effects on anxiety-like and social behavior of adult C57BL/6N mice. The antibiotic-induced dysbiosis model was found to be associated by a significant disruption of the microbial community structure and load in the gut. Furthermore, dysbiosis altered the metabolite profile of the colonic content and plasma without inducing overt inflammation. Whereas anxiety- and depression-like behavior remained unaltered, antibiotic-induced dysbiosis impaired novel object recognition memory but had no impact on spatial memory. This deficit may be originating from changes in neuronal signaling molecules in the brain whose expression was markedly altered in antibiotic-treated mice. Based on pharmacokinetic measurements I conclude that the cognitive deficit and neurochemical alterations in the brain result from gut dysbiosis, but not from a direct action of the antibiotics in the brain. In addition, the changes in the metabolic profile and the cerebral expression of neuronal signaling molecules were to some extent similar to data found in GF mice. Thus, antibiotic-induced gut dysbiosis represents a short-term model with which to study the implication of the gut microbiota on brain function and behavior, a relationship in which a change of circulating metabolites may play a major role.

Further studies are required to elucidate the mechanism of bacterial depletion on cognition

and metabolism in greater detail. These studies will have to identify those components of the intestinal microbiota and the circulating metabolite profile that are of particular relevance to gut-brain signaling. Unlike LPS, the microbial compound flagellin was not able to alter anxiety-like and social behavior, which speaks against a role of this compound in the communication between the gut microbiota and the brain. Prebiotic and probiotic interventions would offer a possibility to investigate microbe-directed therapeutic effects in the current model. Taken together, the findings of this work indicate that an acute disruption of the gut microbial community in adult mice has a substantial impact on brain neurochemistry and function by changing the metabolome and the expression of neuronal signaling molecules of the host.

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6 Appendix

6.1 PCR programs for microbiome analysis

For microbiome sequencing the following PCR program was conducted with the 16S fusion primers F27 and R357 containing Ion Torrent sequencing adapters.

Step	Temperature [°C]	Time [min]	Cycles	Comment
Initial denaturation/incubation	95	05:00	1	
Denaturation	95	00:05	1	
Touchdown	65-55		50	Decrease of 1°C per 2 cycles
Melting curve	55-95		1	Increment of 1°C per 5 s
Final	25	05:00	1	

For qPCR of extracted bacterial DNA the following PCR program was conducted with the 16S primers F515 and R806.

Step	Temperature [°C]	Time [min]	Cycles	Comment
Initial denaturation/incubation	98	02:00	1	
Amplification	98 60	00:05 00:50	50	
Denaturation	95	00:15	1	
Final Extension	55	00:15	1	
Melting curve	55-95		1	Increment of 0.5°C per 5 s

6.2 PCR programs for mRNA (colon, brain)

RNA from colon and brain was reversed transcribed according to the following PCR program.

Step	Temperature [°C]	Time [min]	Cycles
Incubation	25	10:00	1
Amplification	37	120:00	1
Termination	85	00:05	1

Real-time reverse transcription PCR was performed according to the following PCR program.

Step	Temperature [°C]	Time [min]	Cycles
Uracil-DNA Glycosylase incubation	50	02:00	1
Pre-incubation	95	10:00	1
Amplification	95 60	00:15 01:00	40

Two types of negative controls (cDNA amplified without reverse transcriptase; water) were always included and remained negative (at least 7 cycles behind average sample cycle).

6.3 Protein extraction from colon

Frozen tissue was pulverized and suspended in ice-cold 500µl PBS (0.02 mol/L, pH 7.0-7.2). Suspensions were sonicated with the Bandelin Sonopuls (Bandelin, Berlin, Germany) four times for 10 seconds (10 cycles). Samples were centrifuged for 5 min at 5000 × g at 4°C. Protein concentrations of the supernatant were measured with NanoDrop (Peqlab). A quantity of 50 µg protein was used for the TNF-α ELISA, for all other ELISAs 25 µg of protein was utilized.

6.4 Microbiome analysis – data

Table 1 Raw data from microbiome analysis down to the taxonomic rank family

OTU	FDR_P	AB	VEH	Blank
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Microbacteriaceae	0,00117	0,00349	0,00000	0,00000
k__Bacteria;p__Cyanobacteria;c__Chloroplast; o__Streptophyta;f__	0,00117	0,01117	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria; o__Pseudomonadales;f__Pseudomonadaceae	0,00117	0,00817	3,33E-06	0,02819
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;Other	0,00117	0,00475	8,95E-06	0,00758
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Bradyrhizobiaceae	0,00117	0,00603	1,00E-05	0,00793
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Caulobacterales;f__Caulobacteraceae	0,00117	0,00399	3,33E-06	0,00264

k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Comamonadaceae	0,00117	0,14818	2,96E-05	0,04569
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Oxalobacteraceae	0,00117	0,61215	0,00018	0,85170
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Burkholderiaceae	0,00117	0,09422	1,29E-05	0,03593
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__S24-7	0,00139	0,06307	0,75879	3,89E-05
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;Other	0,00145	0,00041	0,00000	0,00019
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Xanthomonadaceae	0,00145	0,00074	0,00000	0,00140
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__[Paraprevotellaceae]	0,00149	0,00056	0,01048	0,00000
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__Rikenellaceae	0,00217	0,00169	0,02278	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; Other;Other	0,00375	0,00123	0,00000	0,00047
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Phyllobacteriaceae	0,00375	0,00107	0,00000	0,00132
k__Bacteria;p__Firmicutes;c__Clostridia; o__Clostridiales;f__	0,00619	0,00865	0,13514	0,00000
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__Prevotellaceae	0,00764	0,00184	0,01952	0,00000
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Burkholderiales;f__Alcaligenaceae	0,00764	0,00655	0,00015	0,01108
k__Bacteria;p__Actinobacteria;c__Coriobacteriia; o__Coriobacteriales;f__Coriobacteriaceae	0,00764	0,00032	0,00195	0,00000
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__Bacteroidaceae	0,00915	0,00041	0,00244	0,00202
k__Bacteria;p__Firmicutes;c__Clostridia; o__Clostridiales;f__Lachnospiraceae	0,00937	0,00165	0,01348	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Lactobacillales;f__Lactobacillaceae	0,01388	0,00279	0,02856	0,00000
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Nocardiaceae	0,01388	0,00067	0,00000	0,00000
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;f__[Odoribacteraceae]	0,01479	0,00024	0,00136	0,00000
k__Bacteria;p__Bacteroidetes;c__Bacteroidia; o__Bacteroidales;Other	0,01958	6,78E-05	0,00028	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Bacillales;f__Staphylococcaceae	0,02761	0,00021	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Brucellaceae	0,02761	0,00086	0,00000	0,00000
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Propionibacteriaceae	0,03129	0,00024	0,00000	0,00105
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria; o__Xanthomonadales;f__Sinobacteraceae	0,03660	0,00040	0,00000	0,00047
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Sphingomonadales;f__Sphingomonadaceae	0,03767	0,00051	0,00000	0,00148
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria; o__Methylophilales;f__Methylophilaceae	0,03767	0,00016	0,00000	0,00031
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Corynebacteriaceae	0,03809	0,00060	0,00000	0,00051
k__Bacteria;p__Firmicutes;c__Clostridia; o__Clostridiales;f__Ruminococcaceae	0,05530	0,00057	0,00336	0,00000
k__Bacteria;p__Deferribacteres;c__Deferribacteres; o__Deferribacterales;f__Deferribacteraceae	0,06008	7,71E-05	0,00049	0,00000
k__Bacteria;p__Cyanobacteria;c__4C0d-2;	0,06246	0,00013	0,00019	0,00000

o__YS2;f__				
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Pseudonocardiaceae	0,09177	0,00046	0,00000	0,00000
k__Bacteria;p__Tenericutes;c__Mollicutes; o__Anaeroplasmatales;f__Anaeroplasmataceae	0,09553	3,67E-05	0,00013	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Bacillales;f__Bacillaceae	0,15849	0,00022	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Rhizobiaceae	0,15849	0,00032	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria; o__Legionellales;f__	0,15849	0,00014	0,00000	0,00000
k__Bacteria;p__Firmicutes;c__Clostridia; o__Clostridiales;Other	0,19185	0,00015	0,00065	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Lactobacillales;Other	0,26181	0,00113	0,00000	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Lactobacillales;f__Enterococcaceae	0,26181	0,00014	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Methylobacteriaceae	0,26181	0,00024	0,00000	0,00000
k__Bacteria;p__Actinobacteria;c__Actinobacteria; o__Actinomycetales;f__Streptomycetaceae	0,41484	0,00017	0,00000	0,00000
k__Bacteria;p__Firmicutes;c__Bacilli; o__Turicibacterales;f__Turicibacteraceae	0,41484	0,00766	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__	0,41484	0,00021	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Xanthobacteraceae	0,41484	0,00021	0,00000	0,00000
k__Bacteria;p__Cyanobacteria;c__4C0d-2; o__MLE1-12;f__	0,55500	0,00039	6,65E-06	0,00000
k__Bacteria;p__Firmicutes;c__Clostridia; o__Clostridiales;f__Clostridiaceae	0,63234	0,00031	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhizobiales;f__Beijerinckiaceae	0,63234	0,00023	0,00000	0,00000
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria; o__Rhodospirillales;f__Acetobacteraceae	0,63234	9,90E-05	0,00000	0,00000

For the columns antibiotic-treated (AB), vehicle-treated (VEH) mice and blank the values are means. OTU, operational taxonomic unit; FDR_P, false discovery rate adjusted *p*-values; k, kingdom; p, phylum; c, class; o, order; f, family.

6.5 Plasma metabolomics – data

Table 2 Raw data from plasma metabolomics

Metabolite name	AB	VEH	ANOVA
1-Methylhistidine	6586129,406	14396966,46	0,33974
3-methyl-2-oxovaleric acid	1840418456	2260447336	0,23727
Acetylglycine	251081303,2	224707501,7	0,77076
ADMA	38875575,98	33663694,84	0,88191
Alanine	19393137,61	32943118,48	0,55159
Alpha-aminobutyric acid	5117933,668	6652290,826	0,51427
Arginine	1280547781	1076909586	0,47347
Aspartic acid	1469183,634	1892590,681	0,77076
Citrulline	34673839,59	31893906,96	0,65453
Creatine	1417699905	1418198299	0,88053
Creatinine	1954458707	2223015872	0,77076
Glutamic acid	57574670,42	58754024,59	0,86285

Glutamine	244234047,7	394584275	0,40844
Histidine	155906982,8	280185234,5	0,50191
Iso-/Leucine	12041574789	11062892626	0,77076
Kynurenine	27566366,39	19884063,73	0,45632
Lysine	737903835,7	781474194,2	0,90503
Methionine	480594783,9	517764287	0,69383
Ornithine	82783246,75	62092753,79	0,55159
Phenylalanin	4311796480	3632187900	0,59822
Pyroglutamic acid	95341678,5	166530133,4	0,38288
Serine	22234243,45	32438571,24	0,55058
Taurine	289182014,6	317278166,1	0,68242
Threonine	14560059,15	21350522,4	0,77076
Tryptophan	1882968031	1456112111	0,23727
Tyrosine	90645663,89	82615064,28	0,77076
Valine	54641856481	44918746444	0,81169
1,5-Anhydrosorbitol	1747322653	2111872193	0,89236
6-Phosphogluconic acid	5874156,779	4216110,058	0,44148
Gluconic acid	44934263,27	63044998,01	0,77076
Glucuronic acid	6240161,77	10727196,58	0,23727
Glyceraldehyd	2793563175	3412164721	0,80672
Glyceric acid	9852482,49	12347383,95	0,55058
Glycerol	47020280,93	54216321,09	0,64024
Hexose	19135256337	23490066729	0,77076
Hexose mono-phosphate	9764317,838	9984095,957	0,88053
Hydroxybutyric acid, a-Hydroxyisobutyric acid	127015455,6	167547118,7	0,55159
Mannitol, Sorbitol	75378389,93	57251742,73	0,37670
Pentose	242735974,4	307369747,9	0,77076
Trehalose	2092863,627	4275513,185	0,77076
2-Ketobutyric acid	624175613,7	676169405	0,77076
Citric acid	3,62866E+11	2,79345E+11	0,50910
Fumaric acid	74605760,3	78584754,45	0,89236
Glyceryl phosphate	19266605,33	26868350,99	0,32801
Lactic acid	12707279867	16783557832	0,43033
Malic acid	857952492,5	885217918,2	0,96877
Oxoglutaric acid	152797792	136911601,7	0,69560
Phosphocreatine	19081976,68	16355153,08	0,83061
Caprylic acid	101304195,3	86679169,07	0,95849
Eicosapentanoic acid	90050254,83	54611678,51	0,06114
FFA C12:0	122119961,7	140708239,9	0,44148
FFA C12:1	29086743,38	44715104,37	0,13013
FFA C12:2	18470007,48	20542617,1	0,88053
FFA C14:0	730162188,8	766749690,1	0,55821
FFA C14:1	143753995,3	180260262,8	0,43033
FFA C14:2	93167711,56	140798889,6	0,26377
FFA C15:0	106555429,8	94467071,97	0,80610

FFA C15:1	11736230,9	13252146,24	0,52802
FFA C16:0	5918523300	5534608419	0,88053
FFA C16:1	1721267607	2294567904	0,52802
FFA C16:2	46115914,14	44431774,85	0,83061
FFA C16:3	24552812,45	23300233,16	0,88893
FFA C17:0	90049200,99	64071485,13	0,64024
FFA C17:1	57216327,27	47289802,63	0,93787
FFA C18:0	1261949020	903504202,1	0,06114
FFA C18:1	3247690915	3613495660	0,65150
FFA C18:2	3832234982	3261027817	0,88893
FFA C18:3	547425134,8	490689263,9	0,89415
FFA C18:4	8431022,297	9580714,96	0,89236
FFA C19:0	9843027,016	9400970,383	0,90503
FFA C19:1	9041875,727	8695991,766	0,90503
FFA C20:0	22020820,78	21448832,94	0,88053
FFA C20:1	88188605,44	97520458,17	0,55428
FFA C20:2	51638269,83	47376004,39	0,90503
FFA C20:3	81844081,01	73023145,06	0,81945
FFA C20:4	467239696	331332502,6	0,25469
FFA C22:6	372938617,8	330466069,6	0,75967
Phytanic acid	22020820,78	21448832,94	0,88053
Valeric acid	14085486,88	10597851,66	0,33678
Deoxycholic acid, Chenodeoxycholic acid		15068199,17	
LPC 16:0	1621090327	2042301201	0,64024
LPC 16:1	114855443,1	142262368,6	0,33974
LPC 18:1	513635668,2	693593367,9	0,29345
LPC 18:2	1810495369	1954984779	0,81150
LPC 18:3	173467128,7	223118361	0,33974
LPC 20:2	9317148,651	11868292,51	0,64024
LPC 20:3	122368882,3	179033350,7	0,03529
LPC 20:4	710279195,1	802402862,6	0,77076
LPC 20:5	212401761,2	207805627,7	0,86285
LPC 22:5	24521922,84	23009178,06	0,58854
LPC 22:6	190626754,3	266432545,2	0,33974
LPE 16:0	124141008,6	149388580,6	0,50665
LPE 18:0	81993551,09	70651727,98	0,81817
LPE 18:1	63337229,16	59648848,6	0,92065
LPE 18:2	172279852,4	134945700,3	0,33974
LPE 20:4	293323787,2	278533590,6	0,88053
LPE 20:5	40823816,68	32136853,02	0,33974
LPE 22:5	6573038,732	7162430,965	0,77076
LPE 22:6	206097075,6	220799951,4	0,38415
PC 32:0	192027780,8	154132637,7	0,32071
PC 32:1	158206884,8	189840771,6	0,64024
PC 32:2	48087498,01	51678966,06	0,90503

PC 32:4	24208589,9	21107644,94	0,48160
PC 34:1	5188760152	7187776437	0,24986
PC 34:3	646247089,2	591646985,1	0,42464
PC 36:2	9534717585	7438395051	0,01858
PC 36:3	2793480286	2455613328	0,33678
PC 36:4	4879379828	4545334146	0,37670
PC 36:5	631936063,3	595771241,2	0,26377
PC 38:3	847205505,4	772976313	0,43033
PC 38:4	4311459299	3058693830	0,08476
PC 38:5	1388909491	949944173,3	0,02467
PC 38:6	3960627415	3736469331	0,81945
PC 38:7	266976487,6	239188344,2	0,25469
PC 40:4	40334515,53	35493803,67	0,38288
PC 40:5	222298754,2	177087924,8	0,03529
PC 40:6	920276622,8	877946676,6	0,47347
PC 40:7	549766903,1	446455911,2	0,22633
PC 40:8	188474694,6	119344344,8	0,01844
PE 34:2	4684946,161	8755709,555	0,65150
PE 36:2	44808917,86	35541440,81	0,31443
PE 36:3	6410412,881	4964946,91	0,88053
PE 36:4	53649320,88	71948343,36	0,08476
PE 38:2	319542975,8	218533993,5	0,08476
Phosphocholine	36073618,98	37401884,75	0,89081
PI 34:2	38053130,71	32783607,33	0,44148
PI 36:3	34597551,41	31300268,41	0,77076
PI 36:4	134015341,2	130111464,1	0,72730
PI 38:3	101742778	92297947,4	0,72730
PI 38:4	692654297,2	672403944,8	0,86285
PI 38:5	66139036,85	44688054,55	0,04231
PS 38:0	133013913,5	173778399,5	0,55428
PS 38:1	746731272,1	765858669,1	0,88053
PS 40:2	110724335,6	99566189,89	0,36038
PS 40:3	291083320	287183308,6	0,64024
SM 34:1	396616687,1	285902029,8	0,00467
SM 34:2	68903469,42	59698889,66	0,80672
SM 36:1	17123766,71	8623782,972	0,24986
Taurocholic acid	451731811	104911457,5	0,86285
Taurodeoxycholate, Taurochenodeoxycholate	19860038,49	49499543,66	0,32801
Adenine	13858575,32	23055449,27	0,33974
Adenosine	167709822,5	169260172,8	0,89081
ADP	6703498,617	743347,6409	0,65150
Allantoin	3083024299	3625970481	0,77076
ATP	1786026,334	121582,273	0,52711
Cytidine	165637864,7	164717900,2	0,84390
Cytosine	242627989,8	247164729,1	0,88053

Deoxyuridine	38197102,8	62518138,69	0,29345
Guanine	11399267,25	16534868,76	0,10919
Inosine	7153952,329	16884397,15	0,26377
Oxypurinol	893974,9686	518069,1064	0,89081
Thymidine	22169034,47	22048044,01	0,81945
Thymine	75401310,92	79091752,43	0,90503
Uracil	478410045,7	457685395,7	0,90503
Uric acid	238034071,8	203358160,6	0,65150
Uridine	484101299,6	495138204,7	0,92065
2-Oxovaleric acid	394134883,3	485718674,7	0,33678
Bilirubin	14869240,31	17262318,66	0,43033
Corticosterone	27472639,37	4629872,791	0,04231
N1-Acetylspermine	33328727,24	23904711,23	0,77076
p-Cresyl glucuronide	28178140,5	25236766,29	0,88053
p-Cresyl sulphate	11803241,18	104453505,8	0,00000
Thymol	10832905,74	6602614,443	0,82958
Trimethylamin-n-oxid	398352,2601	36136393,45	0,00000
Alpha-Tocopherol	5391309,117	3174374,035	0,25469
Ascorbic acid	618582,071	1494564,421	0,33974
Carnitine	934929768,4	1378107133	0,06560
Niacinamide	1687948888	1314620134	0,77076
Palmitoylcarnitine	28687838,02	30398645	0,64024
Riboflavin	5682591,979	5240394,517	0,88053

For the columns antibiotic-treated (AB) and vehicle-treated (VEH) mice values are medians. ANOVA *p*-values were adjusted according to Benjamini-Hochberg. ANOVA, analysis of variance.