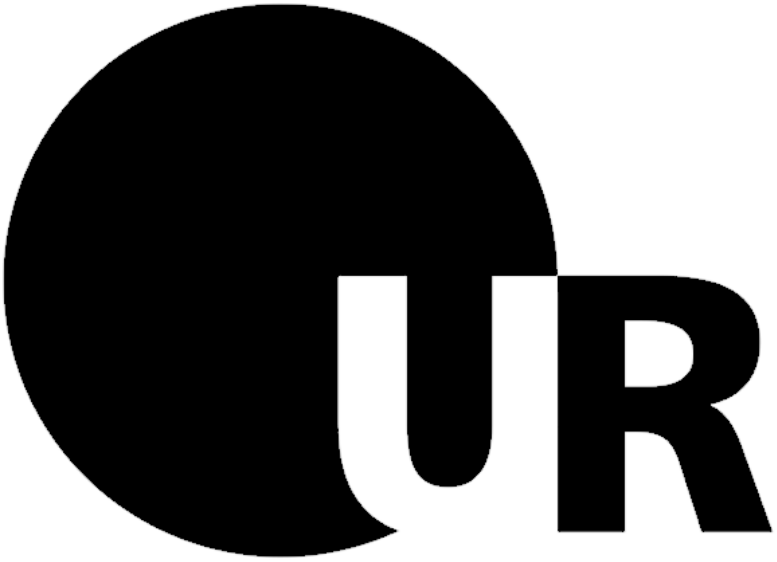
New views on an old antibiotic: Effects of minocycline on innate *versus* stress-induced behavioural, immunological, and microbiome changes



DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

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Dissertation

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Für alle meine Lieben

“Ui schauts – A Doktorarbeit”

Summary

Excessive states of sensations like fear, anxiety, risk assessment, and others, and the resulting psychiatric disorders, render humans incapable of directing their own life. Psychiatric disorders like generalized anxiety disorder (GAD) and major depressive disorder (MDD) are the sixth and the leading cause of disability worldwide, respectively, and represent a major burden for both patients and society. The risk for the development of these disorders is twice as high in women as in men and the available treatment options, while effective for many, are imperfect. In fact, 30 % of patients are classified as treatment-resistant. Unfortunately, the underlying causes and mechanisms remain largely unknown, which has impeded the development of new drugs. In recent years, several studies showed a regulatory role of commensal gut microbiota on the development of CNS functioning and behaviour, which has led to the concept of a microbiota-gut-brain (MGB) axis. This describes a network of different systems as modulator for behaviour and its dysregulation as a cause for psychiatric disorders. In addition, a dysregulation of the peripheral immune system and the immune system of the brain, especially of microglia, was linked in numerous clinical and preclinical studies to the development of psychiatric disorders like GAD or MDD. The well-established link between gut microbiota and the immune system, as well as the role of the immune system in the MGB axis, introduced a complex interplay of systems whose deciphering and specific manipulation could give rise to novel treatment targets in psychiatric disorders. Hence, current research needs to concentrate on a different rationale to meet the medical necessities, ranging from augmentation strategies of available medication to innovative approaches directly targeting the immune system and / or the MGB axis. The recent discovery of pleiotropic effects of the second-generation tetracycline antibiotic minocycline not only on bacteria, but also on the immune system, CNS functioning, and behaviour, gave rise to a promising multimodal approach as a novel treatment. Therefore, using two different animal models for psychiatric disorders, selective breeding and chronic psychosocial stress, the present thesis aimed to identify the potential beneficial effects of minocycline. First, the influence of minocycline on a model of innate anxiety- and depressive-like behaviour, rats selectively bred for high anxiety-like behaviour (HAB), was analysed in comparison to rats non-selected for anxiety-like behaviour (NAB). In a battery of behaviour tests, the behavioural effects of minocycline, the selective serotonin reuptake inhibitor escitalopram, or a combination of both substances as augmentation regimen, on male and female HAB rats were characterized. To unravel potential underlying mechanisms associated with the immune system and / or the MGB axis, these tests were followed by the analysis of microglial density in the prelimbic and infralimbic prefrontal cortex (PFC), brain regions highly affected in MDD, and cecal microbiota composition. The obtained results demonstrate that under untreated conditions, HAB rats display a sustained highly anxious and depressive behavioural phenotype irrespective of sex. Male and female HAB rats also showed a reduced microglial density in the PFC and altered gut microbial composition compared to NAB rats independent of treatment. Three weeks of minocycline treatment alleviated the depressive-, but not anxiety-like, phenotype and further reduced microglial density in the

PFC exclusively in male HAB rats, while female HAB rats did not respond to the treatment. Escitalopram was able to decrease anxiety-like behaviour in the light-dark box in male NAB rats only, while the combinatory treatment did not result in any behavioural effect. Moreover, minocycline reduced plasma cytokine concentrations and induced a robust shift in gut microbiota composition in both HAB and NAB males. Detailed taxonomic analysis revealed a marked increase in the relative abundance of Lachnospiraceae and Clostridiales Family XIII, two bacterial families known to produce butyrate. Correspondingly, plasma concentrations of 3-OH-butyrate were elevated and positively correlated with the respective bacterial abundance in a trait-dependent manner. Thus, these results validate HAB rats for treatment-resistant to conventional antidepressants, as well as inflammation-associated, depressive-like behaviour and suggest that the antidepressant effect of minocycline is sex- and trait-dependent. The multimodal effects of minocycline also support the hypothesis of the MGB axis being a potential target in the treatment of MDD.

In addition, the present thesis aimed to evaluate the influence of minocycline on stress-induced behavioural and physiological alterations in comparison to innate behaviour. This model provides a different approach to psychiatric disorders and allows to better identify the mode of action of minocycline. Humans face stressors daily that comprise both a chronic and psychosocial component, the type of stressor that represents the most acknowledged risk factor for psychiatric and somatic disorders. The chronic subordinate colony housing (CSC) paradigm is a mouse model of chronic psychosocial stress that closely mimics those challenges and induces harmful behavioural, physiological, and immunological changes. The fact that the CSC paradigm specifically induces anxiety-, but not depressive-like, behaviour, offers a unique opportunity to dissect distinct effects of minocycline, including innate *versus* stress-induced anxiety. However, as the laboratory where the experiments were conducted relocated to a new facility, it was crucial to reproduce a valid and robust CSC-induced phenotype. This was confirmed by elevated anxiety-like behaviour, and increased adrenal, but decreased thymus, weight in male CSC mice. Based on these results, a potential beneficial effect of minocycline on stress-induced behavioural and physiological symptoms in the newly validated CSC paradigm was evaluated. Eight days after stressor termination, CSC mice showed robust stress-induced anxiety-like behaviour as well as increased spleen weight and an altered HPA axis activity compared to single-housed controls. Interestingly, minocycline was not able to ameliorate CSC-induced symptoms. Hence, under the applied treatment conditions, the CSC-induced behavioural and physiological phenotype appears to be independent of any target of minocycline. However, time- or dose-dependent effects need to be examined in future studies.

Overall, my findings provide a closer insight into the interplay between the immune system, gut microbiota, and behaviour in two different models of psychiatric disorders. They further extend the knowledge about specific treatment conditions and a distinct pattern of action for minocycline. On a behavioural level, minocycline showed a specific antidepressant, but not anxiolytic, effect in male HAB

rats. In line with the available literature, these results indicate that the sex-dependent antidepressant effect of minocycline requires an *a priori* depressive-like phenotype. In addition, minocycline was suggested as anxiolytic after acute stress or trauma. Here, an anxiolytic effect could not be confirmed in the two animal models of anxiety-like behaviour. These findings propose that minocycline had no or only a delayed effect on innate or chronic stress-induced anxiety-like behaviour. Thus, my experimental data demonstrate that minocycline requires specific conditions for a behavioural effect and acts only antidepressant under the applied experimental conditions. Further, minocycline is proposed to exert its mechanism of action *via* its anti-inflammatory effects. Fittingly, male HAB rats showed an inflammatory phenotype that was altered by minocycline. Female HAB rats expressed a similar inflammatory phenotype, though, and the CSC procedure is known to induce a strong inflammatory response. The high inflammatory phenotype in CSC mice did not facilitate a behavioural effect of minocycline, indicating that the mechanism of action of minocycline is not based in a pro-inflammatory status *per se*. Thus, minocycline might require inflammation-induced depression for a behavioural effect.

In summary, the results garnered from my thesis advance the knowledge about the behavioural influence of minocycline and show a specific effect on depressive-like behaviour dependent on sex, the inflammatory state, and the potential underlying cause of the disease. A future application of minocycline in psychiatric disorders needs close consideration of the patients' circumstances and emphasizes that minocycline represents rather a promising medication of tailored and personalized treatment than as a broad-spectrum antidepressant.

Zusammenfassung

Die Basis psychischer Erkrankungen sind grundlegende und evolutionär essentielle Emotionen, die in einem exzessiven und unangepassten Zustand ein pathologisches Ausmaß annehmen. Dadurch sind Patienten oftmals nicht mehr in der Lage, ihr Leben in geregelten Bahnen zu führen. Die generalisierte Angststörung (GAD) und Depressionen (MDD) zählen zu den wichtigsten Gründen für Erwerbsunfähigkeit, wobei GAD den sechsten und MDD den führenden Platz als Ursache weltweit einnimmt. Dadurch sind diese Krankheiten nicht nur verheerend für den Patienten, sondern haben auch großen Einfluss auf die Gesellschaft. Das Risiko, eine psychische Erkrankung zu entwickeln, ist in Frauen beinahe doppelt so hoch wie in Männern. Trotz der Verfügbarkeit verschiedener Medikamente mit erwiesenen Wirkung in vielen Patienten, reagieren 30 % der Patienten nicht auf die Medikation und werden letztendlich als behandlungsresistent eingestuft. Da die grundlegenden Mechanismen und die Ursachen psychischer Krankheiten weitestgehend unbekannt sind, ist die Entwicklung neuer Medikamente stark eingeschränkt. In den letzten Jahren wurden erstaunliche Fortschritte in der Erforschung zweier verschiedener Systeme erzielt. Die Bakterien der Darmflora, genannt Darm Mikrobiota, haben ungeahnten Einfluss auf das zentrale Nervensystem und das Verhalten. Dies führte zu der Begründung eines neuen Konzepts, der Mikrobiota-Darm-Hirn Achse, welche das Verhalten beeinflusst und, im Falle einer Dysregulation, zur Entwicklung psychischer Erkrankungen beitragen kann. Zudem wurde in einigen Patienten eine Fehlregulation des peripheren Immunsystems sowie des Immunsystems im Gehirn – insbesondere bei Mikroglia – festgestellt, die in unmittelbarem Zusammenhang zu psychischen Erkrankungen wie GAD oder MDD stehen. Das Zusammenspiel zwischen der Mikrobiota-Darm-Hirn-Achse und dem Immunsystem ist allgemein anerkannt und eröffnet ein komplexes System und Wechselspiel, dessen Entschlüsselung und spezifische Manipulation einen neuen Ansatz für Therapiemöglichkeiten bietet. Daher konzentriert sich die aktuelle Forschung auf die Entwicklung neuer Behandlungsstrategien. Diese decken ein weites Spektrum, beginnend bei dem Add-On von Substanzen zu gängigen Antidepressiva bis zur Entwicklung neuer Medikamente mit der Mikrobiota-Darm-Hirn Achse sowie dem Immunsystem als potentielles Ziel, ab. Kürzlich wurde bei einem herkömmlichen Antibiotikum eine Reihe von zusätzlichen, pleiotropen Funktionen entdeckt. Minozyklin ist ein Tetrazyklin der zweiten Generation, das neben seiner antibakteriellen Funktion auch das Immunsystem, das Gehirn und Verhalten beeinflusst und demzufolge mit seinem multimodalen Wirkungsspektrum einen neuen, vielversprechenden Ansatz für die Behandlung psychischer Erkrankungen bietet. Daher war es Ziel der vorliegenden Doktorarbeit, den Einfluss auf Minozyklin in zwei verschiedenen Tiermodellen für psychische Krankheiten, selektive Zucht und chronisch psychosozialer Stress, zu untersuchen. Zunächst wurde die Wirkung von Minozyklin auf verschiedene Verhaltensweisen in einem Tiermodell für angeborenes hohes Angstverhalten und depressions-ähnliches Verhalten, Ratten die selektiv auf hohes Angstverhalten gezüchtet wurden (HAB), untersucht. Um geschlechtsspezifische Effekte zu zeigen, wurde dies auf weibliche HAB Ratten und als Kontrolle auch auf männliche und weibliche Ratten, die nicht aufgrund ihres Verhaltens selektiert wurden (NAB),

ausgeweitet. Dazu wurde Escitalopram, ein selektiver Serotonin Wiederaufnahmehemmer, als Positivkontrolle eingesetzt, sowie eine Kombination beider Substanzen, da in Patienten Minozyklin nicht als Monotherapie, sondern als Add-On Medikation genutzt wird. Um mögliche grundlegende Wirkungsmechanismen von Minozyklin zu identifizieren, wurde sowohl die Anzahl der Mikroglia im infralimbischen und prelimbischen prefrontalen Cortex (PFC) ermittelt, einer Gehirnregion, die stark von MDD beeinflusst ist, als auch die Zusammensetzung der Darm Mikrobiota untersucht. Die vorliegenden Ergebnisse demonstrieren einen stabilen Phänotyp in unbehandelten männlichen und weiblichen HAB Ratten und eine reduzierte Dichte an Mikroglia im PFC in beiden Geschlechtern im Vergleich zu NAB Ratten. Zudem zeigen männliche HAB Ratten grundlegend eine andere Zusammensetzung der Darmflora als NAB Ratten. Die Behandlung der Tiere mit Minozyklin für 3 Wochen erzielte eine Verbesserung des depressions-ähnlichen Verhaltens, jedoch nicht des Angstverhaltens, und eine Reduzierung der Mikroglia Anzahl exklusiv in männlichen HAB Ratten, während weibliche HAB Ratten nicht auf die Behandlung reagierten. Escitalopram verringerte das Angstverhalten von männlichen NAB Ratten, während die Kombination beider Substanzen keinerlei Effekte zeigte. Zudem reduzierte Minozyklin die Konzentration zweier pro-inflammatorischen Zytokine im Plasma und veränderte merklich die Darm Mikrobiota Zusammensetzung in männlichen HAB und NAB Ratten. Eine detaillierte Analyse der Taxonomie zeigte, dass Minozyklin die relative Häufigkeit der beiden Butyrat-produzierenden Bakterienfamilien Lachnospiraceae und Clostridiales Familie XIII erhöhte. Diese Familien sind bekannt für ihre Produktion des Metabolits Butyrat. Dem entsprechend konnten auch erhöhte Plasma 3-OH-Butyrat Konzentrationen festgestellt werden, die mit der jeweiligen Bakterienhäufigkeit in Abhängigkeit des Phänotyps positiv korrelierten. Diese Ergebnisse validieren HAB Ratten nicht nur als Modell für behandlungsresistente Angsterkrankungen und MDD, sondern auch als Modell für entzündungs-assoziierte MDD, und implizieren einen geschlechts- und phänotyp-abhängigen Effekt von Minozyklin. Die multimodale Manipulation von Minozyklin und ihr Behandlungserfolg in HAB Ratten unterstreicht zudem die Mikrobiota-Darm-Hirn-Achse als potentielles Ziel für neue Therapieansätze in psychischen Erkrankungen.

Zudem war es Ziel der vorliegenden Doktorarbeit, den Einfluss von Minozyklin auf stress-induziertes Verhalten und auf die Physiologie als Vergleich zu angeborenem Verhalten zu untersuchen. Das genutzte Modell stellt eine alternative Herangehensweise für psychische Krankheiten dar und ermöglicht somit eine Erweiterung des Wissens über Minozyklin und seine Wirkungsweise. Es ist heutzutage allgemein anerkannt, dass Stress mit einer chronischen und psychosozialen Komponente ein starker Risikofaktor für die Entwicklung von stressbedingten somatischen und psychischen Erkrankungen wie MDD und GAD ist. Das Mausmodell der chronisch subordinierten Koloniehaltung (CSC) ist ein geeignetes Tiermodell, das verhaltensbedingte, physiologische und immunologische Veränderungen in Mäusen induziert, die vergleichbar mit den psychischen, somatischen und / oder gastrointestinalen Erkrankungen bei chronisch gestressten Menschen sind. Das CSC Modell erhöht zudem spezifisch das Angstverhalten, aber nicht das

depressions-ähnliche Verhalten in den Mäusen. Daher ermöglicht dieses Modell, die Wirkung von Minozyklin spezifisch auf Angstverhalten zu untersuchen, insbesondere im Vergleich von Stress-induziertem und angeborenem Angstverhalten. Als Voraussetzung für diese Versuche war es essentiell, das CSC Modell nach dem Umzug der Verhaltenslaboratorien in ein neues Forschungsgebäude neu zu etablieren und einen validen und robusten Phänotyp zu reproduzieren. Dieser Phänotyp wurde durch erhöhtes Angstverhalten, vergrößerte Nebennieren und einen verkleinerten Thymus bestätigt. Basierend auf dieser Grundlage wurden danach Mäuse, die für 20 Tage dem CSC Modell ausgesetzt waren, mit Minozyklin behandelt. 8 Tage nach Beendigung des CSC Modells zeigten die Mäuse noch immer ein erhöhtes Angstverhalten sowie ein erhöhtes Gewicht der Milz und eine fehlregulierte Aktivität der HPA-Achse. Minozyklin war nicht in der Lage, die stress-induzierten physiologischen und verhaltensbedingten Veränderungen zu verbessern. Dies deutet darauf hin, dass der CSC-induzierte Phänotyp unabhängig von einem Wirkungsbereich von Minozyklin entsteht, wobei aber nicht ausgeschlossen werden kann, dass eine längere Behandlungsdauer und eine höhere Minozyklin Dosis andere Effekte zeigen würde.

Somit konnte die vorliegende Doktorarbeit einen detaillierten Einblick in die Wechselwirkungen zwischen dem Immunsystem, den Darm Mikrobiota, und Verhalten liefern und enthüllte spezifischere Wirkungsbedingungen für Minozyklin in zwei verschiedenen Tiermodellen für psychische Erkrankungen. Betrachtet man die Verhaltensergebnisse, zeigen die vorliegenden Versuche einen spezifischen Effekt von Minozyklin auf depressions-ähnliches Verhalten, aber nicht Angstverhalten, in männlichen HAB Ratten. Diese Ergebnisse implizieren, gemeinsam mit der aktuell bekannten Literatur, einen geschlechtsabhängigen Effekt von Minozyklin, sowie die Notwendigkeit einer vorliegenden Verhaltensstörung. Zusätzlich wurde eine angstlösende Wirkung von Minozyklin vorgeschlagen. Dies basierte auf Studien, die akuten Stress oder physiologisches Trauma nutzten. Da die Ergebnisse der vorliegenden Doktorarbeit keinen angstlösenden Effekt von Minozyklin nachweisen konnten, impliziert dies einen verzögerten oder sogar keinen Einfluss von Minozyklin auf Angstverhalten, das durch chronischen Stress verursacht wurde oder angeboren ist. Der Verhaltenseffekt von Minozyklin ist allgemein postuliert als eine Auswirkung der anti-inflammatorischen Wirkung des Antibiotikums. Männliche HAB Ratten zeigen ein verändertes Immunsystem im Gehirn und reagieren auf eine Minozyklin Behandlung. Allerdings zeigen auch weibliche HAB Ratten ein verändertes Immunsystem und das CSC Modell ist bekannt für seine immun-modulatorischen Effekte. Daher deuten diese Ergebnisse darauf hin, dass Minozyklin für seine Wirkung nicht *per se* ein verändertes Immunsystem als Voraussetzung benötigt, sondern auch hier spezielle Bedingungen notwendig sind. Minozyklin könnte daher einen entzündungs-induzierten depressiven Phänotyp für einen Behandlungseffekt benötigen.

Zusammenfassend erweitern die gesammelten Ergebnisse der vorliegenden Arbeit das Wissen über das Wirkungsspektrum von Minozyklin auf Verhalten und zeigen einen spezifischen antidepressiven Effekt, der geschlechtsabhängig ist und auf dem inflammatorischen Status sowie der potentiell

zugrundeliegenden Ursachen beruht. Eine zukünftige Anwendung von Minozyklin bei Patienten mit psychischen Erkrankungen muss sorgfältig durchdacht werden, da Minozyklin vielmehr als ein Medikament für individuelle, patientenbezogene Behandlung, denn als ein allgemeines Antidepressiva erscheint.

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Introduction

1.1 Psychiatric disorders

"*If the human brain were so simple that we could understand it, we would be so simple that we couldn't*"
– Emerson M. Pugh; Quoted by George E. Pugh 1977, The Biological Origin of Human Values

The complexity of the brain is what makes us human – it shapes personality and emotions and determines whom we are. Unfortunately, that very complexity prevents us from understanding how exactly the brain forms a certain behaviour. Scientific research has tried to unravel the underlying networks for human emotions for centuries. However, the output seems sparse compared to the effort. This statement is particularly pertinent for diseases of the brain, and a greater understanding is crucial, not only regarding neurological aberrations, but in terms of psychiatric disorders. Most psychiatric disorders are characterized by emotional and cognitive dysfunctions. Therefore, a unified classification defined mental disorders as significant disturbances of cognition, emotional regulation, or behaviour that reflect a dysfunction in the developmental, biological or psychological process underlying mental functioning and impair daily life. This is often accompanied by distress or disability in social or occupational activities (American Psychiatric Association, 2013). Today, the global lifetime prevalence of mental disorders is estimated between 12.2 % and 48.6 % in adults. 17.4 % of global years lived with disability (YLDs) are attributed to psychiatric disorders. Mental disorders encompass a plethora of diseases that are divided in different categories like psychotic, neurodevelopmental, substance-related, mood or anxiety disorders, to only name a few, the two latter being discussed in more detail below. Additionally, mental disorders show a high degree of comorbidity. Approximately 30 % of patients suffer from a second comorbid mental disorder and about 18 % experience three or more disorders at the same time (American Psychiatric Association, 2013; GBD 2015 Disease and Injury Incidence and Prevalence Collaborators, 2016).

1.1.1 Major depressive disorder

Recently, major depressive disorder (MDD) has been described as the "cancer of the 21st century" (Holden, 2000). Fittingly, despite the fact that the WHO in 2012 estimated that MDD would become the second leading cause of disability by 2020; by 2017 it was already classified as leading cause of disability worldwide (World Health Organization, 2012, 2017). MDD is categorized as a depressive disorder by the *Diagnostic and Statistical Manual of Mental Disorders 5* (DSM-5; for other depressive disorders see Fig. 1). Depressive disorders are generally characterized by sadness, feeling of emptiness or irritable mood, and loss of interest and pleasure (anhedonia), accompanied by somatic and cognitive changes. The division into the different subtypes is dependent on duration, timing, presumed aetiology, as well as severity (American Psychiatric Association, 2013).

As the psychiatric disorder with the highest lifetime prevalence, MDD occurs in approximately 17 % of the population (Kessler *et al*, 2005) with a genetic heritability of about 40 % (American Psychiatric Association, 2013). In university students, the prevalence of MDD is even higher at roughly 30.5 % (Ibrahim *et al*, 2013). Globally, about 322 million people suffer from MDD (12 % in the European region) and depression was ranked as single largest contributor to YLDs (7.5% in 2015) as well as a major contributor to suicide of approximately 800 000 per year (American Psychiatric Association, 2013; World Health Organization, 2017). Importantly, a strong sex difference can be found with approximately 1.5- to 3-fold higher risk to develop MDD in women (American Psychiatric Association, 2013; Duman *et al*, 2016). MDD is characterized by pronounced changes in affective, cognitive, and neuro-vegetative functions for at least 2 weeks nearly every day either as single episode (rare) or recurrent as in the majority of cases. The underlying aetiology of MDD is highly complex and includes environmental, psychosocial, genetic, epigenetic, neuroendocrine, neuro-immunological, and even dietary factors (American Psychiatric Association, 2013; Dash *et al*, 2015; Gururajan *et al*, 2016). The multifactorial nature of MDD is especially evident through the high comorbidity rates with various other somatic and affective diseases. Thus, the prevalence for depression is several times higher in patients suffering from chronic pain (Kim *et al*, 2012), bowel disorders (Fuller-Thomson and Sulman, 2006; Slyepchenko *et al*, 2016), or autoimmune (Martin-Subero *et al*, 2016), and inflammatory diseases (Dantzer and Capuron, 2017) than in the general population. Further, about 90 % of MDD patients suffer from an additional anxiety disorder like generalized anxiety disorder (GAD) or social phobia (Alonso *et al*, 2004; Gorman, 1997). Unfortunately, this high rate of comorbidity can mask disease symptoms and complicates accurate diagnoses (Krishnan and Nestler, 2008).

From a neurological point of view, numerous brain regions are involved in the pathophysiology of depression, like the hippocampus, amygdala, prefrontal and cingulate cortices, striatum, and the mesolimbic dopaminergic circuit (Berton and Nestler, 2006; Heshmati and Russo, 2014; Krishnan and Nestler, 2008; Ressler and Mayberg, 2007). Patients suffering from MDD show reduced grey-matter volume and glial density in the prefrontal cortex (PFC) and the hippocampus. Both regions are hypothesised to mediate the cognitive aspects of depressive symptoms (Krishnan and Nestler, 2008; Rajkowska, 2003; Rajkowska *et al*, 1999). Interestingly, the reduction in PFC grey-matter volume correlates with the severity of MDD symptoms only in men, but not in women (Carlson *et al*, 2015). Functional magnetic resonance imaging studies in women also revealed an association between the severity of MDD and a hypo-connectivity of the amygdala and frontal regions including the dorsolateral PFC (Satterthwaite *et al*, 2016). The reduction in hippocampal volume is mainly found in patients suffering from MDD for at least 2 years with several episodes (McKinnon *et al*, 2009) and is inversely related to the number of episodes (MacQueen *et al*, 2003). Likewise, volumetric changes in the PFC are dependent on number of episodes (Treadway *et al*, 2015; Yucel *et al*, 2008) and negatively correlated with disease

severity and duration (Bludau *et al*, 2016). Moreover, deep brain stimulation of Brodmann Area 25 (infralimbic PFC) in MDD patients improves symptoms (Mayberg *et al*, 2005), proposing a dysregulation in specific areas as one mechanism in MDD.

1.1.2 Anxiety disorders

A second major group of psychiatric disorders are anxiety disorders that are characterized by the underlying emotional concept of excessive and irrational fear and anxiety. Both fear and anxiety describe highly adaptive responses to a threat for the individual's health and homeostasis that overlap and interact. These terms are used interchangeably, though fear is rather described as the emotional response to a real or perceived imminent threat that determines the fight of flight response, whereas anxiety is the anticipation of it (McNaughton and Zangrossi, 2008). In the DSM-5, anxiety disorders include different mental states of excessive fear and anxiety (see Fig. 1) that are mutually highly comorbid but are triggered by distinct stimuli (American Psychiatric Association, 2013). Anxiety disorders have the highest lifetime prevalence for a group of psychiatric disorders of approximately 30 % and are considered the 6th highest contributor to YLDs (3.4%). Today, 264 million people live with anxiety disorders (Kessler and Wang, 2008; Neumann and Slattery, 2016) and, similar to MDD, women have an about 2-fold higher prevalence (4.6 % vs. 2.6 %; American Psychiatric Association, 2013; World Health Organization, 2017). The most common anxiety disorder is specific phobia pronounced as marked fear or anxiety related to a specific object or situation. Also, social anxiety disorder (SAD), which is characterized by persistent fear and avoidance of social situations, is the second most common anxiety disorder with a lifetime prevalence of 13 % (Bandelow *et al*, 2017). To give a conceptual framework for the present thesis, from here on the introduction will focus on GAD and its epidemiology to gain better insight into the underlying neurobiological mechanisms.

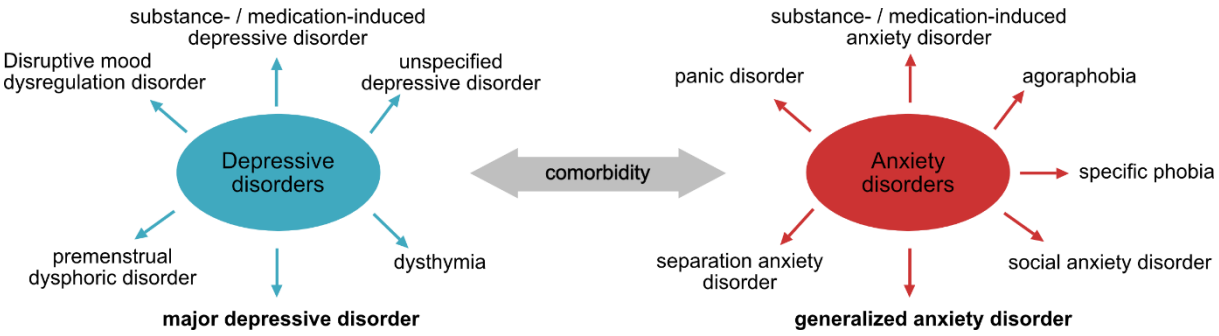


Figure 1. Schematic overview about commonly known kinds of depressive and anxiety disorders as classified by the Diagnostic and Statistical Manual of Mental Diseases DSM-5. The DSM-5 categorizes the abovementioned mental conditions as depressive disorders or anxiety disorders that are characterized by the same main features in mood. Depressive disorders and anxiety disorders share a high rate of comorbidity (American Psychiatric Association, 2013). Of note, the following subtypes of depressive disorders are not mentioned: depressive disorder due to another medical conditions and other specified depressive disorder. Regarding anxiety disorders, selective mutism in children, anxiety disorder due to another medical condition, other specified anxiety disorder, and unspecified anxiety disorder are not named.

GAD is the third most prevalent anxiety disorder with a lifetime prevalence of approximately 6 %. It is characterized by persistent, excessive, and uncontrollable anxiety and worry / apprehensive expectation about various aspects of life for the majority of days over at least 6 months. These psychological symptoms are accompanied by physical symptoms like restlessness, difficulties to concentrate, irritability, or sleep disturbances (American Psychiatric Association, 2013; Kessler and Wang, 2008; Neumann and Slattery, 2016; Somers *et al*, 2006). The majority of GAD cases are chronic, but with fluctuating severity, and a full remission is rarely achieved despite several treatment options (see section 1.1.3 for description; American Psychiatric Association, 2013). Moreover, GAD is a highly comorbid disorder with about 62 % of patients experiencing a MDD episode in the past year (Coplan *et al*, 2015). Similar to depressive disorders, a key brain region involved in anxiety-related disorders is the amygdala together with, but not exclusively, the PFC, hypothalamus, ventral hippocampus, the nucleus accumbens, and other brain regions (Lüthi and Lüscher, 2014; Ressler and Mayberg, 2007; Tovote *et al*, 2015). In line, GAD increases amygdala and dorsomedial PFC volume in women which correlates with symptom severity (Schienle *et al*, 2011). GAD patients also show a greater activation of the ventromedial PFC (Monk *et al*, 2006) as well as abnormal amygdala and PFC activation. This was accompanied by increased grey-matter volume and reduced connectivity between those structures (Hilbert *et al*, 2014), proposing region-specific functional changes in GAD. Further, the amygdala seems less while the anterior cingulate cortex is more responsive in women suffering from GAD, which could predict treatment outcome (Blair *et al*, 2008; Whalen *et al*, 2008).

1.1.3 Conventional treatment for psychiatric disorders

A serendipitous finding in the late 1950s opened the door to a new world of treatment options and introduced the first medications for MDD. Iproniazid, commonly used as an antitubercular agent, concurrently improved depressed mood (Crane, 1956; López-Muñoz and Alamo, 2009). Around the same time, an antihistamine gave rise to a second antidepressant. Imipramine is a tricyclic antidepressant (Feighner, 1999) characterized by a 3-ring molecular structure (Kuhn, 1957, 1958). As both iproniazid and imipramine increase noradrenaline and serotonin (5-hydroxytryptamine, 5-HT) concentrations in the synaptic cleft, the first theory of catecholamine deficiency in depression was born (Schildkraut, 1965). In the 1970's, the "second generation" of antidepressants was introduced like selective serotonin reuptake inhibitors (SSRIs; including citalopram or paroxetine), serotonin and noradrenaline reuptake inhibitors (SNRIs; e.g. reboxetine or venlafaxine), or selective dopamine uptake inhibitors (SDRI) like bupropion in the late 1980s (reviewed in López-Muñoz and Alamo, 2009; Ramachandraih *et al*, 2011; Slattery *et al*, 2004). Over generations, not only clinical efficacy, onset of action, and tolerance were improved, but also undesirable side effects like body weight gain, insomnia, or the toxicity in overdose were reduced (Millan, 2004; Ravindran and Stein, 2010). The broad spectrum of current antidepressants still mainly targets monoaminergic neurotransmission. Beside, positive results are obtained from antidepressants targeting different systems. These encompass strategies such as the melatonin receptor agonist agomelatine, glutamatergic modulators like ketamine or riluzole, anticholinergic modulators (e.g. scopolamine), or anti-inflammatories like infliximab (for review see Berton and Nestler, 2006; Papakostas and Ionescu, 2015).

Antidepressants are effective in the treatment of anxiety disorders, too, corresponding to the high comorbidity between those two diseases (Millan, 2004). Beside tricyclic antidepressants and monoamine oxidase inhibitors, benzodiazepines, anticonvulsants, or atypical antipsychotics are used (Hoffman and Mathew, 2009). Benzodiazepines like diazepam or lorazepam are powerful positive allosteric modulators of the γ-aminobutyric acid $(GABA)_A$ receptor. They potentiate the inhibitory function of GABA, leading to a rapid onset and strong anxiolytic effects. However, as this mechanism easily induces physical dependency, this option is chosen only in patients with an urgent need for treatment (Cloos and Ferreira, 2008; Paulus *et al*, 2005; Ravindran and Stein, 2010). Interestingly, anticonvulsants and atypical antipsychotics are proposed rather as adjunctive treatment in case of treatment-resistance. In general, first choice treatment for anxiety disorders are SSRIs and SNRIs due to a high clinical efficacy (Hoffman and Mathew, 2009; Ravindran and Stein, 2010).

In addition to pharmacotherapy, a number of other therapeutic options are approved. These include cognitive behavioural or interpersonal therapy (Matthews *et al*, 2005), bright light therapy (Oldham and Ciraulo, 2014), and physical exercise. Also, device-based therapy like transcranial magnetic stimulation or

deep brain stimulation (Mayberg *et al*, 2005; Papakostas and Ionescu, 2015) are applied to alleviate symptoms of both MDD and anxiety disorders.

1.1.4 Selective serotonin reuptake inhibitors and alternative strategies

SSRIs were the first antidepressants developed with the premise to counteract depression, initially starting with fluoxetine followed by citalopram and others (López-Muñoz and Alamo, 2009). Citalopram, the highly selective SSRI, is a racemic mixture containing two enantiomers that differ in configuration. The active component of citalopram is the S-enantiomer, whereas the R-enantiomer rather diminishes its function (Hyttel *et al*, 1992). S-citalopram or escitalopram, chemically known as (S)-(+)-1-[(3-dimethyl-amino)propyl]-1-(p-fluorophenyl)-5-phthalancarbonitrile oxalate, shows a unique binding to the 5-HT transporter (SERT). It is capable of interacting with SERT at two different binding sites: a primary high-affinity binding site, which is generally bound by SSRIs, and a secondary lower-affinity allosteric site that is proposed to stabilize and prolong binding to the primary site (Burke, 2002; Chen *et al*, 2005; Murdoch and Keam, 2005). Clinical studies provide evidence that escitalopram has a faster onset of action (1-week vs. 2-3 weeks) and a greater improvement of symptoms compared to other antidepressants. It also shows a high tolerability and few adverse side effects like insomnia, diarrhea, or dizziness (Burke, 2002). Escitalopram has comparable or even superior efficacy in patients suffering from MDD or GAD (Burke, 2002; Davidson *et al*, 2004; Goodman *et al*, 2005; Montgomery *et al*, 2001; Murdoch and Keam, 2005; Ravindran and Stein, 2010; Sánchez *et al*, 2004). These clinical results are supported by preclinical evidence of reduced anxiety- and depressive-like behaviour. In rats, chronic escitalopram treatment ameliorates depressive-like behaviour in the forced-swim test (FST; Jayatissa *et al*, 2006; Sánchez *et al*, 2003) and sucrose preference test (Montgomery *et al*, 2001). Moreover, microinfusion of citalopram (containing about 50 % escitalopram) into the infralimbic PFC of rats alleviated depressive-like symptoms in the FST (Gasull-Camós *et al*, 2017). In mice, repeated administration of escitalopram reversed anxiety- and depressive-like behaviour (Mombereau *et al*, 2010).

Despite the availability of numerous antidepressants, only about 50 % MDD of patients respond sufficiently to the first antidepressant and even after repeated treatment, 30 - 40 % suffer persistently from symptoms (Ferrier, 1999; Hennings *et al*, 2009). Therefore, these patients are categorized as treatment-resistant. As not all substances that facilitate monoaminergic neurotransmission act anti-depressive (like cocaine) and the current monoaminergic antidepressants show a delayed onset of action (2 – 3 weeks), secondary occurring mechanisms downstream of monoamines might yield promising potential. Thus, various novel theories and targets are relentlessly tested. One effective approach is medication augmentation. In agomelatine, different modes of action are fused and it shows, beside monoaminergic activity, improved sleep quality and sleep-wake rhythmicity (Millan *et al*, 2003). Further,

non-antidepressant medication can be administered as add-on to conventional antidepressants. Here, atypical antipsychotics that inhibit 5-HT receptors or act as partial dopamine (DA) agonists are generally used to augment treatment efficacy. A recent meta-analysis of 43 clinical trials proved an effective augmentation strategy of partial DA agonists in patients suffering from MDD but showed inadequate treatment response. Also, in a double-blinded clinical study in treatment-resistant depression, a 5-HT antagonist successfully improved conventional antidepressant treatment within 1 week (Citrome, 2015; Shelton *et al*, 2001). Notably, a recent meta-analysis of nine clinical trials demonstrated that ketamine, an ionotropic NMDA receptor antagonist, has substantial rapid antidepressant effects. However, these effects seem transient with a high heterogeneity in clinical response that urges for caution (Xu *et al*, 2016; Zhang and Ho, 2016). Preclinical studies also propose metabotropic glutamate receptor antagonists as potential novel treatment (Peterlik *et al*, 2016). Several additional targets are currently investigated like nitrous oxide, opioids, psychedelic drugs like psilocybin, or corticotropin-releasing factor (CRF) receptor antagonists (Berton and Nestler, 2006; Ceskova and Silhan, 2018; Millan, 2004; Peterlik *et al*, 2016), to just name a few. Importantly, a meta-analysis of 20 clinical trials revealed an improvement in depressive symptoms with anti-cytokine treatment (Kappelmann *et al*, 2018; see chapter 1.2.2). This provides strong evidence for a dysregulated immune system in MDD and support an inflammatory theory of psychiatric disorders.

1.2 Inflammation and psychiatric disorders

The immune system is a highly complex network involving multiple cell types and factors that provides a comprehensive protection to maintain body homeostasis. By means of inflammation, it responds to the invasion of exogenous material like external threats (invading pathogens) or internal causes like ischaemia or infected cells. The body has three essential levels of defence with the initial protection provided by epithelial barriers. Invading pathogens that pass the epithelium encounter two additional levels: the innate (natural) and the acquired immune systems. The central nervous system (CNS) bears its own line of defence mainly conducted by microglia. Though previously regarded as separate systems, nowadays a tight communication between the peripheral immune system and the immune system of the brain *via* short- and long-range interactions is commonly known. This bidirectional communication allows the immune system to recruit the whole body in immune regulation, thus engaging in a plethora of mechanisms. However, alterations in those communication pathways can account for various diseases that are, at the first glance, not expected to be inflammation-based.

1.2.1 The immune system

Exogenous material is recognized by immune cells due to a protein structure on the cellular surfaces called antigens. The peripheral immune system generally responds in two different manners: The immediate, but unspecific, innate immune response that always reacts to the same extent. Second, the slower adaptive immune response that displays a highly specific recognition of antigens but requires a previous contact. The innate immune response is largely based on numerous cells performing tissue-specific phagocytosis that originate and differentiate from bone marrow progenitor cells. The major cells involved in peripheral phagocytosis are macrophages and dendritic cells that travel to lymph nodes for antigen presentation to T and B cells. The thereby initiated adaptive immune response provides a tailored and specific response to pathogens. It also forms an immunological memory comprising pathogen-specific antibodies to facilitate host defence during a future insult (Medzhitov and Janeway, 2002; Young *et al*, 2014). Beside direct cell-to-cell contact, communication between immune cells is conducted *via* inflammatory mediators like acute-phase proteins, chemokines, or cytokines. The latter are soluble low molecular weight glycoprotein messengers, not only within the immune system but also within other systems of the body, forming an integrating network. They can be classified as pro- or anti-inflammatory as well as hematopoietic cytokines. Pro-inflammatory cytokines (e.g. interleukin (IL)-1, IL-6, tumour necrosis factor (TNF), or interferon (IFN)) are responsible to initiate the immune response while anti-inflammatory cytokines like IL-3, IL-4, or IL-10, block or dampen it. Hematopoietic cytokines (IL-3 or IL-5), on the other hand, stimulate the differentiation of hematopoietic progenitor cells into red and white blood cells (Delves and Roitt, 2000; Yarlagadda *et al*, 2009).

In the past, the CNS has been viewed as an immune-privileged organ protected from excessive peripheral signalling and pathogen or cell invasion by the blood brain barrier (BBB). Today, a close communication between the peripheral immune system and the immune system of the brain is known. Transduction of inflammatory signals is executed *via* three different routes: the humoral, neural, and the cellular route. The circumventricular organs like the choroid plexus, lacking the BBB and, thus, providing an unprotected entry site (Giunti *et al*, 2003), were long assumed as only access to the brain. However, this passage seems to be predominantly a relay station for signals. Circumventricular organs contain immune cells that detect circulating cytokines (Ericsson *et al*, 1995; Nadeau and Rivest, 1999; Vallières and Rivest, 1997). Upon recognition, cytokines are released into the perivascular compartment from which they are transported to the brain. There, cytokines can directly interact with glia cells and neurons to facilitate an inflammatory response (Ericsson *et al*, 1995; Quan *et al*, 1997; Stitt, 1990). In addition, BBB endothelial cells contain cytokine receptors (Ek *et al*, 2001) and specific saturable active transporters (Banks, 2005) to enable immune-to-brain signalling *via* the humoral route. Using the neural route, cytokines are proposed to stimulate vagal or trigeminal afferent fibres directly *via* short-range interactions to relay visceral information to the brain. In line, afferent dorsal root ganglia in rats also express IL-1β receptors (Obreja

et al, 2002), ultimately affecting behaviour. Finally, activated peripheral immune cells can migrate into the brain utilizing the cellular route (Kerfoot *et al*, 2006). In the brain, immune cell-produced neuroendocrine mediators and peripheral cytokines can modulate different brain circuits including the hypothalamus-pituitary-adrenal (HPA) axis (Fig. 2; Banks, 2005; Chrousos, 1995; Cunningham and De Souza, 1993; Dantzer, 2018; Dantzer *et al*, 2000; Hopkins, 2007; Morimoto and Alexopoulos, 2011; Schedlowski *et al*, 2014; Silverman *et al*, 2005; Yarlagadda *et al*, 2009)

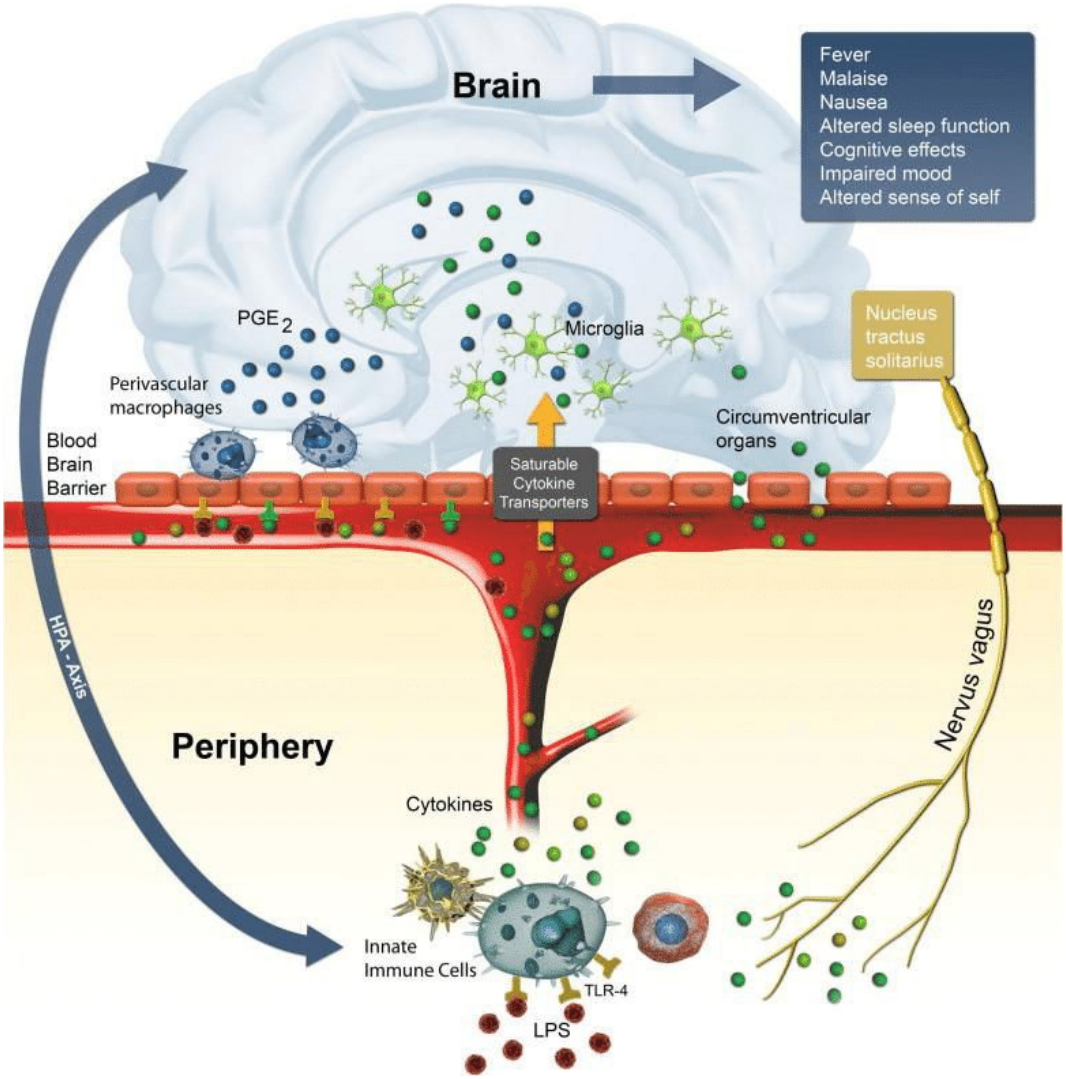


Figure 2. Schematic overview of humoral and neural routes of communication between the peripheral immune system and the immune system of the brain. Activation of innate immune cells by microbiota antigens like lipopolysaccharide (LPS), recognized by the toll-like receptor 4 (TLR-4), leads to the release of cytokines. Peripheral cytokines are not able to cross the blood brain barrier *via* diffusion due to a high molecular weight. They enter the brain either through circumventricular organs or by active saturable cytokine transporters in the brain endothelium. In addition, perivascular macrophages can interact with brain endothelial cells to induce the release of prostaglandin E2 (PGE_2), which activates neuronal regulation of the hypothalamo-pituitary-adrenal (HPA) axis and other systems. The HPA axis can in turn affect the inflammatory reaction of innate immune cells. Cytokines and PGE_2 also stimulate microglia to induce a neuroinflammatory reaction and a subsequent release of inflammatory mediators. As neural pathway, primary afferent nerves like the vagus nerve respond to peripheral cytokines and project to

several brain regions like the nucleus of the solitary. Ultimately, the complex immune-to-brain signalling regulates body homeostasis in numerous systems in the periphery and the brain and, thus, behaviour (adapted from Dantzer *et al*, 2000; Hopkins, 2007; Schedlowski *et al*, 2014).

The innate immune system of the brain

The innate immune system of the brain is mediated mainly *via* resident microglia, but also perivascular macrophages and astrocytes are immunocompetent and contribute to immune functioning. Microglia constitute about 10 % of cells in the CNS and are of mesodermal origin arising from the yolk sac, colonizing the CNS during early development until the BBB is formed. In humans, from the middle of the first until early second trimester and, correspondingly, in rodents from embryonic day 10 to 19, microglia colonize the brain. During postembryonic homeostasis, microglia proliferate and represent an independent self-renewing population (Kettenmann *et al*, 2011; Marin and Kipnis, 2017). A second phase of microglial originating from bone marrow colonizes the brain during the early postnatal development (Kettenmann *et al*, 2011). However, microglia do not colonize as mature cells, but as immature progenitors that develop together with the organism through three distinct stages. Global profiles of transcriptional stages in mice revealed that microglial development starts with early microglia until embryonic day 14, followed by pre-microglia until postnatal day 9. During this time, microglia show a highly diverse transcriptomic profile. They are considered as being adult from the organism's age of 4 weeks onwards with a less heterogeneous transcriptional pattern (Hammond *et al*, 2019; Matcovitch-Natan *et al*, 2016; Thion *et al*, 2018). Importantly, adult mouse microglia show a sex dimorphism. Microglia of females express higher levels of genes associated with the inflammatory response, apoptosis, and lipopolysaccharide (LPS) response compared to males, suggesting a more immune-activated state in line with a previously proposed stronger immune response in females (Klein and Flanagan, 2016; Thion *et al*, 2018). Male and female mice demonstrate a similar distribution of microglia throughout the brain (Thion *et al*, 2018), though region- and sex-specific density has been reported in rats (Schwarz *et al*, 2012). Infection during pregnancy can disrupt microglia maturation and proper immune functioning (Matcovitch-Natan *et al*, 2016). Also, a lack of microbiota – commensal bacteria colonizing the gastrointestinal tract (GIT) – during development can not only prevent microglial maturation (see chapter 1.3.2; Erny *et al*, 2015), but leads to sex-specific alterations in gene expression. Thus, at late embryonic stages, e.g. embryonic day 19, microglia of male germ-free (GF) embryos show alterations in the expression of genes linked to translation and metabolism. In contrast, female GF embryonal microglia remain largely unaffected at this age. Interestingly, microglia of adult female GF mice exhibit a dysregulation in genes linked to morphogenesis, adaptive immune response, and cell migration, while microglia of male GF adults remain unchanged. This differential temporal susceptibility to the absence of microbiota proposes males as susceptible during *in utero* development while females show a stronger reaction during adulthood (Thion *et al*, 2018).

Microglial dynamics

Microglia, the macrophages of the brain, regulate various processes like early brain wiring, synaptic pruning, transmission, and plasticity, as well as neurogenesis (Hu *et al*, 2015; Paolicelli *et al*, 2011; Salter and Beggs, 2014; Thion and Garel, 2017; Tremblay *et al*, 2011; Walker and Yirmiya, 2016; Yirmiya and Goshen, 2011). Additionally, they protect the brain from invading pathogens and integrate peripheral immune signalling, leading to a neuroinflammatory response (Dheen *et al*, 2007; Garden and Möller, 2006). To execute these functions, adult microglia show pronounced morphological and functional plasticity. Under "resting" conditions, ramified microglia have a small round cell body and numerous motile processes and branches. They occupy and surveil individual territories of surrounding tissue to detect potential activating stimuli like pathogens or components of the immune system (Askew *et al*, 2017; Sousa *et al*, 2017). Upon detection, within 1 h microglia can adopt different stages of activation, depending on the nature of the stimuli, and migrate to the site of injury for phagocytosis (Davalos *et al*, 2005). Those stages of activation can be characterized morphologically as well as by functional and molecular properties. Morphologically, microglia can be categorized into three different stages after activation, ranging from primed (ellipsoid-like soma and highly ramified), to reactive (amoeboid cell body with lesser processes), and amoeboid or phagocytic with not more than a few unbranched processes (Torres-Platas *et al*, 2014). Activation of microglia is accompanied by enhanced proliferation and, thereby, an increase in microglial number (Kettenmann *et al*, 2011), followed by apoptosis (Liu *et al*, 2001). This temporally controlled rate of proliferation and apoptosis ensures a stable level of microglia under healthy conditions (Askew *et al*, 2017; Garden and Möller, 2006). On a molecular level, microglial activation leads to either an M1 (pro-inflammatory) or an M2 (anti-inflammatory) state (Hu *et al*, 2015) that entails the production and secretion of cytokines to stimulate other microglia and immunocompetent cells in the brain (Hammond *et al*, 2019). The M1 state is characterized by the synthesis and secretion of pro-inflammatory cytokines, e.g. IL-1β, IL-6 or TNF-α, and the inducible nitric oxide synthase, acting antimicrobial through a classical inflammatory reaction. On the other hand, the M2 phenotype expresses arginase-1, as well as anti-inflammatory mediators like IL-10, and is more associated with tissue repair and homeostasis (Fig. 3; Cherry *et al*, 2014; Hu *et al*, 2015; Rock *et al*, 2004; Sousa *et al*, 2017).

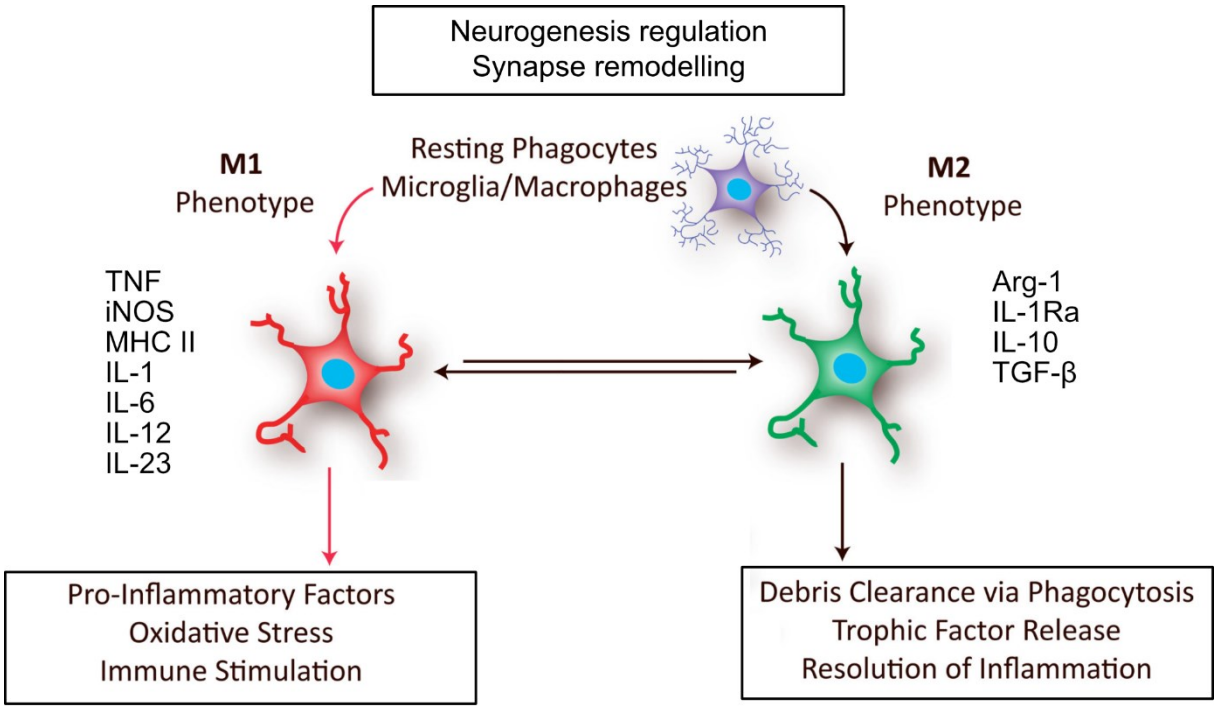


Figure 3. Polarization stages of microglia. Under physiological conditions, resting phagocytes like microglia remain ramified and survey the surrounding tissue to regulate neuronal homeostasis. Upon stimulation, microglia adopt different stages of activation dependent on the stimuli. Microglia then assume a more amoeboid morphology and acquire either the M1 or the M2 stage. M1 microglia express pro-inflammatory cytokines (like tumour necrosis factors (TNF), the inducible nitric oxide synthase (iNOS), the major histocompatibility complex II (MHC II), interleukin (IL)-1, IL-6, IL-12, or IL-23) and stimulate the immune system. M2 microglia are characterized by anti-inflammatory cytokine expression (e.g. arginase-1 (Arg-1), IL-1 receptor antagonist (IL-1Ra), IL-10, or the transforming growth factor TGF-β) and a subsequent resolution of inflammation. Microglia are able to switch between the M1 and M2 stage (adapted from Hu *et al*, 2015).

Thus, at the initial stage of tissue injury, the M1 microglial type dominates to eliminate dead tissue or pathogens and is later replaced by the M2 phenotype for tissue repair (Kigerl *et al*, 2009). To identify the morphological and functional stage of microglia, several membrane and intracellular protein markers beside the aforementioned are utilized. The M1 stage can also be identified using the cluster of differentiation molecules (CD) 11b (CD11b) or CD68, while M2 microglia express CD86 (for an overview see Kettenmann *et al*, 2011). Although these proteins enable the identification of activation stages, general marker for microglia are a useful tool to get a broader overview about cell populations. In this sense, in 1996 the ionized calcium-binding adaptor molecule 1 (Iba-1) was isolated from monocytes of the brain and is nowadays commonly recognized as a rather specific marker for microglia. Iba-1 is constantly expressed in microglia and upregulated in response to activation to regulate calcium homeostasis, mobility, and phagocytosis (Imai *et al*, 1996; Ito *et al*, 1998; Ohsawa *et al*, 2000). This bears some limitations, though, as peripheral macrophages that translocate to the brain are also monocytes and express Iba-1. Further, a response-dependent upregulation in protein levels might exacerbate the valid interpretation of intensity measurements that are used to identify cellular density in

immunohistochemically stained brain slices (Imai *et al*, 1996; Imai and Kohsaka, 2002). Recently, a novel marker for microglia was isolated from human, rat, and mouse immortalized microglia. In a series of control experiments, these publications showed that the transmembrane protein 119 indeed specifically marks microglia but not macrophages independent of cellular activity (Bennett *et al*, 2016; Bohlen *et al*, 2017; Satoh *et al*, 2016), and might represent a reasonable alternative to Iba-1.

Characterization of microglial states with those markers showed that a balanced microglial functioning in the brain is crucial for a healthy organism. A dysregulation, like a failed M1-M2 transition after microglial activation, resulting in a prolonged M1 pro-inflammatory state, is associated with detrimental effects on health (Cherry *et al*, 2014; Liao *et al*, 2012). Several lines of evidence link abnormal microglial dynamics to diseases. A loss-of-function mutation in microglia leading to neurodegenerative diseases like leukodystrophy (Rademakers *et al*, 2012). On the other hand, an over-activation is associated to various disorders like pathological pain (Walker and Yirmiya, 2016), Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, or multiple sclerosis (Butovsky and Weiner, 2018; Cherry *et al*, 2014; ElAli and Rivest, 2015; Salter and Beggs, 2014; Tang and Le, 2016).

1.2.2 Inflammatory theory of psychiatric disorders

Understanding the causes of mental disorders is a main goal in psychiatric research. In 1991, a new theory based on clinical observations was published as potential mechanism in the development of MDD: the "macrophage hypothesis of depression" (Smith, 1991) that connects both the peripheral and the brain immune system to depression. This hypothesis was soon promoted by Maes and colleagues providing evidence for an altered immune system activation in depression (Maes, 1995; Maes *et al*, 1992, 1995a, 1995b) and numerous studies support an inflammatory theory of psychiatric disorders.

In agreement, elevated levels of pro-inflammatory cytokines, like the acute-phase C-reactive protein (CRP), IL-6, or TNF-α, are found in the blood or cerebrospinal fluid (CSF) of patients suffering from autism spectrum disorder (ASD), SAD, or GAD (Hoge *et al*, 2009; Kim *et al*, 2018; Vargas *et al*, 2005; Vogelzangs *et al*, 2013). Activated T cells of GAD patients secrete correspondingly higher levels of pro-inflammatory cytokines (Vieira *et al*, 2010). In MDD patients, elevated levels of IL-6, TNF-α, or CRP (Dowlati *et al*, 2010; Köhler *et al*, 2016; Raison, 2014; Young *et al*, 2014), and their soluble receptors in the plasma (Maes *et al*, 1995a) as well as CSF (Levine *et al*, 1999) can be found, concluding the existence of an exaggerated systemic immune (re)activity in psychiatric disorders. Interestingly, a comprehensive study on cytokine plasma concentrations revealed both increased and decreased levels in MDD patients. After 12 weeks of treatment, in responding patients pro-inflammatory cytokine levels stabilized, but remained unchanged in non-responders (Syed *et al*, 2018). In the brain, MDD patients show enhanced concentration of TNF in the PFC (Dean *et al*, 2010) and activated microglia in one in six patients (Bayer *et al*, 1999). Importantly,

the dysregulated immune response in mood disorders shows a sex dependency. Thus, women suffering from GAD, SAD, or panic disorder do not express elevated levels of plasma CRP (Vogelzangs *et al*, 2013). In the orbitofrontal cortex of MDD suicide victims, *IL-4* mRNA seems upregulated in women, whereas in men *IL-13* mRNA is elevated (Tonelli *et al*, 2008). Conversely, the stimulation of inflammation has potential negative effects on mood and facilitates the development of psychiatric disorders. An immune challenge in healthy volunteers can coincide with the manifestation of depression and anxiety symptoms (Grigoleit *et al*, 2011; Schedlowski *et al*, 2014). Chronic administration of IFN-α, commonly used in hepatitis C or multiple sclerosis, dose-dependently induces depressive and anxiety symptoms in up to 45 % of treated patients (Friebe *et al*, 2010; Malek-Ahmadi, 2001; Zheng *et al*, 2015). In addition, though a low-dose injection of *Salmonella abortus* does not affect physical sickness symptoms, the elevated plasma cytokine concentrations correlate with endotoxin-induced levels of anxiety and depressed mood (Reichenberg *et al*, 2001). The high rate of comorbidity of MDD with inflammation-associated disorders like asthma (de Miguel Díez *et al*, 2011), rheumatoid arthritis (Covic *et al*, 2012), or autoimmune (Benros *et al*, 2013; Martin-Subero *et al*, 2016) and inflammatory diseases (Dantzer and Capuron, 2017; see chapter 1.1.1), strengthens a causal role of the immune system in mood disorders.

This inflammatory theory of mood disorders led to the assumption that treatment outcome is affected by the inflammatory state and anti-inflammatory agents might augment antidepressant therapy. In support, in MDD patients plasma CRP concentrations negatively correlate with the treatment success of escitalopram (Uher *et al*, 2014). Contrary, antidepressant augmentation with the TNF-α antibody infliximab was more successful in patients with higher plasma CRP concentrations (Raison *et al*, 2013). Non-steroidal anti-inflammatory drugs like aspirin or celecoxib, or anti-cytokine medication also successfully augment the effects of antidepressant medications (Kappelmann *et al*, 2018; Köhler *et al*, 2014; Mendlewicz *et al*, 2006; Müller *et al*, 2006).

Preclinical studies using inflammatory animal models of psychiatric disorders, such as maternal immune activation or sickness behaviour induced by an immune challenge with LPS or IFN-α, provide additional evidence for a crucial role of over-activated peripheral and brain inflammation in mood disorders. In this sense, enhanced cytokine concentrations due to maternal immune activation infiltrate the brain (Golan *et al*, 2004; Lin *et al*, 2003) and induce autism-like (Patterson, 2009), depressive-, and anxiety-like behaviour in adult mice (Babri *et al*, 2014; Khan *et al*, 2014). An immune challenge in adult mice causes depressive-like behaviour, an effect that is accompanied by increased microglial M1 morphology (Henry *et al*, 2008; Zheng *et al*, 2015). Further, genetic activation of microglia exacerbates LPS-induced depressive-like behaviour in mice (Corona *et al*, 2013). Chronic stress is a commonly acknowledged risk factor for psychiatric disorders that is accompanied by an over-activated immune system and chronic low-grade inflammation (for review see Langgartner *et al*, 2019). Fittingly, mice exposed to the chronic, unpredictable mild stress (CUMS) model develop a depressive- and anxiety-like phenotype concomitant

with an upregulation of pro-inflammatory cytokines (Goshen *et al*, 2008). In turn, caspase-1 knockout (KO) or IL-1 receptor KO acts protective in stress-induced anxiety- and depressive-like behaviour (Goshen *et al*, 2008; Wong *et al*, 2016). Stress-induced behavioural alterations are also accompanied by increased microglial M1 morphology (Burke *et al*, 2014; Kreisel *et al*, 2014; Xu *et al*, 2017; Yirmiya *et al*, 2015), occurring in various brain regions like the PFC (Hinwood *et al*, 2012, 2013; Tynan *et al*, 2010). Interestingly, glial loss in the PFC of rats, as well as a stress-induced reduction in hippocampal microglia density of mice, induces depressive-like behaviour (Banasr and Duman, 2008; Gong *et al*, 2018; Kreisel *et al*, 2014; Tong *et al*, 2017). In line with clinical studies, infliximab can prevent stress-induced depressive-like behaviour (Liu *et al*, 2016), and an IL-1 receptor antagonist reverses ischemic stroke-induced depressive symptoms (Craft and Devries, 2006) in mice. Moreover, in previously treatment-resistant rats, augmentation of fluoxetine with aspirin reverses CUMS-induced depressive-like behaviour (Wang *et al*, 2011).

Overall, the comprehensive body of evidence demonstrates a role of the inflammatory system in psychiatric disorders (Anderson *et al*, 2012; Berthold-Losleben *et al*, 2009; Dantzer, 2018; Griez *et al*, 2015; Hayley, 2011; Köhler *et al*, 2016; Miller *et al*, 2009; Robson *et al*, 2017; Salim *et al*, 2012; Yirmiya *et al*, 2015; Young *et al*, 2014). It is important to note that not all clinical studies show increased levels of pro-inflammatory cytokines (Kubera *et al*, 2000; Young *et al*, 2014) but report even reduced levels in depression (Lehto *et al*, 2010b, 2010a). Thus, rather an inflammatory dysregulation, not necessarily an over-activation, and in particular a deviation from microglial homeostasis, contributes to psychiatric disorders. Considering this, effects of immune-modulatory medication might be restricted to a subset of patients and require the definition and analysis of biomarkers to achieve a personalized and tailored treatment. Of note, in MDD an auto-immune reaction against the serotonergic system was indicated that might be supported by pro-inflammatory cytokines (Maes *et al*, 1995b; Robson *et al*, 2017; Sluzewska *et al*, 1997). Therefore, augmentation with anti-inflammatory agents might increase the efficacy of antidepressants. A possible agent is the anti-inflammatory tetracycline antibiotic minocycline that is currently under evaluation as treatment for psychiatric disorders.

1.2.3 The antibiotic minocycline

Since the discovery of penicillin (Chain *et al*, 1940), antibiotics have been developed and various different classes emerged. Tetracyclines are bacteriostatic antibiotics composed of a four-ring core essential for the antibiotic function and different attached side groups (Fig. 4). They inhibit protein synthesis by binding to the 30S bacterial ribosome (Goldman *et al*, 1983; Tritton, 1977). Dependent on the year of discovery, tetracyclines are subdivided into three generations (Chopra and Roberts, 2001).

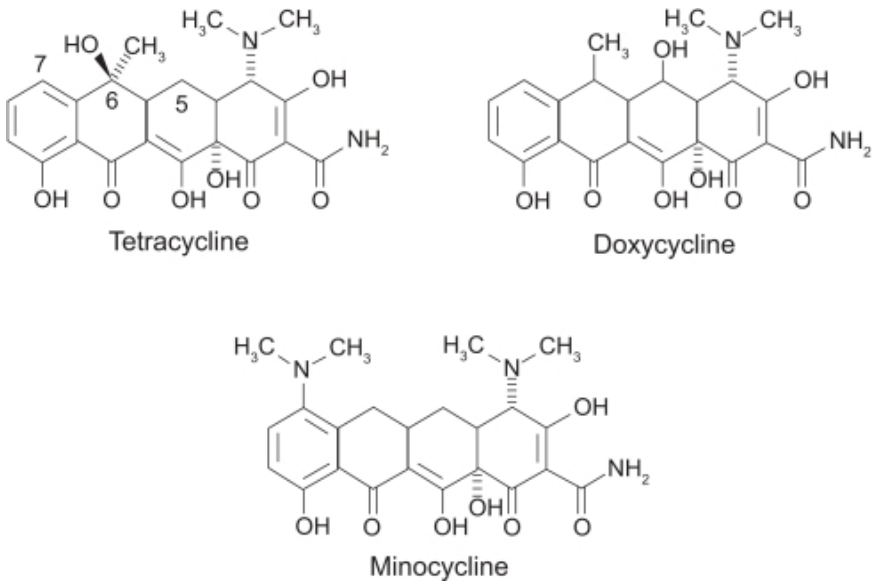


Figure 4. Chemical structure of tetracycline, doxycycline, and minocycline. (adapted from Valentín *et al*, 2009).

The first tetracycline discovered was the broad-spectrum antibiotic chlortetracycline, isolated from *Streptomyces aureofaciens* in 1948 (Duggar, 1948), that targets both gram-negative and gram-positive bacteria as well as atypical organisms. Soon after, the naturally occurring tetracycline (first-generation), and the semisynthetic second-generation doxycycline and minocycline were identified. The latter (7-dimethylamino-6-demethyl-6-deoxytetracyline) was synthesised in 1972 and is distinguishable by a modification on three sides, leading to a prolonged half-life of 12 – 18 h compared to 6 – 10 h of tetracycline (Agwuh and MacGowan, 2006; Noble *et al*, 2009). Besides their anti-microbial functions, tetracyclines caught attention due to pleiotropic effects in inhibiting matrix metalloproteinases, the ability to scavenge reactive oxygen species, anti-apoptotic and anti-inflammatory effects, as well as protective effects in the neurodegenerative disorders Huntington's and Alzheimer's disease (Chopra and Roberts, 2001; Griffin *et al*, 2010; Sloan and Scheinfeld, 2008). In addition, they act beneficial on nociceptive, inflammatory, and neuropathic pain (reviewed in Bastos *et al*, 2012; Burke *et al*, 2014).

Minocycline appears to be almost completely absorbed from the stomach and small bowel (Agwuh and MacGowan, 2006; Saivin and Houin, 1988) into the blood stream and can easily cross the BBB due to structural high lipophilicity (Agwuh and MacGowan, 2006; Barza *et al*, 1975). So far, it shows a low propensity for antibiotic resistance and a low incidence of side effects. Therefore, it is commonly used for the treatment of acne vulgaris and infections of the respiratory tract, Lyme disease, cholera, or syphilis (Chopra and Roberts, 2001; Gump *et al*, 1977; reviewed in Noble *et al*, 2009). Minocycline a potent regulator of neuroplasticity in the brain (Choi *et al*, 2007; reviewed in Plane *et al*, 2010; Quintero *et al*, 2006) with neuroprotective effects demonstrated by reducing tissue damage after spinal cord injury in rats (Festoff *et al*, 2006) and in multiple sclerosis in humans (Zhang *et al*, 2008). It facilitates neurogenesis

and neuronal survival in animal models of ischemia, restores hippocampal neurogenesis, and induces the recovery of neural stem cell populations after infection (Das *et al*, 2011; Ekdahl *et al*, 2003; Liu *et al*, 2007; Yrjänheikki *et al*, 1998). The pleiotropic effects of minocycline also encompass a beneficial influence on oxidative stress, glutamate excitotoxicity, and the attenuation of decreased DA, and norepinephrine levels (reviewed in Noble *et al*, 2009; Plane *et al*, 2010; Soczynska *et al*, 2012). Importantly, minocycline has strong anti-inflammatory properties. In rodents, minocycline attenuates inflammatory responses by inhibiting peripheral and pro-inflammatory cytokine production in the brain *in vivo* and *in vitro* (Kobayashi *et al*, 2013; Levkovitz *et al*, 2015; Naura *et al*, 2013; O'Connor *et al*, 2009b; Singh *et al*, 2014a; Wang *et al*, 2017, 2018). Furthermore, minocycline inhibits microglial proliferation (Seabrook *et al*, 2006; Tikka *et al*, 2001) and reduces inflammation-promoting T cells-microglia interactions *in vitro* (Giuliani *et al*, 2005). These studies are supported by several groups showing an anti-inflammatory effect of minocycline on microglia *in vivo* (Bye *et al*, 2007; Ekdahl *et al*, 2003; Neigh *et al*, 2009; Pabreja *et al*, 2011; Tikka and Koistinaho, 2001; Wang *et al*, 2017; Wu *et al*, 2002; Yrjänheikki *et al*, 1998; Zheng *et al*, 2015). Further, minocycline specifically abolishes microglia M1 polarization (Kobayashi *et al*, 2013) as well as induction of M1 microglia and cytokine production in the PFC in chronic pain (Burke *et al*, 2014; Xu *et al*, 2017), providing evidence for a direct effect of minocycline on microglia in the brain. Although the exact mechanisms underlying the beneficial effects of minocycline remain not fully understood, these studies strongly suggest the inhibition of the activation and proliferation of a variety of immune cells as well as positive effects on neuronal signalling as causal.

Interestingly, and potentially due to its anti-inflammatory effects and the inflammatory theory of psychiatric disorders, minocycline gained traction in the psychiatric area. In rodents, minocycline can alleviate cardiac arrest/CPR- (Neigh *et al*, 2009) and stress-induced anxiety (Levkovitz *et al*, 2015; Wang *et al*, 2018; Wong *et al*, 2016), and restores LPS- (Henry *et al*, 2008) and stress-induced social avoidance (Kreisel *et al*, 2014). However, most studies focus on minocycline as a potent novel treatment for depression. In naïve rats, minocycline decreases depressive-like behaviour (Molina-Hernández *et al*, 2008b, 2008a) and prevents LPS- as well as streptozotocin-induced cytokine secretion and depressive-like behaviour in mice (Henry *et al*, 2008; O'Connor *et al*, 2009a). In the learned helplessness paradigm, a single intracerebroventricular (icv) infusion of minocycline enhances escape attempts, indicative of decreased depressive-like behaviour (Arakawa *et al*, 2012). Likewise, subchronic treatment in rats prevents a stress-induced depressive-like phenotype (Wang *et al*, 2017; Xu *et al*, 2017). In both rats and mice, chronic minocycline treatment is effective in various tests. It reverses stress- or inflammation-induced decreased sucrose consumption in the sucrose preference test, elevates the time spent struggling in the FST, and decrease the time spent immobile in the tail-suspension test (TST). All of these behavioural effects are accompanied by a diminished inflammatory response (Burke *et al*, 2014; Tong *et al*, 2017; Wang *et al*, 2018; Wong *et al*, 2016; Zheng *et al*, 2015).

In keeping with these promising preclinical studies, minocycline is tested in clinical trials as adjunctive medication in the treatment of psychiatric disorders. In patients with Schizophrenia, minocycline could improve both positive and negative symptoms (Ghanizadeh *et al*, 2014; Kelly *et al*, 2015a; Levkovitz *et al*, 2010; Miyaoka *et al*, 2008), an effect that appears to be dependent on an inflammatory status in the patients (Deakin *et al*, 2018). Currently, it is under evaluation as a preventive treatment for at-risk mental states in schizophrenia and psychosis (Qurashi *et al*, 2017). Further, it modulates decision making (Kato *et al*, 2012; Watabe *et al*, 2012, 2013) and alleviates symptoms of ASD (Ghaleiha *et al*, 2016). In 1996, the first effective treatment of MDD was reported in a case study (Levine *et al*, 1996). Thereafter, several studies show that minocycline is beneficial on depressive symptoms and general well-being (Dean *et al*, 2017; Miyaoka *et al*, 2012; Soczynska *et al*, 2017). Additionally, it is effective in improving symptoms of HIV-induced mild-to-moderate depression (Emadi-Kouchak *et al*, 2016) and is evaluated in bipolar depression (Husain *et al*, 2016). A meta-analysis of ten studies reveals a moderate antidepressant effect of minocycline in MDD (Rosenblat and McIntyre, 2017) and in treatment-resistant depression, an improvement is likewise indicated (Husain *et al*, 2017; Raison *et al*, 2013). The surprisingly small clinical effect seen in single studies supports the aforementioned suggestion that anti-inflammatory agents in general and minocycline in particular might be only effective in a subset of patients with a distinct inflammatory profile. Of note, there are opposite treatment indications for minocycline. It can worsen symptoms of Parkinson's disease in both a monkey and a mouse model (Diguet *et al*, 2004) and induces a depersonalization disorder in a patient treated for acne (Cohen, 2004).

1.3 Microbiota theory of psychiatric disorders

Bacteria are found on all external and internal surfaces of the body, including the skin, saliva, oral mucosa, conjunctiva, and the GIT with a high intra- and interindividual diversity. This diversity is defined as number and abundance of distinct types of organisms within one organism (alpha diversity) or between organisms (beta diversity; Human Microbiome Project Consortium, 2012). The vast majority of commensal bacteria is found in the gut (Gill, 2006) followed by the skin and the remaining body with approximately the same amount (Berg, 1996). It has been postulated several times within the last 50 years that bacteria outnumber the cells of the human body by 10:1. However, revisiting these estimations and considering all cells in the human body including blood cells, the ratio actually appears closer to 1:1 (Sender *et al*, 2016).

Although microorganisms are universally present, research targeting the interaction of gut microbiota and their microbiome (collective genome) on the brain and, subsequently, on behaviour has only recently been recognized. To gain better insight, several different approaches are utilized in this context, like the

simple but efficient model of GF animals (Luczynski *et al*, 2016). Furthermore, depletion *via* antibiotics or manipulation of the composition by administering specific bacteria are applied (Sekirov *et al*, 2010). Those approaches enable research to reveal a strong impact of microbiota on behaviour and neuroendocrinology in rodents (Bercik *et al*, 2011a; Bravo *et al*, 2011; Messaoudi *et al*, 2011; Sudo *et al*, 2004; Turnbaugh *et al*, 2006). The initiation of large-scale metagenomics projects like the 'Human Microbiome Project' (NIH HMP Working Group, 2009) further contributes to unravel the complex host-microbiota interplay. Thus, microbiota not only affect metabolism (Le Chatelier *et al*, 2013) and growth (Blanton *et al*, 2016), but also behaviour and neuroendocrinology, as shown in rodents (Bercik *et al*, 2011a; Bravo *et al*, 2011; Messaoudi *et al*, 2011; Sudo *et al*, 2004; Turnbaugh *et al*, 2006).

1.3.1 The gut microbiota

"Bad digestion is the root of all evil" – has been postulated already as early as 400 B. C. by Hippocrates (Hawrelak *et al*, 2004), and underlines the importance of a balanced microbiota composition for an healthy organism. Three main factors shape the individual microbiota composition: initial colonization, age, and diet (Gill, 2006; Wu *et al*, 2011). In mammals, the sterile maternal uterus restricts gut colonization to the exposure to a complex microflora during delivery. The hygienic conditions at birth and the following days (Hanson *et al*, 1990), the mode of delivery, as well as breastfeeding critically determines the initial composition of the microbiota and ensures the formation of a healthy and stable community (Bäckhed *et al*, 2015; Brunel and Gouet, 1993; Guarner and Malagelada, 2003; Human Microbiome Project Consortium, 2012; Redondo-Lopez *et al*, 1990). During the first 4 month of life, the dynamic microbiota adapts to dietary changes and then gradually evolves and matures within the first 3 years. The majority of bacterial strains colonize during that time and, once acquired, are retained for decades with only varying in the relative abundance of each community. Thus, the adult mammalian gut hosts an immensely complex and diverse ecosystem within the nine-meter GIT with a surface area of approximately 250 – 400 m^2 (Hawrelak *et al*, 2004; Sonnenburg *et al*, 2004). The small intestine, caecum, and large intestine (colon) present three distinct microbial habitats that drive a spatial longitudinal heterogeneity by chemical and nutrient gradients as well as compartmentalized host immune activity. They are inhabited by 10^{13} to 10^{14} microorganisms (Gill, 2006) with 500 to 1000 species whose microbiome encompass approximately 2 to 4 million genes (Hooper and Gordon, 2001). These numbers were derived from faecal samples, though, and it has recently been suggested that the actual number of species reaches at least 35,000 (Frank *et al*, 2007). In rats and mice, similar as in non-human primates and humans, the gut microbiota is dominated by the two bacterial phyla of *Bacteroidetes* and *Firmicutes*, whereas other phyla present only a minor

proportion (Fig. 5; Benson *et al*, 2010; Eckburg *et al*, 2005; Gu *et al*, 2013; Ley *et al*, 2005; Nagpal *et al*, 2018).

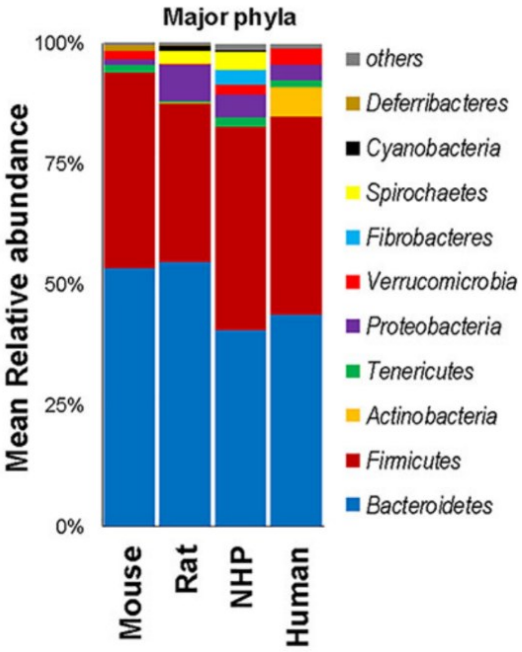


Figure 5. Relative abundance of major gut bacterial phyla in mice, rats, non-human primates (NHP), and human subjects. In all species, Bacteroidetes and Firmicutes are the dominant bacterial phyla. In rodents, Bacteroidetes prevailed Firmicutes abundance, while in NHP and humans it remained comparable. Other phyla present a minor proportion in gut microbiota composition with the highest abundance of Proteobacteria und rats and of Actinobacteria in human samples (adapted from Nagpal *et al*, 2018).

The small intestine contains a low bacterial diversity (Frank *et al*, 2007), while in the caecum and colon a high diversity and density in bacterial communities with distinct nutrient niches can be found (for review see Donaldson *et al*, 2015; Lee *et al*, 2013; Sekirov *et al*, 2010). Diet strongly determines microbiota composition in the gut. Comparison of American vs. Venezuelan and Malawian (Yatsunenko *et al*, 2012), or European and Burkina Faso populations (De Filippo *et al*, 2010), reflected culturally-defined differences in diet by their microbiota composition. Despite these differences, a core microbiota has been identified in nearly half of human subject samples as well as rodents (Benson *et al*, 2010; Ley *et al*, 2006; Qin *et al*, 2010; Tap *et al*, 2009) and adult diet-induced changes seem to be fast but dynamic. In mice, a first alteration can be observed after 1 to 4 days of dietary change that are mostly reversible (Carmody *et al*, 2015). In humans, changes were observable already after 1 day with a fast recovery to the original microbiota phenotype 2 days after diet termination (David *et al*, 2014; Wu *et al*, 2011). Of note, an impact of genetics on gut microbiota composition is discussed controversially (Benson *et al*, 2010; Carmody *et al*, 2015; Goodrich *et al*, 2016; Khachatryan *et al*, 2008; Parks *et al*, 2013; Turnbaugh *et al*, 2009; Wen *et al*, 2008).

Microbiota and the immune system

Reciprocally, the gut microbiota plays a crucial role for the development and proper functioning of the GIT (Stappenbeck *et al*, 2002) by regulating energy intake, absorption and storage, and utilizing calories from otherwise indigestible polysaccharides (Bäckhed *et al*, 2004, 2007; Carmody *et al*, 2015; Turnbaugh

et al, 2008). Most importantly, it mediates development and fine-tuning of the immune system. The large interface between gut epithelium and microbiota allows for countless confrontations with antigens of bacteria recognized by several pattern recognition receptors like toll-like receptors (TLRs) or NOD-like receptors (NLRs) (reviewed in Creagh and O'Neill, 2006; Wells *et al*, 2011). TLRs are the primary receptors recognizing microbial structures as well as viruses, fungi and protozoans. Upon recognition of a microbe-associated molecular pattern, TLRs induce the pro-forms of IL-1β and IL-18 and initiate intracellular signalling to "prime" the system for an inflammatory response (Creagh and O'Neill, 2006; Guo *et al*, 2015; Kahlenberg *et al*, 2005; Su *et al*, 2016). Simultaneously, NLRs detect bacteria and, as part of the inflammasome complex like the NLRP3 inflammasome, cleave cytokine pro-forms into their bioactive forms (Creagh and O'Neill, 2006; Wells *et al*, 2011). TLRs are also located on macrophages and microglia and detect circulating microbe-associated molecular pattern in the whole body (Dantzer *et al*, 2008; Su *et al*, 2016). Therefore, the unique GIT immune system integrates information and regulates an appropriate homeostasis between immune tolerance and inflammatory reaction (reviewed in Macpherson and Harris, 2004; Schenk and Mueller, 2008). GF mice provided the first evidence for the impact of microbiota on host immunity, presenting – beside impairments in gut functionality - abnormal numbers of immune cells and cytokines, as well as deficits in lymphoid structures. Both re-colonization with microbiota from healthy mice (Bouskra *et al*, 2008; Ishikawa *et al*, 2008; MacPherson and Uhr, 2004; Rook and Stanford, 1998; Shanahan, 2002) or distinct microbial populations successfully induced immune cell maturation and differentiation in GF mice (Christensen *et al*, 2002; Ivanov *et al*, 2008; Mazmanian *et al*, 2005). The crucial role of bacteria in immunoregulation is also a cornerstone of the 'hygiene', or 'old friends' hypothesis, stating that a boost in inflammatory diseases nowadays is associated to a failure in immunoregulation. This failure is potentially attributed to a lacking exposure to a widespread microbial environment and diversity and, thereby, insufficient maturation and differentiation of immune cells. Consequently, this leads to a dysregulated pattern of cytokines that potentially promotes susceptibility to inflammatory and, thus, mental diseases (Blaser, 2017; Langgartner *et al*, 2019; Lowry *et al*, 2016; Rook *et al*, 2013; Rook and Lowry, 2008).

Microbiota perturbation

The gut microbiota composition is highly susceptible to perturbations during pregnancy as well as early postnatal periods. Changes during this particular time can alter HPA-axis responsiveness, impair memory, as well as a increase the for disorders like ASD or inflammatory bowel disease (IBD; David *et al*, 2014; Faith *et al*, 2015; Fischbach and Segre, 2016; Golubeva *et al*, 2015; Gur *et al*, 2019; Jasarevic, *et al*, 2015; O'Mahony *et al*, 2009; Zerbo *et al*, 2015; Zijlmans *et al*, 2015). A recent study in the Bangladeshi population demonstrated a disturbed and immature microbial composition in young children with

moderate or severe acute malnutrition. Food intervention could briefly restore the composition, however, long-term effects remain elusive (Subramanian *et al*, 2014). Moreover, antibiotic treatment during bacterial infection strongly affects microbiota. Though beneficial for health, several studies show the negative effects of antibiotics on commensal bacteria in both humans (Dethlefsen *et al*, 2008; De La Cochetière *et al*, 2008) and animals (Croswell *et al*, 2009; Sekirov *et al*, 2008). In humans, only 5 days of antibiotic treatment decrease microbiota diversity, richness and evenness (Dethlefsen *et al*, 2008). Despite a recovery of the original structure after approximately 4 weeks, some taxa hardly recover even within 2 years (Jernberg *et al*, 2007). In addition, long-lasting side effects like antibiotic-associated diarrhoea or prolonged pathogen colonization are demonstrated in both humans (McFarland, 2006) and mice (Brandl *et al*, 2009) with profound effects on the immune system found in the latter (Hill *et al*, 2010). Antibiotic-induced microbiota depletion in mice induces and retains signatures of inflammation even after microbiota recovery (Lichtman *et al*, 2016). Eventually, these long-term effects entail the chance of developing numerous somatic and psychiatric disorders irrespective of species (Lurie *et al*, 2015; for review see Sekirov *et al*, 2008; Ubeda and Pamer, 2012).

Further, physiological as well as social stress alters microbiota composition and enhances immune activity in mice (Bailey *et al*, 2010, 2012; Langgartner *et al*, 2017b; Tannock and Savage, 1974). Chronic psychosocial and physiological stress increase secretion of pro-inflammatory cytokines in mouse mesenteric lymph nodes but decrease immune reactivity in the colon. This is accompanied by a significant histological damage of the colon and a spontaneous colitis with aggravated symptoms after a chemical challenge, underlining the impact of stress on colon homeostasis and inflammation (Jarillo-Luna *et al*, 2007; Reber *et al*, 2006, 2007, 2008). Importantly, these stress effects seem mediated *via* a compromised intestinal barrier function (termed leaky gut) as a broad-spectrum antibiotic treatment blocks colonic inflammation in mice (Reber *et al*, 2011). Also, rural upbringing acts stress-protective in humans, presumably *via* an environmental microbiota-mediated downregulation of the immune system (Böbel *et al*, 2018). These microbiota-immune system interactions are bidirectional, as an infection modulates microbial composition in humans (Khachatryan *et al*, 2008).

1.3.2 Microbiota -gut-brain axis and its implication in psychiatric disorders

The gut-brain axis was initially introduced as a concept to describe a complex reflex network that includes the CNS, the neuroendocrine and immune system, the sympathetic and parasympathetic arm of the autonomic nervous system, and the enteric nervous system. In this multidirectional communication network, efferent fibres from the brain, like the vagus and pelvic nerve, induce a top-down physiological response through motor, sensory, and secretory aspects of the GIT, smooth muscles, and glands. Also, emotions are transmitted from the limbic system, modulating the endocrine system and ANS. Bottom-up

signalling from the GIT reaches mainly cortical areas like the cingulate and insular cortex and the amygdala to modulate brain functioning (reviewed in Grenham *et al*, 2011; O'Mahony *et al*, 2011). The role of enteric microbiota, which have been regarded as isolated system without direct influence on body function, has only recently been recognized and integrated into this network and its impact on the now called microbiota-gut-brain (MGB) axis remains to be studied.

Communication between the host and gut microbiota can occur *via* various key routes (Fig. 6). On the one hand, microbial changes can be translated into behaviour through the vagus nerve. In line, chronic colitis-induced anxiety (Bercik *et al*, 2011b) and anxiolytic and antidepressant effects of the probiotic *Lactobacillus rhamnosus* (Bravo *et al*, 2011) are absent after vagotomy. Similarly, a subclinical dose of pathogenic bacteria induces anxiety-like behaviour in the absence of gut inflammation (Goehler *et al*, 2005; Lyte *et al*, 2006). Gut microbiota can also produce soluble substances like the neurotransmitter 5-HT or DA and metabolites like short-chain fatty acids (SCFAs; see chapter 1.3.3). These substances circulate in the body and alter several systems (O'Mahony *et al*, 2011, 2015; Sherwin *et al*, 2016; Stilling *et al*, 2016). Interestingly. microbiota are proposed to modulate tryptophan levels, thereby potentially influencing 5-HT signalling (Dinan *et al*, 2015). Immune-to-brain signalling is another important part of the MGB axis. Using the neural, humoral, and the cellular route, inflammatory signals from the periphery are able to modulate brain function (see chapter 1.2.1). In the brain, pro-inflammatory cytokines like IL-1 or IL-6 can induce the release of corticotropin-releasing hormone (CRH), which in turn activates the HPA-axis (Silverman *et al*, 2005; Webster *et al*, 1998). Together with an immunological modulation, gut microbiota determine HPA axis development while the HPA axis influences gut microbiota composition and intestinal barrier function (Kelly *et al*, 2015b; Sudo *et al*, 2004).

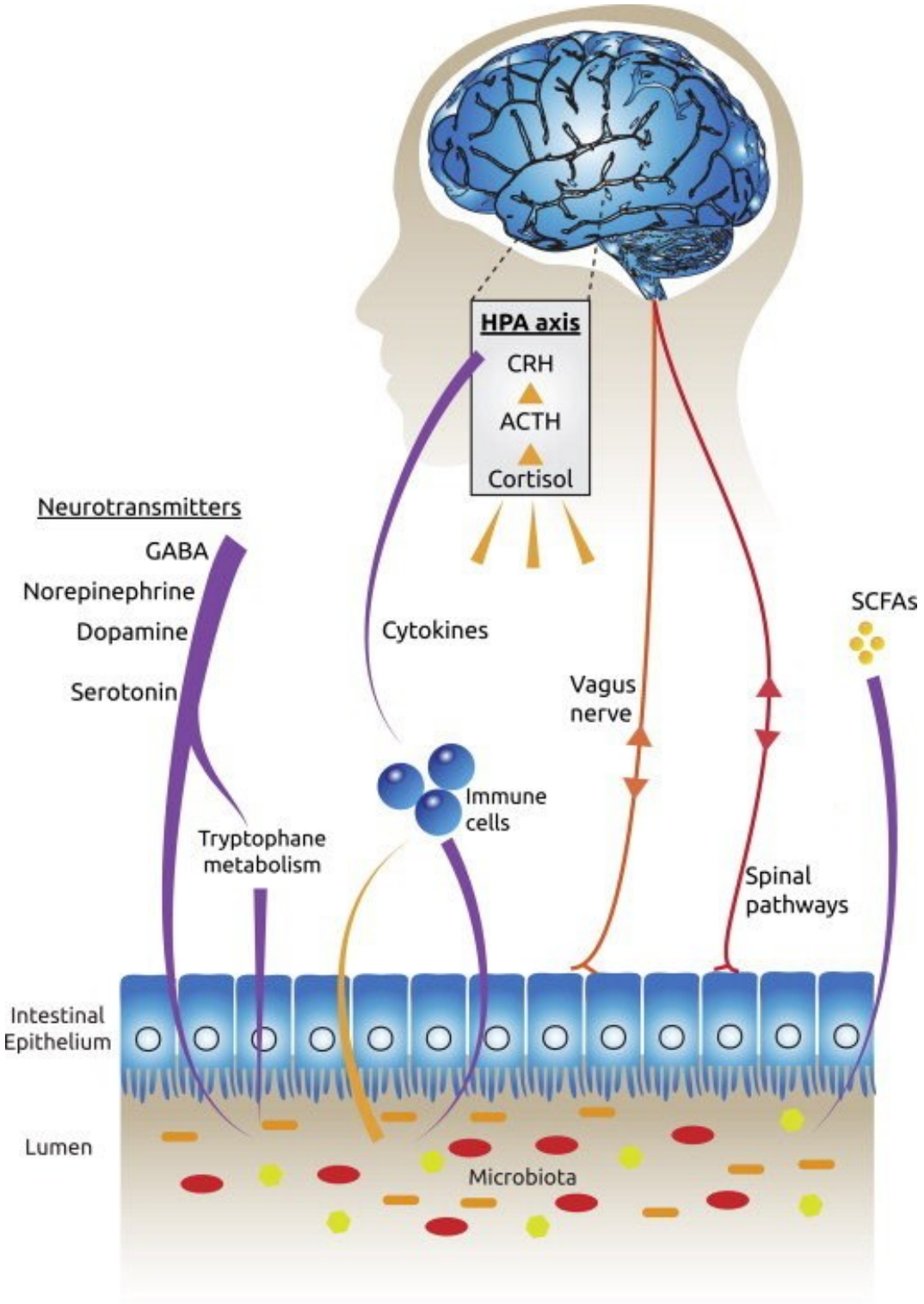


Figure 6. Schematic illustration of the complex and bidirectional communication network comprising the microbiota-gut-brain axis. Several lines of communication are active in the microbiota-gut brain axis. Neuronal transmission *via* the vagus nerve and spinal pathways links gut microbiota and the brain. Soluble factors like short-chain fatty acids (SCFAs), neurotransmitters, and cytokines produced by immune cells and gut microbiota circulate in the blood, eventually reaching and affecting the brain. Peripheral cytokines also modulate function of the hypothalamic-pituitary-adrenal axis that in turn affects immune cells (adapted from Dinan *et al*, 2015).

Providing a direct causal evidence for the influence of gut microbiota on brain and behaviour has been proven difficult. The first landmark study in 2004 demonstrated that in GF mice, the HPA axis reactivity is enhanced in response to stress (Clarke *et al*, 2013; Neufeld *et al*, 2011; Sudo *et al*, 2004). The same phenomenon is seen in GF rats (Crumeyrolle-Arias *et al*, 2014), demonstrating the importance of gut microbiota in the development of an appropriate stress response. Further, GF mice present a variety of behavioural alterations, like decreased anxiety- and depressive-like behaviour (Clarke *et al*, 2013; Heijtz *et al*, 2011; Luczynski *et al*, 2016; Zeng *et al*, 2016; Zheng *et al*, 2016) and altered social preference (Arentsen *et al*, 2015; Desbonnet *et al*, 2014). Comparable results are obtained after antibiotic application (Bercik *et al*, 2011a; Desbonnet *et al*, 2015). Curiously, in GF rats an increase in anxiety-like behaviour was observed (Crumeyrolle-Arias *et al*, 2014). GF mice also show an increased permeability of the BBB (Braniste *et al*, 2014) and changed neurotransmitter signalling as well as brain-derived neurotrophic factor expression in the limbic system (Arentsen *et al*, 2015; Clarke *et al*, 2013; Matsumoto *et al*, 2013; Neufeld *et al*, 2011; Sudo *et al*, 2004). After an immunological challenge, GF mice exert a blunted peripheral immune response (Clarke *et al*, 2013; Erny *et al*, 2015) and also brain immune function seems impaired. They present an immature microglia phenotype but increased microglial numbers and activity in various brain regions like the cortex and hippocampus, which can be replicated by antibiotic administration in normal mice (Erny *et al*, 2015). Interestingly, female GF mice show the same behavioural and immunological alterations as males but unchanged brain biochemistry (Clarke *et al*, 2013; Erny *et al*, 2015; Neufeld *et al*, 2011). Fascinatingly, Bercik and colleagues could show that anxiety-like behaviour can be transmitted *via* microbiota (Bercik *et al*, 2011a). In this study, microbiota samples are transferred from highly anxious NIH Swiss mice to GF BALB/c mice that initially showed normal anxiety-like behaviour, but adopt the behavioural phenotype after the transfer. Behavioural abnormalities, as well as a hyperactive HPA axis, could be partially reversed by early colonization with microbiota of healthy mice. However, this strongly dependent on the time point of application (Clarke *et al*, 2013; Sudo *et al*, 2004). In addition, impaired microglial functioning and maturity is restored after the application of a mixture of SCFAs (Erny *et al*, 2015), underlining the crucial regulatory role of gut microbiota on MGB axis functioning.

As GF mice show prominent alterations in both behaviour and physiology, the question arose if similar connections are found in the presence of psychiatric disorders. In a rat model of depressive-like behaviour, olfactory bulbectomy, rats show increased depressive-like behaviour accompanied by a shift in microbial composition (Park *et al*, 2013). In humans, a strong comorbidity between psychiatric disorders and gut-associated diseases is known. In detail, comorbidities of anxiety and depression in patients suffering from chronic gut disorders are frequent. 50 to 90 % of patients show symptoms of both and IBD patients experience a 3 times higher rate of depression (Fuller-Thomson and Sulman, 2006; Walker *et al*, 2008; Whitehead *et al*, 2002). MDD patients show a distinct alteration in microbial composition together with alterations in cytokine levels (Jiang *et al*, 2015; Kelly *et al*, 2016). Importantly, upon transplantation

of microbiota from depressed patients into microbiota-depleted rats, depressive-like symptoms occurred (Kelly *et al*, 2016), providing evidence for the importance of microbiota in the regulation of behaviour.

Considering the association between MGB axis functioning and psychiatric disorders, probiotic bacteria are proposed as a potential treatment option. In this context, the term 'psychobiotics' was coined, describing bacteria with beneficial effects on health (Dinan *et al*, 2013). Preclinical studies show promising results of the probiotics *Bifidobacterium longum* or *Lactobacillus rhamnosus* on anxiety- and depressive-like behaviour (Bravo *et al*, 2011; reviewed in Foster and McVey Neufeld, 2013; Mayer *et al*, 2015) as well as immune function (Desbonnet *et al*, 2010) and HPA axis activity (Ait-Belgnaoui *et al*, 2012, 2014) in rats and mice. However, in clinical studies the success is rather small. Aside a mixture of both bacteria alleviating psychological distress in healthy volunteers (Messaoudi *et al*, 2011), a meta-analysis of ten trials using psychobiotics in psychiatric disorders reveals only a general improvement in well-being (Dinan *et al*, 2013; Romijn and Rucklidge, 2015). Indeed, it was indicated that a stronger manipulation of microbiota might be necessary to achieve behavioural alterations in humans. Antimicrobials like minocycline (see chapter 1.2.3) might represent a valid alternative.

1.3.3 The short-chain fatty acid butyrate

SCFAs are monocarboxylic acids that are produced by microbiota. They are the main group of gut microbiota metabolites and important messengers in the MGB axis (Roy *et al*, 2006). Acetate, a C2 body, propionate, a C3 body, and butyric acid, a C4 body, occur in an approximate ratio of 60:20:20 in colon and faeces (Hallert *et al*, 2003; Hamer *et al*, 2008; Mortensen and Clausen, 1996; Topping and Clifton, 2001). The chemical structure of butyric acid is dependent on the surrounding pH. In the colon with a pH higher than 4.8, butyric acid dissociates almost completely into butyrate and H^+ (Fallingborg, 1999; Stilling *et al*, 2016). Therefore, in the present thesis it will be referred to as butyrate. Bacteria producing butyrate represent a functional rather than a phylogenetic group of gram-positive bacteria that are located almost exclusively in the colon. The two most important groups are the Clostridial Cluster IV and XIVa comprising Clostridium, Eubacterium, Lachnospiraceae, and multiple others. However, as these bacteria are anaerobic in nature, cultivation and identification of them has been proven rather difficult. Therefore, the mentioned bacterial cluster might be only a fraction of identifiable genera of butyrate-producing bacteria (for review see Louis and Flint, 2009; Pryde *et al*, 2002). Under physiological conditions, butyrate is synthesized almost exclusively by bacterial fermentation and utilized by surrounding bacteria. About 95 % is rapidly absorbed into adjacent tissue for energy metabolism and the blood stream at micromolar concentrations, though, limiting the site of action not only to the GIT (Canani *et al*, 2011; Duncan *et al*, 2009; Pryde *et al*, 2002). SCFAs utilize different transporters that are expressed on multiple organs including the GIT. Moreover, they are expressed on cells of the BBB and on neurons, astrocytes,

oligodendrocytes, and microglia in the brain (Bergersen *et al*, 2002; Cuff *et al*, 2002; for review see Ganapathy *et al*, 2008; Kim *et al*, 2014; Lee *et al*, 2012; Moreira *et al*, 2009; Vijay and Morris, 2014). High levels of butyrate appear to facilitate the production of the structurally related β-hydroxybutyrate (3-OH-butyrate; Hird and Symons, 1962; Iriki *et al*, 2009). 3-OH-butyrate is generally synthesized by host cells under conditions of ketogenic diet or fasting (Seyfried and Mukherjee, 2005), but also by astrocytes (Seyfried *et al*, 2005), and exploits the same transporters as SCFAs (Vijay and Morris, 2014). At target sites, SCFAs and 3-OH-butyrate bind to four different receptors (Kimura *et al*, 2011; Offermanns and Schwaninger, 2015; Singh *et al*, 2014b; Yonezawa *et al*, 2013).

Butyrate plays an essential role as a key regulator of colonic homeostasis and the main source for energy metabolism in intestinal epithelial cells, mediating a symbiotic relationship between host and microbiota (for review see den Besten *et al*, 2013; Clausen and Mortensen, 1995). In support, butyrate enhances epithelial barrier integrity and its producers are underrepresented in patients with IBD (Frank *et al*, 2007; for review see Galvez *et al*, 2005; Scheppach and Weiler, 2004). SCFA receptors are also found on numerous immune cells and well known for their immunomodulatory function (Kim *et al*, 2014), like suppressing colonic inflammation (Singh *et al*, 2014b) as well as IL-12 production in monocytes (Säemann *et al*, 2000). Importantly, butyrate was identified as a potent histone deacetylase inhibitor as it was shown that sodium butyrate (SB) enhances expression of genes involved in metabolism, cell proliferation, migration and differentiation, and considerably more (for review see Bourassa *et al*, 2016; Canani *et al*, 2012; Cavaleri and Bashar, 2018; Li *et al*, 2012; Stilling *et al*, 2016). Interestingly, histone deacetylase inhibitors have been hypothesized as therapeutic agent in mood disorders based on potent anti-depressive effects in preclinical studies (reviewed in Machado-Vieira *et al*, 2011). In recent years, butyrate came into focus in neuroscience research due to its versatile character and expression pattern of both transporters and receptors, demonstrating a passage into the brain. In the brain, butyrate and 3-OH-butyrate act neuroprotective and beneficial in learning and memory, and stimulate neurogenesis (Kim *et al*, 2009; for a detailed overview see Stilling *et al*, 2016; Zou *et al*, 2009). They exert their anti-inflammatory properties in the brain also by maintaining a mature microglial phenotype (Erny *et al*, 2015), inhibiting microglial activation or attenuating an overactivation (Fu *et al*, 2015; Huuskonen *et al*, 2004; Kim *et al*, 2004; Park *et al*, 2005), and eventually inducing microglial apoptosis (Chen *et al*, 2007). Short-term treatment with SB reduces the time spent immobile in the FST and TST and is able to reverse chronic mild stress- and early-life stress-induced depressive-like behaviour in male rats and mice without affecting locomotor activity (Han *et al*, 2014; Resende *et al*, 2013; Valvassori *et al*, 2015; Yamawaki *et al*, 2012). Chronic SB treatment also ameliorates innate depressive-like behaviour in Flinders Sensitive Line rats (FSL; Wei *et al*, 2015). In mice, chronic SB administration can reverse physiological as well as social defeat stress-induced depressive-like behaviour (Han *et al*, 2014; Tsankova *et al*, 2006) either alone or in combination with fluoxetine (Schroeder *et al*, 2007).

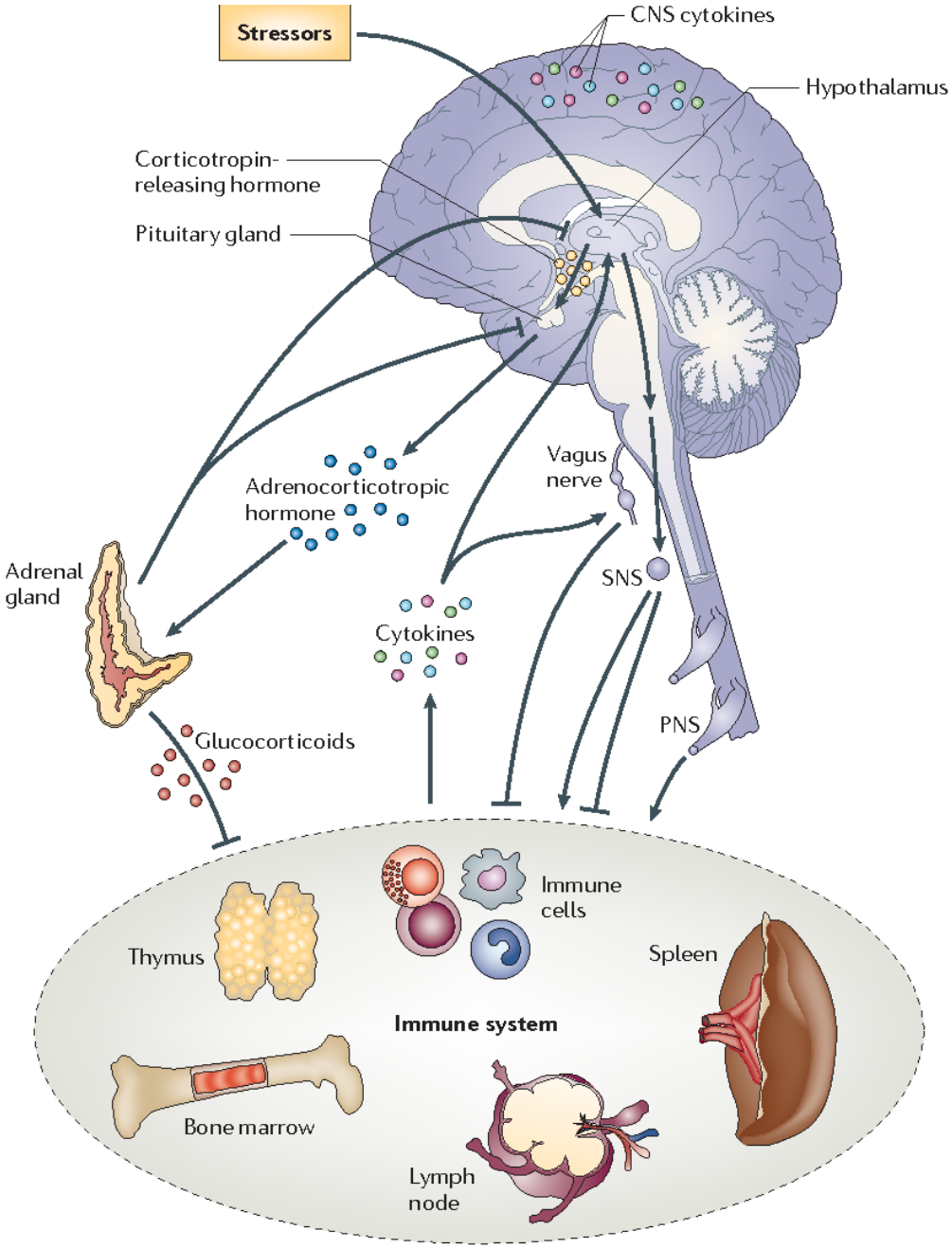
These studies provide evidence for a strong influence of butyrate and 3-OH-butyrate on behaviour in rodents. Considering the anti-inflammatory effects of butyrate, an immunomodulatory mechanism for behavioural manipulation seems likely. Thus, a well-considered application of butyrate might be a potential novel treatment option for psychiatric disorders.

1.4 Stress theory of psychiatric disorders

1.4.1 Stress and its responsive systems

The definition of a stressor states it as anything that disrupts the physiological balance of the body, independent of whether it is an actual or perceptual disruption, or threat to the intrinsic complex and dynamic equilibrium termed homeostasis (Goldstein and McEwen, 2002). Those stressors can be of physical or psychological nature, describing external challenges or anticipated disruptions of homeostasis, respectively. Therefore, stress describes a state in which homeostasis is threatened and the body adapts in behaviour and physiology to re-establish balance (Bartolomucci, 2007; Chrousos, 2009; McEwen, 2004). This reaction involves mainly two efficient and highly conserved physiological systems – the sympathetic nervous system and the HPA axis. The sympathetic nervous system provides an exclusively neuronal and immediate response to stressor exposure within seconds. It comprises neurons originating from the thoracolumbar regions of the spinal cord that project to effector organs and chromaffin cells in the medulla to trigger the release of adrenaline or noradrenaline (reviewed in Ulrich-Lai and Herman, 2009). In contrast, the hormonal driven HPA axis takes minutes to elicit its role. In response to a stressor, CRH and arginine vasopressin (AVP) are secreted from parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) into the portal blood stream to reach the anterior pituitary gland and trigger the synthesis of adrenocorticotropic hormone (ACTH). ACTH is released into the peripheral blood stream and by stimulating the cortex of the adrenal glands, synthesis and secretion of glucocorticoids (GC) into the blood stream is induced. Termination of this response is mediated *via* a negative feedback loop. GCs can cross the BBB and bind to glucocorticoid and mineralocorticoid receptors at different levels like the hypothalamus and the hippocampus (Harris *et al*, 2013; Lupien *et al*, 2009). Beside those two main systems, the immune system is an important mediator during stress as both pro- and anti-inflammatory cytokines regulate and are regulated by the stress response (Fig. 7; McEwen, 2008; Sternberg, 2006; Turnbell and Rivier, 1999). In more detail, cytokines like IL-1 can enter the brain and initiate CRH release in the hypothalamus to activate the HPA axis (Berkenbosch *et al*, 1987; Sapolsky *et al*, 1987; Turnbell and Rivier, 1999). On the other hand, GC can regulate multiple aspects of the immune response. Thus, GC can shift T cells from a pro- to an anti-inflammatory profile (Agarwal and Marshall Jr., 2001) and inhibit LPS-induced cytokine production in the periphery (Ma *et al*, 2004) and in the brain (Tanaka *et al*, 1997). By

inhibition of the expression of cell-adhesion molecules, GC also prevent immune cell migration (Pype *et al*, 1999).



F**igure 7. Schematic illustration of the hypothalamo-pituitary-adrenal (HPA) axis and its effects on the immune system.** Stressor exposure activates parvocellular neurons in the paraventricular nucleus of the hypothalamus that secrete corticotropin-releasing hormone (CRH) into the portal system of the median eminence. CRH reaches the anterior pituitary and induces synthesis and secretion of adrenocorticotropic hormone (ACTH) into blood vessels for systemic circulation. Upon reaching the inner adrenal cortex, ACTH initiates the synthesis and release of glucocorticoids into the blood stream. Cortisol modulates immune organs and cells and thus secretion of pro-inflammatory cytokines and their effects on brain functioning. Additionally, glucocorticoids act as a negative feedback loop at several levels to terminate the stress response and return to homeostasis. In addition, the sympathetic nervous system can activate or inhibit peripheral immune function (adapted from Sternberg, 2006).

Stress is a condition that accompanies daily life independent of species. Every threat to body homeostasis is answered by a stress response, pronounced as increased attention and arousal, energy mobilization, and increased cardiovascular and respiratory rates, whereas digestive and reproductive functions are inhibited. As the immune system is also inhibited by GCs, it enables the organism to react quickly to any change, a condition called "allostasis" (McEwen, 1998, 2008). Keeping this balance increases the chance of an individual's survival. However, if the essentially beneficial acute stress response exceeds a certain severity or temporal threshold, meaning it is inadequate, prolonged, or excessive (also called "allostatic load or overload"), it has detrimental consequences on behaviour and physiology. These are caused by changes in the brain and a resulting impaired ability to appropriately regulate and terminate the stress response (for review see de Kloet *et al*, 2005; McEwen, 2008). Evidently, the stress response is meant to be of a limited duration and intensity, the parameters that generally determine the definition of stress. Acute stress is described to last minutes to hours whereas chronic stress persists for days or even months (Dhabhar, 2000). Further, stress intensity is recognizable in physiological parameters like heart rate, blood levels of GC (cortisol in humans and corticosterone in rodents; hereafter CORT) and catecholamines, or levels of cytokines. In acute stress, an increase in plasma GC levels after 15-30 min will return to baseline after 60 to 120 min (de Kloet *et al*, 2005). Contrary, in chronic stress a persistent elevation (hypercorticism) or reduction (hypocorticism) of CORT is noticed (Albeck *et al*, 1997; Heim *et al*, 2000; Schmidt *et al*, 2010b). Interestingly, also unchanged basal plasma CORT levels are found after chronic stress (Reber *et al*, 2007) accompanied by a sensitized reaction to a heterotypic stressor (Uschold-Schmidt *et al*, 2012). In general, the HPA axis adapts to chronic or repeated exposure to a homotypic stressor as a protective mechanism of the body from harmful long-term CORT stimulation. These adaptions are found on the level of the adrenal gland (Uschold-Schmidt *et al*, 2012) as well as a compromised negative feedback loop of the HPA axis (Aguilera, 1994).

1.4.2 Stress in psychiatric disorders

Acute and chronic stress have both been accepted as potent risk factors for somatic and mental disorders (Langgartner *et al*, 2019; Lupien *et al*, 2009; Masis-Calvo *et al*, 2018; McEwen, 2007). The exposure to a stressor, even if only for a short period, can lead to the development of allergic manifestations, migraines, hyper- or hypotensive attacks, panic attacks, and psychotic episodes. On the other hand, chronic stress has been shown to cause severe somatic and mental diseases like cardiovascular diseases, IBD, anxiety, depression, and neuroendocrine and immune dysregulation (Chrousos, 2009; Langgartner *et al*, 2015). Today, most of the challenges humans face on a daily basis are work-related and in a social context. In this type of chronic stress, internal believes of worthlessness, performing insufficiently, and competition for resources and social rank are combined with stressful social situations (Cohen *et al*, 2007; Tamashiro *et al*, 2005). Thus, chronic psychosocial stress, the combination of psychological and social stress,

represents a potent natural stressor that has been accepted as one of the major risk factors for the development of a variety of disorders (Lupien *et al*, 2009; Masis-Calvo *et al*, 2018).

A mechanism common throughout stress-induced diseases is reduced GC signalling. Mice exposed to chronic psychosocial stress reliably show a reduced HPA axis activity and a GC insensitivity of adrenals, but a hyperactive HPA axis in response to a heterotypic stressor (Langgartner *et al*, 2015). Further, in a model of innate depression and anxiety, rats selectively bred for low- or high anxiety, a pathological dexamethasone suppression test is shown (Keck *et al*, 2002). This phenomenon also visible in MDD patients and a sign of a dysregulated negative feedback loop (Holsboer, 2000). Patients suffering from stress-related diseases like geriatric depression or fibromyalgia also show hypocorticism (Heim *et al*, 2000) which correlated with the onset of IBD (Reber, 2012). In MDD patients, the most consistently reported abnormality is an elevation in plasma cortisol and CSF CRH levels. In addition, they present a blunted stress reactivity and an impaired recovery, as well as increased levels of *CRH* mRNA and protein in limbic brain regions (Burke *et al*, 2005; Dinan and Cryan, 2016; Merali *et al*, 2004). Interestingly, *in vitro* and *in vivo* stimulation revealed a reduced GC response in those patients, pointing rather towards a GC resistance that is probably caused by reduced receptor expression and / or functionality (Holsboer, 2000; Langgartner *et al*, 2015; Pariante and Miller, 2001). These changes in GC signalling show the significance of a balanced stress response to react appropriately to a disruption of homeostasis. Moreover, GC resistance and thus reduced CORT signalling potentially leads to a disinhibition of immune function and a pro-inflammatory state in patients. GCs are well accepted as modulators of the immune response (Dhabhar, 2009; Kadmiel and Cidlowski, 2013; Pruett, 2003) and a GC resistance of certain immune cells has been proposed to contribute to chronic stress-induced inflammation in both rodents and humans (Foertsch *et al*, 2017; Miller and Raison, 2016). This inflammatory overactivation or chronic low-grade inflammation has been shown several times in both clinical and pre-clinical studies as connected to mental disorders (Engler *et al*, 2017; Eraly *et al*, 2014; Hodes *et al*, 2014; Kivimäki *et al*, 2014; Langgartner *et al*, 2015; Rohleder, 2014; Stefanski and Engler, 1998). Importantly, in a landmark study, Hodes and colleagues could provide evidence that in mice, psychosocial stress-induced inflammation is causative for the development of anxiety- and negative affective-related responses (Hodes *et al*, 2014). Contrary, IL-6 KO mice appear to be resilient to stress (Hodes *et al*, 2014) and immunomodulation prior to chronic stress can act stress-protective (Reber *et al*, 2016b, 2016a). Given the aforementioned association between inflammation and psychiatric disorders (see chapter 1.2.2), an interplay between chronic stress and the immune system as underlying pathophysiology of psychiatric disorders seems highly likely.

1.5 Rodent models of psychiatric disorders

Animal models are essential experimental tools to study and potentially unravel the mechanisms underlying a particular pathophysiology. In general, three strategies are utilized to develop animal models for research in psychiatric disorders: genetic manipulation, selective breeding to obtain a particular phenotype, and environmental manipulations that can be combined (reviewed in Neumann *et al*, 2011; Slattery and Cryan, 2014). However, to obtain an appropriate animal model, three basic criteria have to be fulfilled - construct, face, and predictive validity. These three aspects state that a valid animal model should have the same causative mechanisms and underlying theories (construct validity), a similar phenotype (face validity), and a comparable reaction to manipulation (predictive validity) as the human disease it should mimic (Cryan *et al*, 2002; Geyer and Markou, 1995; Slattery and Cryan, 2014). Most animal models fulfil at least face and predictive validity. Genetic manipulation is performed mainly in mice and an endless number of knockout (KO) lines has been developed and characterized. Those line target selected genes potentially involved in anxiety- and depression-related behaviour, and stress-sensitivity (for review see Neumann *et al*, 2011). Selective breeding represents a more natural approach as the animal itself is not externally manipulated. Hence, it enables the research of underlying mechanisms of idiopathic diseases without the influence of a previous manipulation. Among many others, the FSL rats are commonly used as a model for depressive-like behaviour (Overstreet, 1993; Overstreet and Wegener, 2013). Similarly, rats or mice selectively bred for high (HAB) and low (LAB) anxiety-like behaviour are a valid model for anxiety-like behaviour with a concomitant depressive-like phenotype (Landgraf *et al*, 2007), to only name two. Utilizing an environmental approach, the chronic subordinate colony housing is a mouse model of chronic psychosocial stress that, unlike other stress models, leads specifically to the development of anxiety- but not depressive-like behaviour. Therefore, it offers the unique opportunity to dissect those two psychiatric disorders and their underlying pathophysiological mechanisms.

1.5.1 Rats selectively bred for high and low anxiety-like behaviour

An excellent example for successful selective breeding to gain a better insight and reveal neuroendocrine, neuronal, and neurogenetic parameters involved in anxiety-related behaviour, are HAB and LAB rats. In 1993, commercially available Wistar rats were tested for their anxiety-like behaviour on the elevated plus-maze (EPM) at the age of 9 to 10 weeks and classified according to the percentage of time spent on the open arm as HAB (< 10 %) or LAB (> 50 %) rats (Landgraf and Wigger, 2002; Liebsch *et al*, 1998a). Continuous and bidirectional breeding eventually resulted in the HAB and LAB breeding lines. The highly anxious phenotype was shown in several behavioural tests like a reduced centre time in the open field (Liebsch *et al*, 1998b), increased time in the dark compartment in the light-dark box (Slattery and

Neumann, 2010), less time on the board in the modified hole board test (Ohl *et al*, 2001) and impaired fear extinction in cued fear conditioning (Muigg *et al*, 2008). The specific phenotype of those lines was shown to be stable over years and independent of season (Beiderbeck *et al*, 2012; Liebsch *et al*, 1998b; Neumann *et al*, 2010). Furthermore, the phenotype remained unchanged independent of sex (Bosch and Neumann, 2008; Landgraf *et al*, 2007) and age (Landgraf and Wigger, 2002) across several European laboratories (Salome *et al*, 2002; Veenema *et al*, 2007). Concomitant with high anxiety-related behaviour, this breeding line yields other behavioural abnormalities. Compared to LAB rats, HAB rats showed reduced locomotor activity in the open field (Liebsch *et al*, 1998b) an on the EPM but unchanged baseline locomotion in the home cage (Liebsch *et al*, 1998a; Slattery and Neumann, 2010), increased time spent floating during the FST, indicative for a depressive-like phenotype, and increased risk assessment (Ohl *et al*, 2001). Further, these two breeding lines differ in social and aggressive behaviour. LAB rats spend less time in social contact with cage mates (Ohl *et al*, 2001), showed impaired abilities in a social discrimination task (Landgraf and Wigger, 2002), and even a lack of social preference in the social preference test (Beiderbeck *et al*, 2014). The low anxiety-like phenotype in male LAB rats is also accompanied by abnormal levels of aggression (reviewed in Neumann *et al*, 2010). HAB rats show normal aggressive and social behaviour in general. However, during lactation HAB dams display higher levels of maternal aggression and maternal care (reviewed in Bosch, 2011). On a physiological level, HAB rats show an altered stress reactivity (Liebsch *et al*, 1998b, 1998a). Thus, a hyper-responsive HPA axis activity potentially caused by a hyperdrive of the AVP system (Keck *et al*, 2002; Landgraf *et al*, 1999) and a pathological dexamethasone/CRH challenge test are observed (Keck *et al*, 2002). In addition, they show vegetative and sleep dysregulations under basal conditions and in response to an acute stressor (Carnevali *et al*, 2016; Landgraf and Wigger, 2002). The exact underlying mechanism remains unclear, however a single a nucleotide polymorphism (SNP) located in the AVP promoter region (Murgatroyd *et al*, 2004) as well as increased neuropeptide S functional activity, due to a synonymous SNP in the gene coding for its receptor (Slattery *et al*, 2015), in HAB rats strongly propose a genetic background. As a consequence, HAB rats show increased synthesis and release of AVP in several brain regions compared to LAB rats (Bosch *et al*, 2006; Bosch and Neumann, 2008, 2010; Keck *et al*, 2002; Wigger *et al*, 2004), probably causing the highly anxious behaviour. Moreover, in the PVN of HAB rats increased levels of *CRH* mRNA were found compared to LAB rats. An increased HPA axis reactivity is closely connected to psychiatric disorders like anxiety, which likely contributes to the behavioural phenotype (Bosch *et al*, 2006).

A single injection with the benzodiazepine diazepam is able to reduce the thermal pain threshold and alleviate anxiety-like and depressive-like behaviour in HAB rats and enhance time on the open arm in LAB rats (Jochum *et al*, 2007; Liebsch *et al*, 1998a). When the SSRI paroxetine was administered in a chronic regimen, 8 weeks of treatment improved depressive-like behaviour as well as pain sensitivity and normalized HPA axis reactivity and *AVP* mRNA levels in HAB rats (Keck *et al*, 2003). Another SSRI,

citalopram, alleviates the high-anxiety phenotype in HAB rats after the same treatment regimen (Jochum *et al*, 2007).

The visible differences in behaviour and physiological parameters in HAB rats that are also present in patients suffering from anxiety disorders or depression, propose HAB rats as a valid model to study the underlying mechanisms for innate anxiety and comorbid depression. Further, since 8 weeks of paroxetine treatment were necessary to alleviate symptoms, HAB rats might be a feasible model to study treatment-resistant diseases.

1.5.2 Chronic subordinate colony housing

Traditionally, animal models of chronic non-social stress are composed of repeated and/or permanent applications of uncontrollable and unpredictable stressors like noise, restraint or forced-swim stress, predator cues, shaking, or a combination of these that induce measurable changes on a molecular, behavioural and physiological level (Bartolomucci *et al*, 2005; Willner, 1997). However, these models do not mimic a natural stressful situation for an individual, as the majority of disease-promoting stressful stimuli in humans are of psychological or social nature. Therefore, animal models for chronic psychosocial stress such as submission, social defeat, or social exclusion are of higher clinical relevance. Nowadays, several models are commonly used in research like the chronic social defeat stress model during adolescence (Schmidt *et al*, 2007) or during adulthood (Berton *et al*, 2006), the chronic intermittent social defeat/overcrowding model (Reber *et al*, 2006), or the chronic subordinate colony housing (CSC; Reber *et al*, 2007). Interestingly, even though these models differ in their detailed procedure, like duration and frequency of stress, the outcome is largely comparable with high face and predictive validity. All of these models primarily result in thymus atrophy, adrenal hypertrophy, HPA axis dysregulation, as well as elevated levels of anxiety-related or depressive-like behaviour (Berton *et al*, 1998; Gruver and Sempowski, 2008; Heinrichs *et al*, 1992; Keeney and Hogg, 1999; Reber *et al*, 2007; Stefanski and Engler, 1999).

The recently developed CSC paradigm combines psychological and social aspects of stress continuously for 24h on 20 consecutive days. This very robust and reliable animal model comprehensively mimics the type of health-compromising stressors human face on a daily basis, thereby providing strong construct validity. Importantly, the CSC paradigm is based on natural behaviour in mice utilizing the natural drive of male mice to establish a hierarchical order within a given colony. The largest male will (usually) emerge as dominant and eventually force the other mice into a subordinate position. In the CSC paradigm, four experimental mice (CSC mice) are housed together on day 1 with a larger, dominant resident, resulting in immediate subordination of the intruder CSC mice. To counteract any possible habituation, CSC mice are transferred into the homecage of another dominant resident on day 8 and 15, adding an unpredictable

and uncontrollable component to the model. The formation of the hierarchical structure normally occurs during the first 30 min of the encounter, however a dominant position of the resident is not guaranteed. Thus, this critical timeframe is analysed to confirm the subordinate position of each CSC mouse. Resident males are trained before CSC housing to avoid extensive aggressive behaviour and harmful attacks towards the CSC mice and reliably (>99 % of CSC colonies) obtain the dominant position. This is displayed by aggressive attacks, threatening and offensive behaviour towards CSC mice like chasing or mounting. CSC mice in turn show an subordinate position by defensive behaviour like submissive upright and flight (Reber *et al*, 2007; Reber and Neumann, 2008a). As appropriate controls, single-housed mice are used (SHC; Reber and Neumann, 2008a; Singewald *et al*, 2009). The CSC procedure promotes the long-lasting (at least 8 days after stressor termination; Slattery *et al*, 2012) development of both somatic and affective disorders as well as reduced GC signalling (high face validity), thereby representing a potent model to study the underlying mechanisms of relevant stress-induced pathologies.

In detail, 19 consecutive days of CSC induce several symptoms indicative for chronic stress like altered body weight gain, enlarged adrenal glands, pituitary and spleen, as well as decreased thymus weight (Reber *et al*, 2007; Uschold-Schmidt *et al*, 2012). As reported previously (Choi *et al*, 2006; Razzoli *et al*, 2006, 2011; Zelena *et al*, 1999), during the CSC procedure, both decreased (Peters *et al*, 2012; Reber *et al*, 2007, 2008; Reber and Neumann, 2008a; Schmidt *et al*, 2010a; Singewald *et al*, 2009) as well as unchanged body weight (Füchsl *et al*, 2014; Slattery *et al*, 2012; Veenema *et al*, 2008) are observed. Notably, after the CSC procedure, CSC mice gain significantly more weight compared to SHC controls (Slattery *et al*, 2012), proposing a potential compensatory mechanism to ensure sufficient energy supply in preparation for subsequent stressful events. Beside changes in body weight, the HPA axis is dysregulated in the CSC model, a symptom often seen in patients suffering from MDD or anxiety disorders (Faravelli *et al*, 2012; Lamers *et al*, 2013). Enlarged adrenal glands are mediated by cell hyperplasia jointly with a reduced *in vitro* responsiveness of adrenal explants to an ACTH challenge. This adrenal insensitivity seems not to be restricted to *in vitro* stimulation of adrenal explants. Though morning basal plasma CORT levels remain comparable to those of SHC mice, plasma ACTH levels can be elevated (Reber *et al*, 2007; Uschold-Schmidt *et al*, 2012; Veenema *et al*, 2008). Contrary, CSC mice show hypocorticism in the evening as they appear to be unable to mount the circadian rise in plasma CORT (Reber *et al*, 2007). Interestingly, CSC mice react to a mild heterotypic stressor (like exposure to an elevated platform) with exaggerated plasma CORT levels, while for an increase in ACTH levels the encounter with a strong heterotypic stressor like 6 min of forced-swim stress was necessary (Füchsl *et al*, 2013; Uschold-Schmidt *et al*, 2012). Likewise, an enlarged pituitary is caused by cell hyperplasia and accompanied by the increased capability to produce and secrete ACTH (Füchsl *et al*, 2013). Thus, the HPA axis and GC signalling is dysregulated on several levels, though the feedback loop appears to be uncompromised (Füchsl *et al*, 2013). An exaggerated CORT response is nevertheless present after a heterotypic stressor, proposing the activation of an alternative

mechanism. Exposure to CSC also results in spontaneous colitis (Langgartner *et al*, 2017b; Reber *et al*, 2007, 2011) and aggravated DSS-induced colitis, indicated by an increase in the histological damage score (Reber *et al*, 2008; Veenema *et al*, 2008), and colorectal cancer (Peters *et al*, 2012). Of note, DSS-induced colitis is a frequently used model for IBD which is closely associated with chronic stress and mood disorders and presents a high comorbidity in humans (Fuller-Thomson and Sulman, 2006; reviewed in Hibi *et al*, 2002; Maunder, 2005). Concomitantly, relative mRNA expression of IL-1β, TNF-α, and IFN-γ is increased in the colon (Langgartner *et al*, 2017b). Also, lamina propria mononuclear and mesenteric lymph node cells secrete more cytokines, the colon contains more macrophages, dendritic cells and T helper cells, and a splenomegaly is observed (Foertsch *et al*, 2017; Reber *et al*, 2007, 2008, 2011). This chronic low-grade inflammation after 20 days of CSC is probably mediated by the reduced adrenal sensitivity and a GC resistance in certain immune cell subpopulations like splenocytes. As mentioned in chapter 1.4.2, GC resistance-induced low-grade inflammation is associated with mental disorders, thus supporting the CSC as a valid model for chronic psychosocial stress. In line, the CSC model was shown to induce several behavioural phenotypes. Hence, increased ethanol intake already after 14 days of CSC (Peters *et al*, 2013) as well as hyperactivity during the dark phase is observed one week after stressor termination (Slattery *et al*, 2012). Further, the CSC model profoundly and reliably increases general anxiety-related behaviour as seen in at least five different behavioural tests. CSC mice show a decreased time in the light box of an light-dark box (Peters *et al*, 2013; Reber and Neumann, 2008a) and reduced percentage of time spent on the open arm of an elevated plus-maze (Reber *et al*, 2007, 2008, 2016b; Reber and Neumann, 2008a), as well as reduced time in its distal part during open arm exposure (Singewald *et al*, 2009). During the open field test, CSC mice entered the centre zone and explored a novel object less (Langgartner *et al*, 2017b; Slattery *et al*, 2012; Veenema *et al*, 2008) and spent more time in the centre zone of an elevated platform (Uschold-Schmidt *et al*, 2012). CSC exposure does not induce social anxiety but rather a lack of social preference that is reversed after 7 days. Importantly, the anxiety-like phenotype is not, in contrast to other chronic stress models, accompanied by increased depressive-like behaviour (Slattery *et al*, 2012). Taken together, the CSC model represents a powerful tool to unravel the underlying mechanisms of chronic psychosocial stress-induced somatic and affective pathologies, in particular the highly anxious phenotype as well as systemic low-grade inflammation (Fig. 8), underlining the translational value.

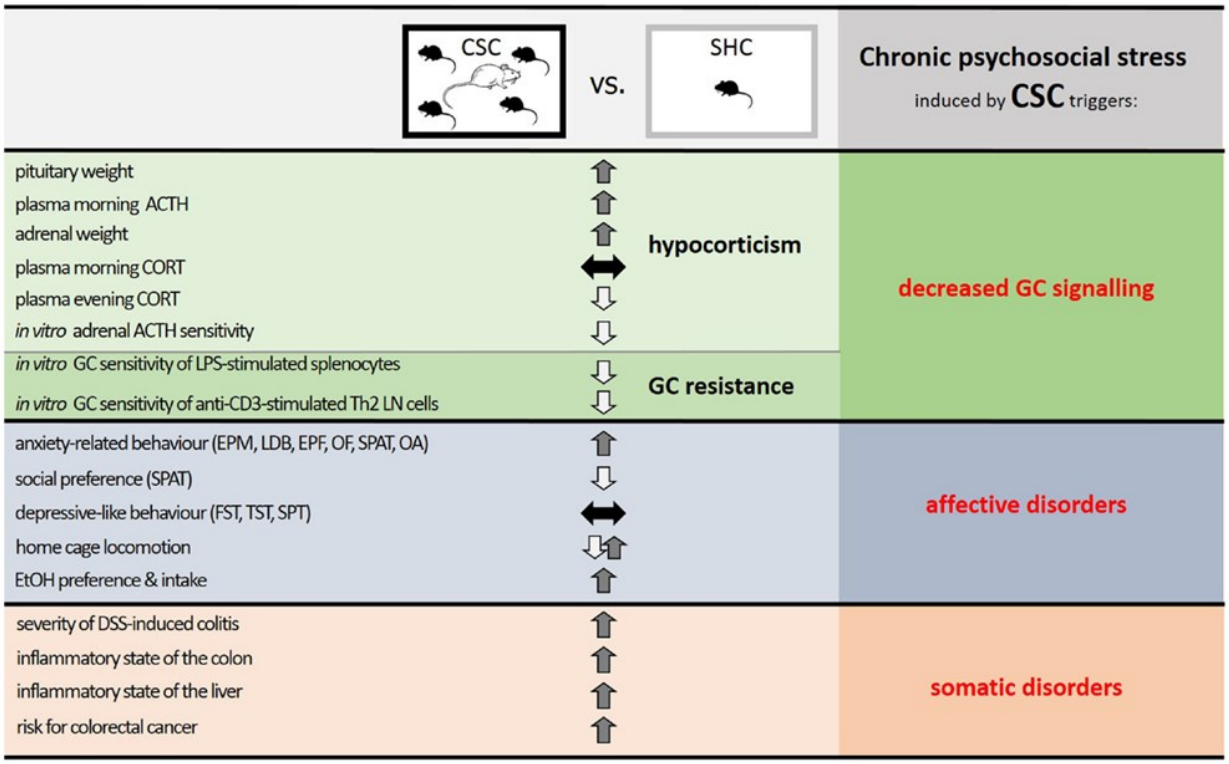


Figure 8. Summary of the main effects of the chronic subordinate colony housing (CSC) paradigm on physiological, behavioural, and immunological parameters in male mice. After 20 days of CSC exposure, CSC mice show, compared to single-housed controls (SHC), alterations in glucocorticoid (GC) signalling as well as affective and somatic alterations. CSC mice develop reduced GC signalling on several levels as well as affective and somatic changes. Given that in humans chronic psychosocial stress can lead to somatic and affective disorders in combination with reduced GC signalling, the CSC model represents a promising tool to mimic stress-related pathologies and unravel the underlying mechanisms (adapted from Langgartner *et al*, 2015).

1.6 Aims of the present thesis

Given the imperfect treatment options in psychiatric disorders with a rate of 30 % non-responders, the twice as high prevalence in women compared to men, and a clear involvement of both the inflammatory system and the MGB axis as novel treatment approaches, the experiments performed during the present thesis aimed to:

1) Evaluate the effects of chronic treatment with minocycline on depressive- and anxiety-like behaviour in HAB and non-selected (NAB) rats in a sex-dependent manner followed by a detailed analysis of the underlying systemic mechanisms.

2) Verify the CSC paradigm in mice and investigate potential beneficial effects of minocycline on chronic stress-induced anxiety-like behaviour and other physiological symptoms induced by chronic psychosocial stress.

1.6.1 Evaluation of minocycline in a rat model of innate anxiety and depression

Advancing research within the last decades paved the way for innovative approaches in the treatment of psychiatric disorders. A number of studies provide striking evidence for a regulatory role of gut microbiota in the development of the brain and peripheral immune system as well as appropriate behaviour, while a dysbiosis in gut microbiota composition has been associated with psychiatric disorders like MDD. At the same time, an imbalanced inflammatory system was strongly connected to the development of affective diseases. Minocycline showed promising results in the treatment of psychiatric disorders, presumably *via* its anti-inflammatory and bactericidal effects, and produced the first clinical results as augmentation treatment. Therefore, 4 main questions emerged:

i) Can the previously indicated antidepressant and anxiolytic effect of minocycline alone or as augmentation to escitalopram ameliorate the behavioural phenotype of HAB rats in comparison to NAB rats?

ii) Are the effects of minocycline comparable in male and female HAB or NAB rats?

iii) Are HAB rats characterized by alterations in the number of microglia within the PFC, and are the behavioural effects of minocycline connected to changes in brain microglia?

iv) Does the gut microbiota composition differ between HAB and NAB rats and does minocycline exert differential effects on their gut microbiome?

These questions were addressed by treating male and female HAB and NAB rats with minocycline, escitalopram, or a combination of both, for 3 weeks. Concomitant with a beneficial behavioural effect, an alteration in PFC microglial density and – due to its antibacterial effect – gut microbiota composition was

predicted. Aiming to connect peripheral and central systems and support a complex interplay as depicted in the MGB axis, peripheral immune responses as well as the gut microbiome and one of its metabolites were analysed.

1.6.2 Verification of the CSC paradigm and potential beneficial effects of minocycline

An essential approach to mimic psychiatric disorders besides selective breeding is environmental manipulation. The CSC paradigm, as an animal model of chronic psychosocial stress, induces concomitant affective and somatic alterations. The specific induction of anxiety-, but not depressive-like, behaviour after CSC exposure offers a unique opportunity to dissect the impact of minocycline on anxiety-like behaviour and on innate *versus* stress-induced parameters. The following research questions emerged in this context:

i) Is the CSC paradigm robust enough and can – as a prerequisite for subsequent experiments – be re-established after relocation of the laboratory into a new facility?

ii) Is treatment with minocycline able to reverse CSC-induced behavioural and physiological symptoms?

To answer these questions, the CSC paradigm was re-established and, subsequently, minocycline was administered for 8 days after termination of the CSC paradigm. Following, selected stress-related parameters as well as HPA axis functionality were assessed.

Material and Methods

The Material and Methods include sections taken, and adapted, from one first author publication: Anna K. Schmidtner, David A. Slattery, Joachim Gläsner, Andreas Hiergeist, Katharina Gryksa, Victoria Malik, Julian Hellmann-Regen, Isabella Heuser, Thomas C. Baghai, André Gessner, Rainer Rupprecht, Barbara Di Benedetto, Inga D. Neumann. **Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner.** *(In preparation)*

Individual contributions of external authors are indicated in the respective method.

2.1 Effects of minocycline, escitalopram, or a combination of both on rats

The following methods were utilized to assess the influence of minocycline, escitalopram, or a combination of both substances on social, anxiety-, and depressive-like behaviour. In addition, the influence on the peripheral immune system, the immune system of the brain, and the microbiome were analysed.

2.1.1 Animals and husbandry

Male and female NAB rats were either purchased from Charles River (200 – 250 g; Sulzfeld, Germany) or, like all male and female HAB rats, bred at the University of Regensburg. All rats were housed in groups of 3 – 4 in standard polycarbonate rat cages according to their experimental group (i.e. sex, trait and treatment). Criteria for selection as a HAB rat was met if the rat spent < 10 % of time on the open arm of an EPM at the age of 9 weeks (Landgraf and Wigger, 2002). Age- and sex-matched rats were used as social stimuli in the social preference / avoidance test (SPAT). All rats were housed under standard laboratory conditions (12 h light : dark cycle, lights on at 06:00 h, 22 ± 1 °C, 55 ± 5 % humidity) with free access to food and tap water (vehicle) or drug solution (minocycline and / or escitalopram) and allowed to habituate to room conditions at least 7 days prior to experiments. The experiments were approved by the Committee on Animal Health and Care of the local government, and performed according to the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz, the ARRIVE guidelines (Kilkenny *et al*, 2010), and recommendations from the NIH. All efforts were made to minimize the number of animals used and their suffering.

2.1.2 Experimental protocol

At the age of 11 – 12 weeks, rats were assigned to the respective treatment and the effects of minocycline hydrochloride (kindly provided by Charité Berlin), escitalopram oxalate (kindly provided by Lundbeck A/S), or a combination of both substances on social, anxiety-, and depressive-like behaviour were evaluated. In the first set of experiments, male and female HAB and NAB rats received 40 mg / kg / day minocycline, 10 mg / kg / day escitalopram, or a combination of both substances with the same concentrations dissolved in tap water for 22 days (n = 8 – 16 per group). The doses were chosen based on previous studies (Hinwood *et al*, 2012, 2013; Jayatissa *et al*, 2006; Liu *et al*, 2007; Raghavendra *et al*, 2003). In a second cohort of animals, 80 mg / kg /day minocycline, based on a recent study (Amorim *et al*, 2017), was administered in the same fashion exclusively to male and female HAB rats (n = 9 – 10 per group). Body weight and fluid consumption were monitored daily to calculate the respective concentrations needed for oral drug

administration *via* drinking water and to prepare fresh solutions daily. On day 15 of treatment, social behaviour was tested in SPAT, followed by the evaluation of anxiety-like behaviour in the light-dark box (LDB; day 17) and on the EPM on day 19 of treatment. To analyse depressive-like behaviour, the pre-swim (day 21) and the FST on day 22 were performed (Fig. 9). The order of tests was chosen to minimize test interactions by introducing the least stressful experiment first (SPAT) and the most stressful one last (FST). Vaginal smears were taken after the EPM and FST to control for oestrus cycle-dependent effects. Immediately after the FST, rats were deeply anesthetized and transcardially perfused. Blood was collected from the right atrium, stored in EDTA-coated tubes (Sarstedt, Nürnbrecht, Germany) on ice and centrifuged at 5000 rpm for 10 min at 4 °C. Plasma was stored at - 20 °C for determination of cytokine and microbiome metabolite concentrations. Liver and fecal boli were collected, snap-frozen using dry ice and stored at - 80 °C until measurement of minocycline concentrations with high-pressure liquid chromatography (HPLC). Cecal samples were collected from the cecal pouch and frozen on - 80 °C for microbiome analysis. All microbial samples were collected carefully under sterile conditions to avoid contamination.

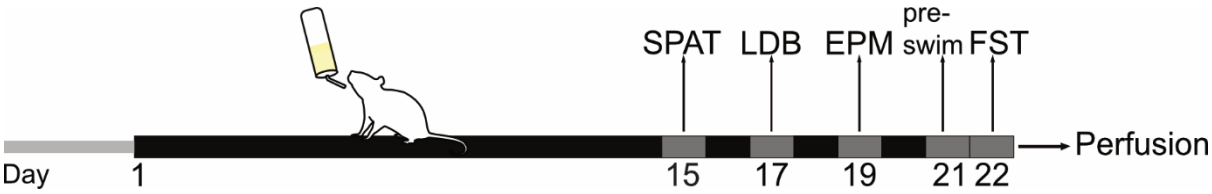


Figure 9. Experimental time course of male and female NAB and HAB rats treated with either vehicle, minocycline, escitalopram, or a combination of both, for 22 days in the drinking water. 15 days after beginning of the treatment, social preference was tested in the social preference / avoidance test (SPAT), followed by the evaluation of anxiety-like behaviour in the light-dark box (LDB) on day 17 and on the elevated plus-maze (EPM) on day 19. Depressive-like behaviour was tested on day 21 in the pre-swim and analysed on day 22 during the forced-swim test (FST). Afterwards, all rats were deeply anesthetized and transcardially perfused.

2.1.3 Behavioural testing

All behavioural experiments were performed during the day to avoid bias of dark-cycle locomotor activity changes (09:00 to 12:00) except the SPAT, which was performed during the socially active phase of rats (19:00 to 21:00; Lukas *et al*, 2011). Behaviour was recorded by an overhead camera and analysed by an observer blind to the treatment or *via* automated scoring using NOLDUS software (Ethovision XT version 12, NOLDUS information technology, Wageningen, Netherlands).

2.1.3.1 Social preference / avoidance test

The SPAT was conducted using a previously described adapted protocol (Lukas *et al*, 2011) to test for social motivation and interaction. Briefly, during the active phase (1 h after lights off) rats were placed into a novel arena (40 x 80 x 40 cm; red light) and granted 30 s of habituation. Afterwards, an empty wire-mesh cage (non-social stimulus; 20 x 9 x 9 cm) was placed at one short sidewall of the arena for 4 min of free exploration. The empty cage was then replaced by an identical one containing an unknown sex- and weight-matched conspecific (social stimulus) for an additional 4 min period. Before each trial, the arena was cleaned thoroughly with water containing a low concentration of detergent. Each rat was recorded and behaviour was analysed using JWatcher behavioural observation software (V 1.0, Macquarie University and UCLA). The time spent with active olfactory investigation of the non-social and social stimulus (investigation time) was used as an indicator for social preference.

2.1.3.2 Anxiety-like behaviour

To assess levels of innate non-social anxiety-like behaviour, two different tests were performed: the LDB and the EPM. These tests are based on a natural conflict between the animal's exploratory drive and the innate fear of open and lit areas. The more anxious an animal, the less time it spends in exposed, potentially threatening areas and *vice versa*.

2.1.3.2.1 Light-dark box

The rat LDB was performed as previously described (Slattery and Neumann, 2010; Waldherr and Neumann, 2007) to evaluate both anxiety-like behaviour and locomotion. It consists of two compartments: a light (40 x 50 cm, 100 Lux) and a dark box (40 x 30 cm, 0 Lux) connected by a small opening (7.5 x 7.5 cm) to enable transitions between the compartments. Rats were placed into the light box facing away from the opening and recorded for 5 min. The time spent in each box was scored live as indicator for anxiety-like behaviour using the plus-maze DOS program (©Ernst Fricke, 1993). As a measure of locomotor activity, total distance travelled was assessed using automated scoring.

2.1.3.2.2 Elevated plus-maze

As the second test for anxiety-related behaviour, rats were tested on the EPM (Pellow and File, 1986; Waldherr and Neumann, 2007). Briefly, the 70 cm elevated plus-shaped maze consists of two closed arms protected by two walls (50 x 10 x 40 cm; 10 lux) and two open arms (50 x 10 cm; 100 lux), connected by a

central neutral zone (10 x 10 cm). Rats were placed into the neutral zone facing one closed arm and recorded for 5 min. The percentage time spent on the open arms as an indicator for anxiety and closed arm entries as indicator for locomotor activity were analysed live using the plus-maze DOS program.

2.1.3.3 Forced-swim test

The FST was conducted according to a previously described adapted protocol to assess active vs. passive stress-coping with the latter being an indicator of depressive-like behaviour (Cryan *et al*, 2005; Detke *et al*, 1995; Slattery and Cryan, 2012). Briefly, for the pre-swim, rats were placed into a round plexiglass cylinder (21 x 46 cm) filled with water (23-25 °C) to a depth of 30 cm for 15 min, dried, and returned to their home cage. After 24 h, rats were replaced into the swim cylinder under the same conditions for 5 min (FST). Water was changed between each rat and both sessions were recorded for subsequent analysis. Using a time sampling technique, the predominant behaviour (struggling, swimming, or immobility) in each 5-s period of the FST was rated, providing 60 scores in total per rat. Struggling behaviour was characterized as upward-directed movement of the forepaws usually along the side of the cylinder, while swimming behaviour was defined as horizontal movement through the swim cylinder including crossing into another quadrant. Immobility was expressed as floating with minimal movements to maintain the head above the water (Cryan *et al*, 2005; Detke *et al*, 1995; Slattery and Cryan, 2012).

2.1.4 Perfusion and brain slicing

Immediately after the FST, rats were euthanized with CO_2 and transcardially perfused at a speed of 20 ml / min using 100 ml 0.01 M phosphate buffered saline (1 x PBS; pH 7.4) and 250 ml 1 x PBS supplemented with 4 % paraformaldehyde (Sigma-Aldrich, Schnelldorf, Germany; pH 7.4). Afterwards, brains were removed, post-fixed for 24 h in 4 % paraformaldehyde solution, and cryo-protected in 30 % sucrose in 1 x PBS at 4 °C. After 3 days, brains were rapidly frozen using 2-methylbutane (Sigma-Aldrich) cooled by dry ice. Whole rat brains were cut in 40 µm coronal cryo-sections in series of 6 and stored free-floating in 30 % ethylene glycol / 30 % glycerol (Sigma-Aldrich) in 1 x PBS at -20 °C until immunofluorescent-immunohistochemistry was performed.

2.1.5 β-hydroxybutyrate assay

3-OH-butyrate concentrations (stimulated by microbial butyrate production) in plasma were determined with a commercial assay kit (Sigma-Aldrich) in 1 / 10 diluted samples. The enzyme reaction results in a

colorimetric product proportional to the presence of the metabolite 3-OH-butyrate. Lower limit of quantification was 4.16 ng / ml.

2.1.6 Luminex ® multiplex cytokine detection

Performed and validated by Martina Toelge (Microbiomix, Regensburg, Germany) in collaboration with Dr. Joachim Gläsner at the Institute of Microbiology (University Hospital Regensburg).

To quantify IFN-γ and IL-12 subunit 40 (IL-12p40) concentrations in rat plasma, a rat cytokine multiplex bead immunoassay (Invitrogen, Darmstadt, Germany) was used. All required reagents were provided with the kit and prepared according to the manufacturer's protocol. The assay was performed in a 96-well filter bottom microplate. Beads were protected from light throughout the whole procedure. The lyophilized standard was reconstituted in an appropriate volume of assay diluent and 3-fold serially diluted to generate a set of 7 standards, while standard diluent alone was used as a blank. The wells of the assay microplate were pre-wetted with 200 µl of wash solution for 30 s and then aspirated using a vacuum. The concentrated bead mix was diluted 20-fold with wash solution, vortexed, and sonicated for 1 min (Bandelin Sonorex TK22) just before adding 50 µl of the solution to each well. The microplate was washed twice by adding 200 µl of wash solution per well, soaking for 30 s, and aspirating. All wells were filled with 50 µl of incubation buffer, and 100 µl of the prepared standard dilutions were added to the appropriate wells. 50 µl of assay diluent were added to each sample well followed by the addition of 50 µl of the sample (pre-diluted in assay diluent if applicable). The microplate was covered and incubated overnight at 4°C on an orbital shaker (600 rpm). Following incubation, the liquid was aspirated and the microplate was washed twice. Then, 100 µl of biotinylated detection antibody (10-fold diluted in biotin dilution buffer) were added, the microplate was covered, and incubated for 1 h at room temperature (RT) with shaking. After washing twice again, 100 µl of R-phycoerythrin-conjugated streptavidin (10-fold diluted in streptavidin dilution buffer) were added, the microplate was covered, and incubated (30 min, RT) with shaking. After washing again 3 x 2 – 3 min, each well was filled with 110 µl of wash solution to suspend the beads. The microplate was covered and placed on an orbital shaker at RT until analysis. Acquisition of raw data was performed by the *Luminex xMAP 100* system (Luminex, Austin, TX, USA). The software was set to acquire data using 75 µl of sample per well and count 100 events per single bead set. Raw data was captured as mean fluorescence intensity (MFI), and the concentration of each analyte in the samples was calculated based on 4- or 5-parameter logistic fit standard curves using the *LiquiChip* Analyzer software (Qiagen, Hilden, Germany). The lower limit of quantification was 2 pg / ml (IFN-γ) and 10 pg / ml (IL-12p40), respectively.

2.1.7 Minocycline tissue extraction and high-pressure liquid chromatography analysis

Performed by Julian Hellmann-Regen at the Department of Psychiatry, Section Clinical Neurobiology (Campus Benjamin Franklin, Charité Berlin).

A sensitive and specific HPLC method was developed and optimized for detection and rapid resolution of minocycline isolated from rat liver tissue and fecal boli. The method was specifically optimized for the detection of rather low tissue levels of minocycline in order to be able to quantify minocycline concentrations following oral ingestion. Samples were homogenized in calcium-free PBS at a ratio of 1:5 (w / v) by 30 strokes in glass-PTFE potter homogenizers. To facilitate extraction of minocycline from the tissue and promote adherence to solid phase columns, all homogenates were subsequently acidified by adding 0.02 vol of orthophosphoric acid (85%, w / v). After centrifugation (1000 x g, 5 min, 4 °C), 1 ml of the resulting supernatant was loaded onto a 30 mg HLB Oasis column (Waters, Milford MA, USA), that was preconditioned by serially rinsing with 3 ml methanol and 3 ml ultrapure water. After loading, columns were washed three times with 1 ml 5% Methanol in ultrapure water, and finally eluted slowly at atmospheric pressure in 1 ml methanol. After elution, samples were placed under a gentle stream of argon and evaporated to dryness in a heat block at 50 °C. After evaporation, the residues were reconstituted in 200 µl of HPLC mobile phase. Following a brief centrifugation (5 min, 15.000 x g), samples were injected directly onto a C8 Inertsil column, 5 µm, 4.6 x 250 mm, protected by a C8 guard column (GL Sciences, Tokyo, Japan). HPLC analysis was performed using a Shimadzu 10-series system with a binary pump, isocratic elution at 1.4 ml / min and UV detection at 350 nm. The mobile phase contained acetonitrile-methanol-ultrapure water-acetic acid (2.5:10:85:2.5, v / v). The HPLC assay showed linearity in the range of 0.05-50 µg / ml. Within a total run time of only 12 min, minocycline was resolved after 3.8 min, not affected by endogenous substances, and the last endogenous peak occurred at 7.5 minutes. The limit of quantitation was 50 ng / ml. Recovery from liver homogenates and fecal boli averaged 95% as compared with compound diluted directly in mobile phase. Intra- and inter-assay coefficient of variation averaged about 5 % and 10 % at 5 µg / ml. All chemicals were purchased from Sigma-Aldrich.

2.1.8 Analysis of microglia

Carried out in collaboration with PD Dr. Barbara Di Benedetto and Dr. Victoria A. Malik at the Institute of Psychiatry and Psychotherapy (University Hospital Regensburg).

2.1.8.1 Immunofluorescent-immunohistochemistry

Three coronal sections from the infralimbic and prelimbic PFC of male and female HAB and NAB rats (AP + 2.76 - 4.3 mm from bregma) were selected and washed 3 x 20 min in 1 x PBS to remove the storage solution. Then, all slices were blocked for 1 h at RT in 1 x PBS supplemented with 2 % normal goat serum

(Vector Labs, USA) and 0.1 % Triton-X 100 (Sigma-Aldrich), followed by incubation with the primary antibody rabbit anti-Iba-1 (1:1000, WAKO, Japan) in the same solution at 4 °C over night. After washing 3 x 10 min with 1 x PBS, slices were incubated for 2 h at RT with the corresponding secondary antibody anti-rabbit Alexa Fluor 488 (1:1000, Invitrogen) and DAPI (1:1000, Sigma-Aldrich) in 1 x PBS containing 2 % normal goat serum. Slices were again rinsed 3 x 10 min with 1 x PBS and mounted with Aqua-Poly/Mount (Polysciences, USA) for confocal analysis.

2.1.8.2 Confocal microscopy and quantification of microglia cells

Confocal microscopy was performed with an Olympus confocal microscope (inverted type IX81, Olympus Europe Holding GmbH, Hamburg, Germany). For each treatment condition, 6 brains were analysed with an average of 5 images (20 optical sections, 1 µm Z-step size) per rat. Pictures were taken randomly from the prelimbic / infralimbic regions of the PFC of at least two coronal sections per brain (see Fig. 16 for location of the infralimbic and prelimbic PFC). Using the "cell counter" plugin from ImageJ (NIMH, Bethesda, MD, USA), total numbers of total cells (DAPI+) and Iba-1-positive (Iba+) cells in each field were separately quantified. Percentage of microglia cells were calculated by normalizing total counts of Iba+ cells on DAPI+ cells per picture, to account for potential differences in cell densities due to perfusion-dependent tissue shrinkages, and averaged per each brain.

2.1.9 Analysis of intestinal microbiome by 16S-rDNA pyrosequencing

Performed by Dr. Joachim Gläsner and Dr. Andreas Hiergeist at the Institute of Microbiology (University Hospital Regensburg).

2.1.9.1 Isolation of DNA from stool specimen

Cecum content was collected from male HAB and NAB rats, immediately cooled on dry ice and stored without any preservative at - 80 °C until processing. After thawing, samples (100 mg wet weight each) were mixed with a pool of three spike bacteria (*Salinibacter ruber, Rhizobium radiobacter, Alicyclobacillus acidiphilus*) containing a defined number of 16S-rDNA copies. Cells were lysed by exposure to S.T.A.R. Buffer (Roche, Mannheim, Germany) / proteinase K, five cycles of freezing in liquid nitrogen and boiling, and repeated bead beating in the TissueLyser II (Qiagen). DNA purification was performed with the MagNA Pure 96 instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Large Volume Kit (Roche). Total nucleic acids and dsDNA was quantified using the NanoDrop 1000 spectrophotometer (ThermoFisher Scientific) and the dsDNA-specific Quant-iT PicoGreen reagent (Invitrogen) in a VICTOR^3 fluorescence reader (PerkinElmer, Waltham, MA, USA), respectively.

2.1.9.2 Quantification of 16S-rDNA copies

In the isolated DNA, 16S-rRNA gene copy numbers of total bacteria were determined by quantitative polymerase chain reaction (PCR) on a LightCycler 480 II Instrument (Roche, Basel, Switzerland). PCR reactions included 1 µM each of universal eubacterial 16S-rRNA gene primers 764F and 907R and the LightCycler 480 SYBR Green I Master kit (Roche). Quantification standards were generated by cloning a complex PCR amplicon mixture derived from a cecal microbiome DNA preparation into the pGEM-T.Easy vector (Promega, Madison, WI, USA). qPCRs were performed over 40 cycles (95 °C for 10s, 60 °C for 15 s and 72 °C for 15 s) with an initial 10-min hot start at 95 °C. To identify errors in DNA isolation before amplification and pyrosequencing, additional spike bacteria-specific qPCRs were performed (data not shown, primers and probes are specified in Table 1).

Table 1. Primers used for quantification of 16S-rDNA copy numbers by qPCR and for pyrosequencing.

Name	DNA sequence (5' - 3')	*Escherichia coli* 16S-rDNA nucleotide position	Purpose of use	Reference
764F	CAAACAGGATTAGATACCC	764	Quantification of total 16S-rDNA copies	(Imase *et al*, 2008)
907R	CCGTCAATTCCTTTRAGTTT	907	Quantification of total 16S-rDNA copies	(Lane, 1991)
341F	CCATCTCATCCCTGCGTGTCTCCGACTCAG <MID>CCTACGGGAGGCAGCAG	341	Pyrosequencing and total 16S quantification standards	(Klindworth *et al*, 2013)
1061R	CCTATCCCCTGTGTGCCTTGGCAGTCTCAG CRRCACGAGCTGACGAC	1061	Pyrosequencing and total 16S quantification standards	(Klindworth *et al*, 2013)
Aacidi-238TM	6FAM-AGCTAGTTGGTGAGGTAACGGCCCACC-BBQ	238	Quantification of 16S-rDNA copies of *Alicyclobacillus acidiphilus*	(Stämmler *et al*, 2016)
Aacidi-193F	GAGGAAAGTTGCAAATGCAACA	193	Quantification of 16S-rDNA copies of *Alicyclobacillus acidiphilus*	(Stämmler *et al*, 2016)
Aacidi-453R	aggagctttccactctccttat	453	Quantification of 16S-rDNA copies of *A. acidiphilus*	(Stämmler *et al*, 2016)
Rradio-166TM	LC670-AATTAATACCGCATACGCCCTACG-BBQ	166	Quantification of 16S-rDNA copies	(Stämmler *et al*, 2016)

			of *Rhizobium radiobacter*	
Rradio-126-F2	GGAACATACCCTTTCCTGCGG	126	Quantification of 16S-rDNA copies of *Rhizobium radiobacter*	(Stämmler *et al*, 2016)
Rradio-197-R2	GCCAATCCTTCCCCGATAAATC	197	Quantification of 16S-rDNA copies of *Rhizobium radiobacter*	(Stämmler *et al*, 2016)
Salini-180TM	LC640-CACGTCGTCTGGATCCCGCATG-BBQ	180	Quantification of 16S-rDNA copies of *Salinibacter ruber*	(Stämmler *et al*, 2016)
Salini-7F	AGAGTTTGATCATGGCTCAG	7	Quantification of 16S-rDNA copies of *Salinibacter ruber*	(Stämmler *et al*, 2016)
Salini-413R	TACGCCCCATAGGGGTGT	413	Quantification of 16S-rDNA copies of *Salinibacter ruber*	(Antón *et al*, 2002)

2.1.9.3 Amplification of V3-V6 16S-rDNA variable region and 454 pyrosequencing

Extracted DNA underwent partial 16S-rRNA gene amplification by PCR using the forward primer 341F containing a 10-bp multiplex identifier (MID) sequence, and the reverse primer 1061R. A total of 10 ng dsDNA was used as a template to amplify the hypervariable regions V3 through V6 of the 16S-rRNA gene. PCR was performed in a final volume of 30 µl containing 0.12 µM of each primer, 2 mM MgCl2, and 1 U Platinum Taq DNA Polymerase (Invitrogen). The PCR amplification was carried out over 30 cycles (30 s at 95 °C, 45 s at 64 °C, 45 s at 72 °C) with an initial 5-min hot start at 95 °C and a final extension step (7 min at 72 °C). The resulting 790-bp amplicons were recovered from gels using the QIAquick Gel Extraction kit (Qiagen) and further purified with Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany). Copy numbers of amplicons suited for pyrosequencing, i. e., containing Lib-L-adaptors, were determined using the KAPA Library Quant 454 Titanium / Lib-L Universal Kit (KAPA Biosystems, Wilmington, DE, USA). For DNA library preparation, equimolar concentrations of 1 x 10^6 adaptor-labeled amplicon molecules / µl for each sample were pooled. This library was re-amplified by emulsion PCR using the GS FLX Titanium LV emPCR kit (Lib-L) applying 0.4 copies per bead and sequenced on a GS FLX+ instrument (454 / Roche) with the GS FLX Titanium Sequencing Kit XL+ using an acyclic flow pattern (Flow Pattern B).

2.1.9.4 16S-rRNA gene sequence processing and operational taxonomic unit clustering

Raw sequencing data was pre-processed with the GS Run Processor v2.9 application (Roche) to remove adapter sequences and low quality reads. Quality filtering was performed by the default "Long Amplicons 3 pipeline" resulting in 917 Mb from 1,398,617 passed filter wells with a median read length of 677 bases. A combination of QIIME (v1.9.1; Caporaso *et al*, 2010) and R (version 3.4.0; R Core Team, 2017) with installed Bioconductor package (Huber *et al*, 2015) was used to further process and analyse the sequence data. Reads were de-multiplexed and filtered for quality using QIIME's *split_libraries.py* script with default parameters except minimum and maximum read length, which were set to 400 bp and 800 bp, respectively. This read length threshold covered 99.99% of all sequencing reads. The filtered reads were mapped to operational taxonomic unit (OUT)s built on the SILVA database (release 128; Quast *et al*, 2013) using QIIME's *pick_closed_reference_otus.py* script with default parameters. The reference database OTUs used here constituted computationally built clusters of the SILVA SSU ribosomal RNA database. The clustering into OTUs at a 97% identity threshold and the classification were achieved by UCLUST 1.2.21 (Edgar, 2010). Raw sequencing data has been deposited in the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/PRJEB30124).

2.1.9.5 Microbial composition and community structure analysis

Classified reads were grouped by taxonomy to generate box plots, showing relative abundances on each taxonomic level. Microbial diversity within a single sample (i. e., number and evenness of taxa; syn.: alpha-diversity) and the diversity between samples (syn.: beta-diversity, characterized by a Bray-Curtis dissimilarity matrix) were calculated and plotted with the *vegan 2.4-1* package of *R*. Taxa box plots were generated using the *ggplot2* package within *R*.

2.1.10 Statistical analysis

Carried out in cooperation with Dr. Andreas Hiergeist at the Institute of Microbiology (University Hospital of Regensburg).

For statistical comparisons of behaviour, immunological parameters, and plasma 3-OH-butyrate levels the software package SPSS (version 12) was used. Behavioural data and 3-OH-butyrate levels were compared using either a two-tailed Student's T-test, one-way analysis of variance (ANOVA; factor treatment), two-way ANOVA (factors trait x treatment) or two-way ANOVA for repeated measures (factors trait x treatment x stimulus) followed by a *post hoc* test using Bonferroni correction for multiple comparisons when appropriate. For analysis of cytokine concentrations, a non-parametric Mann-Whitney U test was applied (for detailed statistical analysis see respective statistics tables and graph descriptions). Data are

presented as mean + SEM. For microbiome data, the Shannon indices of diversity, observed OTU numbers, 16S-rDNA copy numbers, and relative abundances of bacterial taxa were analysed in R by ANOVA with a subsequent Tukey's test. Bray-Curtis distances as depicted in the principal coordinates analysis (PCoA) plot were analysed in R by pairwise multilevel comparison using the vegan package in R followed by a Bonferroni correction. Correlations between bacterial families and plasma butyrate were calculated using Spearman's correlation coefficients in GraphPad Prism (GraphPad software version 6.0, San Diego, California, USA). Significance was accepted at $p \leq 0.05$.

2.2 CSC studies

To assess effects of minocycline on stress-induced behavioural and physiological alterations as well as specify its effects on anxiety-like behaviour, the CSC paradigm was first validated in the new behavioural laboratories. In a second cohort of mice, minocycline was administered subchronically for 8 days after stressor termination.

2.2.1 Animals and husbandry

Male C57BL/6N (hereafter BL6 mice; 20 – 22 g; purchased from Charles River) and CD1 mice (30 – 32 g; Breeding colony University Hospital Regensburg; used as dominant residents) were single-housed in standard polycarbonate mouse cages (BL6 mice) or polycarbonate observation cages (38 x 22 x 35 cm; CD1 residents) upon arrival. All animals were housed under standard laboratory conditions (12 h light – 12 h dark, lights on at 07:00 h, 22 ± 1 °C, 55 ± 5 % humidity, free access to food and tap water (vehicle) or drug solution (minocycline)) and allowed to habituate at least 7 days to room conditions prior to experiments. The experiments were approved by the Committee on Animal Health and Care of the local government, and performed according to the Guide for the Care and Use of Laboratory Animals of the Government of Oberpfalz, the ARRIVE guidelines (Kilkenny *et al*, 2010), and recommendations from the NIH. All efforts were made to minimize the number of animals used as well as their suffering.

2.2.2 Experimental protocols

As the department moved to a new facility, characteristic parameters of the CSC paradigm had to be confirmed *a priori*. Thus, BL6 mice (n = 14 – 17 per group) were exposed to the CSC paradigm, tested for anxiety-like behaviour in the LDB (day 19) and on the EPM (day 20; Fig. 10A), and subsequently rapidly decapitated for sample collection (see below).

To evaluate a possible role of minocycline in stress-induced physiological and behavioural alterations, a second cohort of BL6 mice was exposed to the CSC paradigm. On day 20, SHC and CSC mice were injected intraperitoneal (i.p.; 5 ml / kg) with either 0.9 % saline (Braun, Melsungen, Germany) or 80 mg / kg minocycline dissolved in saline, single-housed, and tested in the LDB after 60 min (n = 12 – 14). For the following 7 days (day 21 to 28), mice were treated continuously either with the same dose of minocycline dissolved in tap water or tap water as vehicle. Body weight and fluid consumption were measured on a daily basis to calculate accurate concentrations for fresh drug solutions and oral administration *via* drinking water. On day 7 of treatment, mice were tested again for anxiety-like behaviour in the open field / novel object recognition test (OF / NOR), followed by the mouse FST on day 8 (see Fig. 10B). The order of tests was chosen to minimize test interactions by introducing the least stressful experiment first (OF / NOR) and the most stressful one last (FST). Afterwards, mice were rapidly decapitated and trunk blood was collected identical as in rats for analysis of plasma ACTH and CORT levels. Left and right adrenal glands, thymus, and spleen were removed, pruned from fat and weighed separately. Adrenal glands were stored in ice-cold 1 x PBS for subsequent *in vitro* stimulation with either saline or ACTH (see chapter 2.2.5). Of note, thymus as well as adrenals were not collected and stimulated in all animals due to operational errors.

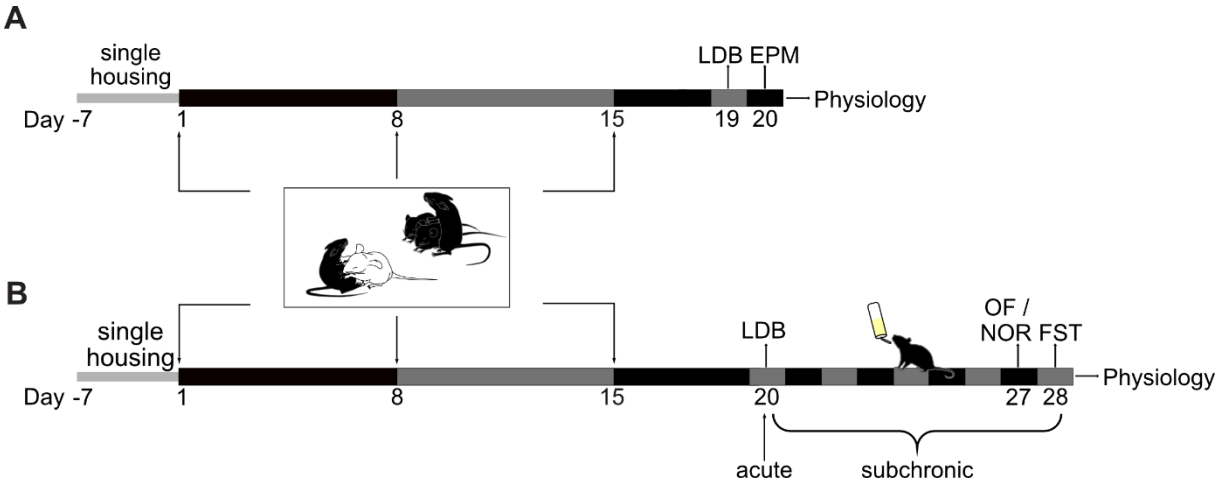


Figure 10. Experimental time course of the chronic subordinate colony housing (CSC) paradigm for 20 days with or without acute and subchronic minocycline treatment. Upon arrival, mice were single-housed for one week and then assigned as either single-housed controls (SHC) or CSC mice. In order to induce chronic psychosocial stress, CSC mice were housed together with a larger, dominant resident for 20 consecutive days. In detail, four CSC mice were placed into the homecage of a dominant resident on day 1 of the CSC procedure for the following 7 days. On day 8 and again on day 15 of CSC, the four CSC mice were transferred into the homecage of another unfamiliar resident to avoid habituation. For confirmation of a valid CSC model, CSC and SHC mice were tested in the light-dark box (LDB) and elevated plus-maze (EPM) on day 19 and 20, respectively. After the EPM, mice were rapidly decapitated to assess stress-induced physiological parameters **(A)**. In order to evaluate the effects of minocycline on CSC-induced parameters, a second cohort of mice was injected acutely with minocycline after 20 days of CSC and tested in LDB 1 h later. A subchronic treatment was continued for 7 days with evaluation of anxiety-like behaviour on day 27 in the open field / novel object recognition test (OF / NOR) and depressive-like

behaviour in the forced-swim test (FST) on day 28, followed by rapid decapitation and analysis of physiological and neuroendocrine parameters **(B)**. Illustration of social defeat adapted from Melanie Royer©, 2019.

2.2.3 Chronic subordinate colony housing paradigm

As described before (Langgartner *et al*, 2015; Reber *et al*, 2007), one week after arrival, BL6 mice were randomly assigned as SHC or CSC mice in a weight-matched manner. Four CSC mice were housed together with a dominant male CD1 resident in its home cage for 20 consecutive days to induce chronic psychosocial stress. To avoid habituation to the stressful situation, the dominant resident was replaced on day 8 and day 15 by an unfamiliar resident. Referring to previous studies demonstrating group-housing to be stressful in male mice *per se* (Singewald *et al*, 2009), SHC mice were used as appropriate control group. SHC mice remained undisturbed in their home cage except for change of bedding and weighing on day 8 and 15. On day 19 and / or 20, anxiety-like behaviour was analysed followed by analysis of physiological and neuroendocrine parameters.

2.2.4 Behavioural testing

All behavioural experiments were conducted during the day to avoid bias of dark-cycle locomotor activity changes (09:00 to 12:00). Behaviour was recorded by an overhead camera and analysed by an observer blind to the treatment or *via* automated scoring using NOLDUS software.

2.2.4.1 Anxiety-like behaviour

To assess levels of innate non-social anxiety-like behaviour, three different tests were performed: the LDB, the EPM, and the OF / NOR. These tests are based on a natural conflict between the animal's exploratory drive and the innate fear of open, lit areas and novel objects. The more anxious an animal, the less time it spends in exposed, potentially threatening areas and in contact with an unknown object, and *vice versa*.

2.2.4.1.1 Light-dark box

As previously described (Costall *et al*, 1989; Reber and Neumann, 2008b), the mouse LDB consists of two compartments: a light (27 x 27 x 27 cm, 300 lux) and a dark box (18 x 27 x 27 cm, 50 lux), separated by a partition wall with a small opening (6 x 7 cm) to allow transitions. Initially, the opening was closed and mice were placed into the dark box for a 30 s habituation period. Afterwards, mice were allowed to

explore the whole LDB freely for 5 min. Using NOLDUS software, time spent in each compartment indicating anxiety-like behaviour and distance travelled as a measure of locomotor activity were automatically analysed.

2.2.4.1.2 Elevated plus-maze

The mouse EPM, as previously described (Reber *et al*, 2007), consists of two open (6 x 30 cm; 100 Lux) and two closed (6 x 30 x 16 cm; 25 Lux) arms connected by a neutral platform (6 x 6 cm) that are elevated 35 cm above the ground. The mouse was placed into the neutral zone facing a closed arm and recorded for 5 min. The percentage time spent on the open arms was analysed live using the plus-maze DOS program as an indicator for anxiety-like behaviour. Closed arm entries were used as an indicator for locomotor activity.

2.2.4.1.3 Open field / novel object recognition test

In the OF / NOR, conducted as previously described (Langgartner *et al*, 2017b), each mouse was placed in the centre of an open field box (40 x 40 x 38.5 cm; 300 lux) and allowed to explore freely for 5 min. Afterwards, an unknown object (metal cylinder; 3.5 cm in diameter, 1.5 cm in height) was placed in the centre of the box for 5 additional min of exploration. Using NOLDUS software, time spent in the centre (20 x 20 cm) and time spent exploring the novel object were measured as an indicator for anxiety-like behaviour. Distance moved during the 10 min of testing was used as a parameter for locomotor activity.

2.2.4.2 Forced-swim test

The mouse FST was performed according to a previous protocol (Porsolt *et al*, 1977; Slattery *et al*, 2012). Briefly, mice were placed into a small plexiglass cylinder (12 cm in diameter, 40 cm in height) filled with 23 ± 2 °C water up to 13 cm for 6 min. The duration of immobility during the last 4 min was analysed using JWatcher behavioural observation software. A mouse was considered immobile when floating motionless except for movements necessary to keep its head above the water.

2.2.5 ACTH stimulation of adrenal explants *in vitro*

Following CSC and 8 days of minocycline administration, stimulation of *in vitro* adrenal explants with ACTH was performed as previously described (Füchsl *et al*, 2014; Peters *et al*, 2014; Uschold-Schmidt *et al*, 2012). After pruning left and right adrenals from fat, adrenals were cut in two halves each containing

cortical and medullary tissue. Both halves were weighed and pre-incubated in 200 ml Dulbecco's Modified Eagle's Medium F-12 (Sigma-Aldrich) supplemented with 0.1 % bovine serum albumin (Sigma-Aldrich) at 37 °C and 5 % CO_2. After 4 h, the medium was replaced and each half of one adrenal was supplemented with either 0.9 % saline (basal; Braun) or 0.9 % ACTH (Sigma-Aldrich) dissolved in saline (100 nM) for 6 h at identical conditions. The supernatant was carefully removed and stored at - 20°C until analysis with an enzyme-linked immunosorbent assay (ELISA) for CORT. CORT concentrations were calculated in relation to the weight of the respective adrenal explants.

2.2.6 ELISA for CORT and ACTH

Plasma samples and supernatant from adrenal *in vitro* stimulation were analysed using a commercially available ELISA for CORT (analytical sensitivity < 1.631 nmol / l; intra-assay and inter-assay coefficients of variation ≤ 6.35 %; IBL International, Hamburg, Germany) or ACTH (plasma samples only; analytical sensitivity 0.22 pg / ml, intra-assay and inter-assay coefficients of variation ≤ 7.1 %; IBL International). Plasma CORT and ACTH concentrations were calculated as percentage in relation to vehicle treated SHC mice.

2.2.7 Statistical analysis

For statistical comparisons the software package SPSS (version 12) was used. Validation of the CSC paradigm was statistically analysed using two-tailed Student's t-tests. Experiments involving minocycline treatment following the CSC paradigm were statistically compared using a two-way ANOVA (factors stress x treatment) followed by a *post hoc* test using Bonferroni correction for multiple comparisons when appropriate. Data are presented as mean + SEM, significance was accepted at $p \leq 0.05$.

Results

The Results include sections taken, and adapted, from one first author publication: Anna K. Schmidtner, David A. Slattery, Joachim Gläsner, Andreas Hiergeist, Katharina Gryksa, Victoria Malik, Julian Hellmann-Regen, Isabella Heuser, Thomas C. Baghai, André Gessner, Rainer Rupprecht, Barbara Di Benedetto, Inga D. Neumann. **Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner.** *(In preparation)*

3.1 Effects of minocycline, escitalopram, and the combination on behaviour and the immune system in rats

Psychiatric disorders show a strong gender bias and an unsuccessful treatment in 30 % of all cases. Several studies propose a manipulation of the inflammatory system and the gut microbiome as promising novel treatment target. Thus, in the present study, the effects of minocycline on an model of innate comorbid anxiety- and depressive-like behaviour were evaluated in a broader range of behavioural experiments, including the SPT, LDB, EPM, and FST in a sex-dependent manner (see chapter 2.1.2 for experimental details). Escitalopram is currently viewed as gold standard of available antidepressants and was used as positive control. Since minocycline is generally applied as augmentation of a conventional antidepressant in clinical studies, it was used to determine the capacity of minocycline as an adjunctive agent. In all analyses, an influence of the female cycle could not be detected in keeping with recent meta-analyses (for review see Beery, 2018). Importantly, the drinking behaviour of male (Fig. 11A) and female (Fig. 11B) HAB and NAB (Fig. 11C & D) rats differed only marginal over time and between treatment groups, except for NAB females that drank less drug solution. A statistical comparison was not conducted as fluid intake was measured on average per cage, causing a very low n number. The drug concentration was calculated daily according to the body weight and drunk volume. Thus, the treatment regimen of minocycline and escitalopram was assumed stable and rats received the appropriate concentration of the respective drug. Behavioural testing on the respective days did not alter fluid intake on the following days (indicated by arrows in Fig. 11).

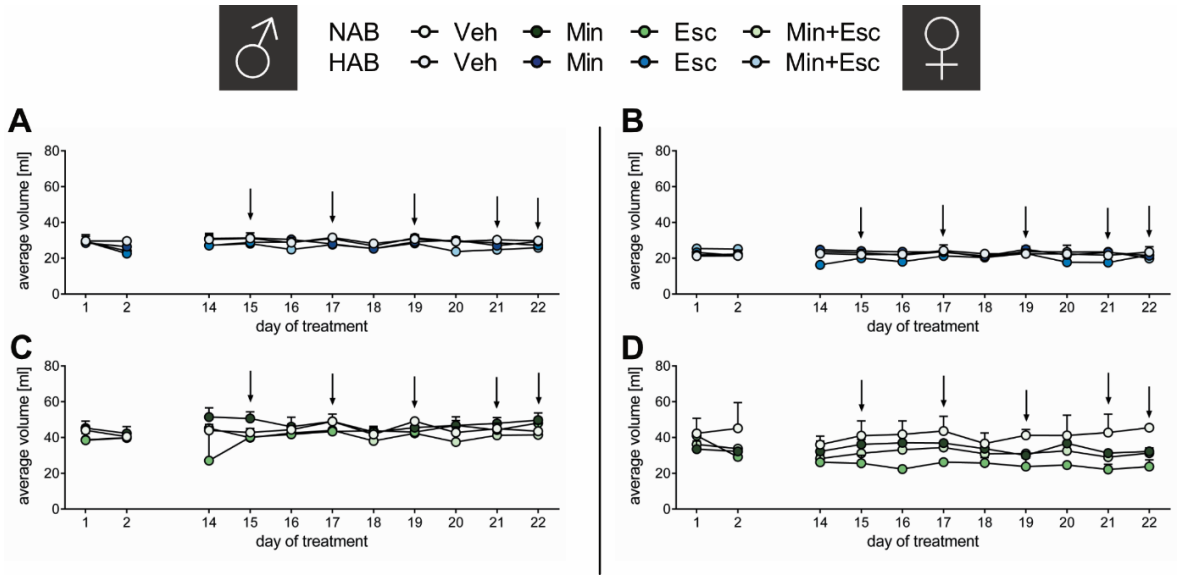


Figure 11. Average drinking volume of male and female HAB and NAB rats treated with either vehicle (Veh), minocycline (Min), escitalopram (Esc), or a combination of both (Min+Esc) on the first two treatment days and during behavioural testing (day 14 -22). Male **(A)** and female **(B)** HAB rats and male NAB rats **(C)** showed comparable fluid intake over 22 days independent of treatment and behavioural

testing. Female NAB rats **(D)** seemed to consume less drug solution compared to Veh-treated NAB females. Arrows indicate days of behavioural testing, which did not have a major influence on drinking volume. Data represents mean + SEM. Statistical comparison was not performed due to a low n number of **(A)** n = 3-5, **(B)** n = 3-4, **(C)** n = 2-4, **(D)** n = 2 per treatment group.

3.1.1 Minocycline facilitates naturally occurring social preference in male and female HAB rats

After 15 days of treatment with either minocycline (40 mg / kg), escitalopram, or a combination of both substances, all rats displayed natural social preference. This was indicated by a higher investigation time of the social (cage with conspecific) vs. the non-social stimulus (empty cage; $p<0.001$ vs. respective non-social stimulus, significance not indicated; Fig. 12A - D). *Post hoc* analysis revealed that minocycline alone, but not escitalopram or the combination, facilitated social approach in male (Fig. 12A) and female (Fig. 12B) HAB rats ($p<0.05$ vs. Veh social stimulus). In NAB rats, social preference remained unchanged by the provided treatment in both sexes (Fig. 12C & D).

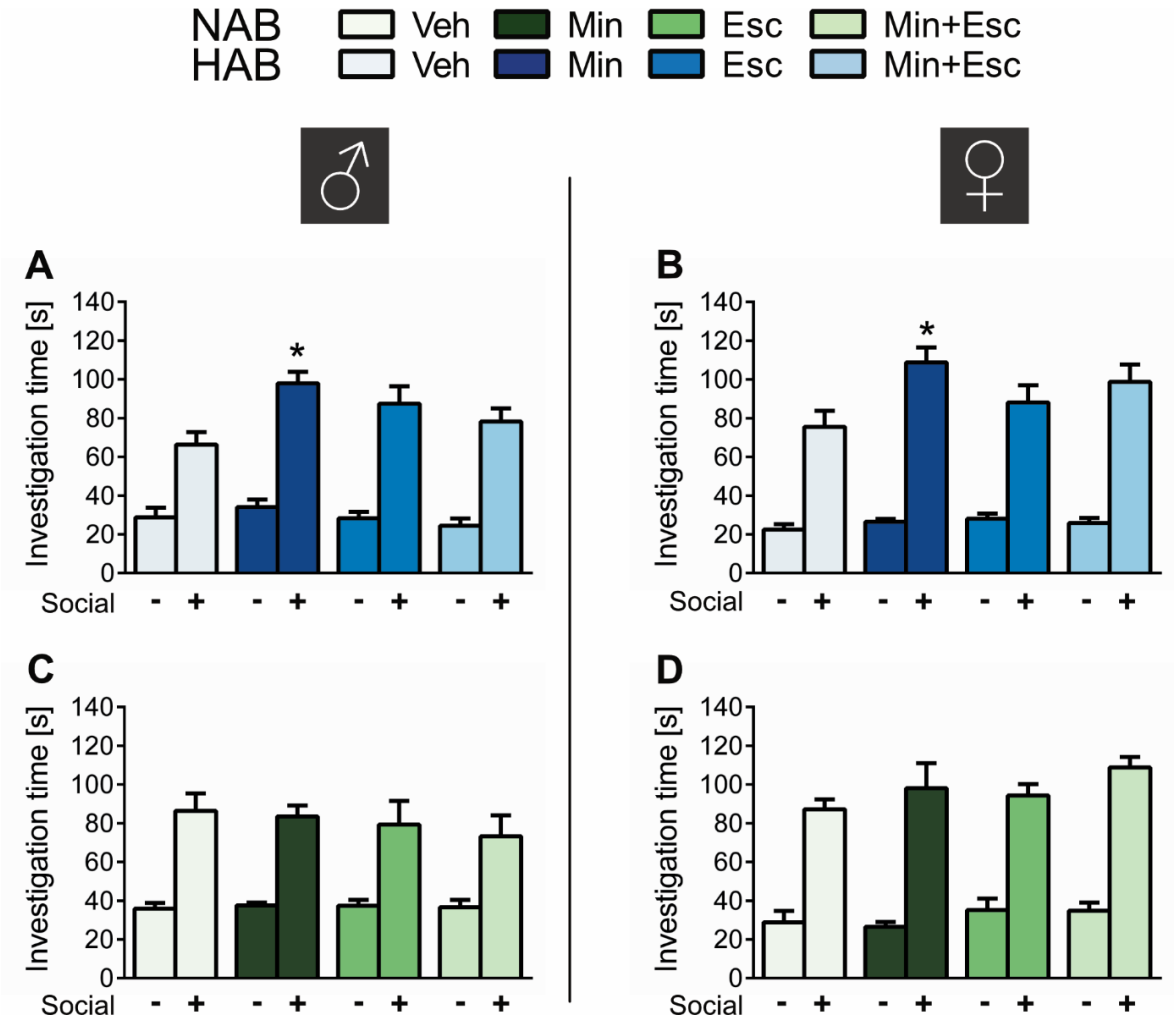


Figure 12. Social preference of male and female HAB and NAB rats with either vehicle (Veh), minocycline (Min), escitalopram (Esc), or a combination of both (Min+Esc) on day 15 of treatment. Social preference is reflected by a significant increase in investigation of the social (+) vs. the non-social (-) stimulus. Male

(A & C) and female **(B & D)** HAB and NAB rats show natural social preference (significance not indicated) which is facilitated in male **(A)** and female **(B)** HAB rats by Min. Data represents mean + S.E.M; * p<0.05 vs. corresponding Veh social stimulus. One-way ANOVA for repeated measures followed by a *post hoc* test using Bonferroni correction; n = 8 - 16 per treatment group.

Chronic minocycline rat study, social behaviour statistics:

sex	Stimulus effect (non-social vs. social stimulus)	Stimulus x treatment
♂ **(Fig. 12A)**	$F_{(1,41)}$ = 169.961; p<0.001 *	$F_{(3,41)}$ = 2.483; p = 0.07 (*)
♀ **(Fig. 12B)**	$F_{(1,42)}$ = 233.512; p<0.001 *	$F_{(3,42)}$ = 2.530; p = 0.07 (*)
♂ **(Fig. 12C)**	$F_{(1,40)}$ = 78.364; p<0.001 *	$F_{(3,40)}$ = 0.36; p = 0.78
♀ **(Fig. 12D)**	$F_{(1,28)}$ = 208.968; p<0.001 *	$F_{(3,28)}$ = 0.805; p = 0.50

3.1.2 Anxiety-like behaviour remains unaffected by minocycline in HAB and NAB rats irrespective of sex

To evaluate potential anxiolytic effects of minocycline (40 mg / kg), escitalopram, or the combination, male and female HAB and NAB rats were tested in two relevant behavioural tests on day 17 and 19 of treatment. In the LDB on day 17, HAB rats displayed the expected sex-independent highly anxious phenotype compared to NAB rats as depicted by a decreased time spent in the light box (p<0.001 vs. NAB; Fig. 13A & B). Male HAB rats (Fig. 13A) and female (Fig. 13B) HAB and NAB rats did not respond to the provided treatment. In male NAB rats, escitalopram increased the time in the light box, indicative of anxiolysis (p<0.05 vs. corresponding Veh; Fig. 13A). The distance travelled in the LDB as a measurement of locomotor activity remained unchanged in male HAB (Fig. 13C) and female (Fig. 13D) HAB and NAB rats, while in male NAB rats, the combination reduced locomotion (p<0.05 vs. corresponding Veh; Fig. 13C). Similarly, on the EPM (day 19) the highly anxious HAB rats spent a reduced percentage of time on the open arm compared to NAB rats independent of sex and treatment (p<0.001 vs. NAB; Fig. 13E & F). Male (p<0.001 vs. NAB; Fig. 13G) and female (p<0.001 vs. NAB; Fig. 13H) HAB rats also showed a decreased number of closed arm entries compared to NAB rats which tended to be increased in male HAB rats after minocycline treatment (p = 0.066 vs. corresponding Veh; Fig. 13G).

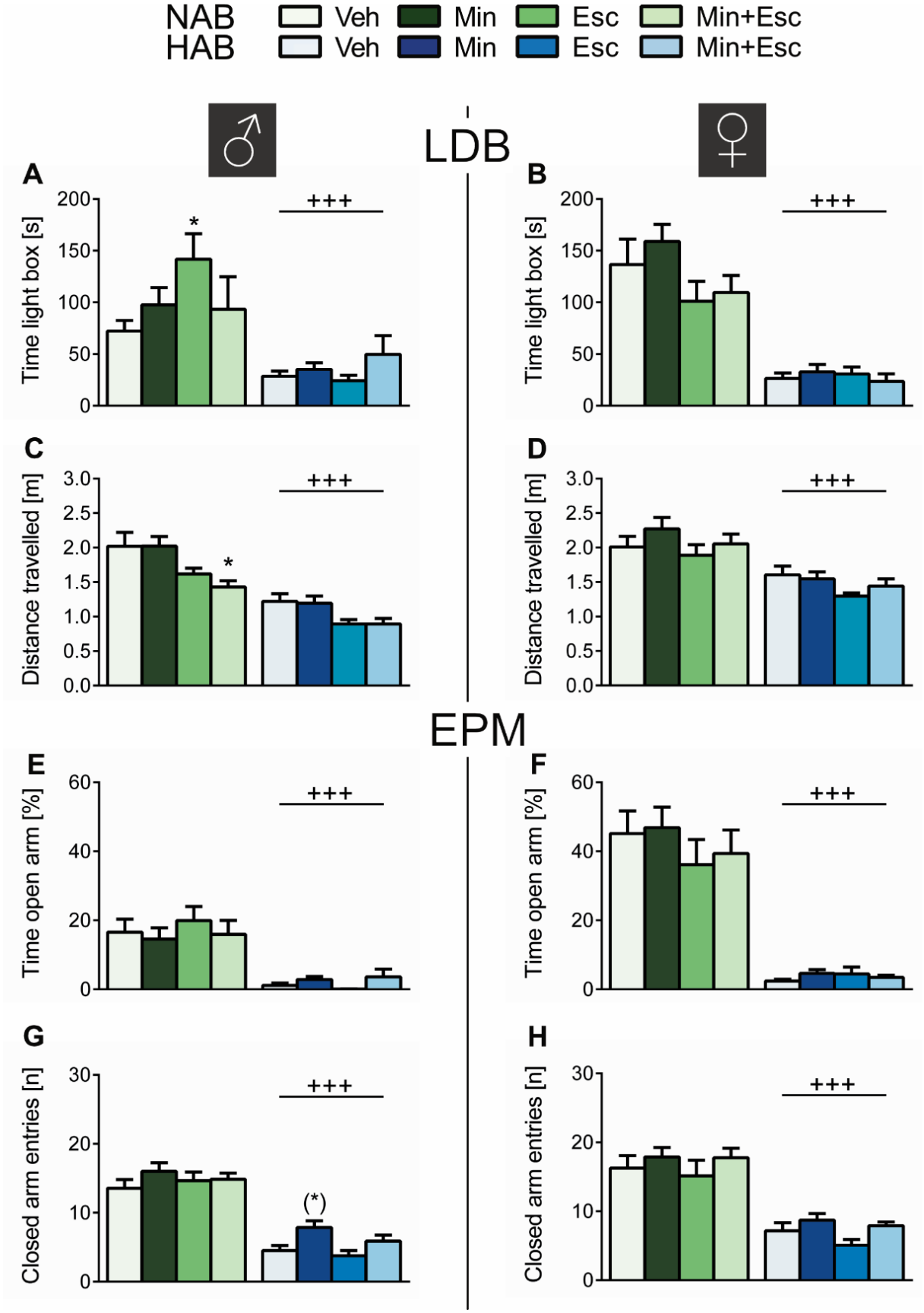


Figure 13. Anxiety-like behaviour of male and female NAB and HAB rats treated with vehicle (Veh), minocycline (Min), escitalopram (Esc), or a combination of both (Min+Esc), in the light-dark box (LDB; day 17) and on the elevated plus-maze (EPM; day 19). Compared to NAB rats, both male **(A & C)** and female **(B & D)** HAB rats showed increased anxiety-like behaviour and decreased locomotor activity in the

LDB. In male NAB rats, Esc increased the time in the light box **(A),** while the combinatory treatment decreased locomotion **(C)**. On the EPM, in both male **(E & G)** and female **(F & H)** HAB rats anxiety-like behaviour was elevated and locomotor activity was reduced compared to NAB rats. In male HAB rats, Min tended to increase closed arm entries **(G)**. Data represents mean + SEM; (*) p = 0.066, * p<0.05 vs. corresponding Veh; +++ p<0.001 vs. NAB. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 8 - 16 per treatment group.

Chronic minocycline rat study, anxiety-like behaviour statistics:

sex	Trait effect (HAB vs. NAB rats)	Trait x treatment
♂ **(Fig. 13A)**	$F_{(1,84)}$ = 35.729; p<0.001 *	$F_{(3,84)}$ = 2.030; p = 0.116
♀ **(Fig. 13B)**	$F_{(1,68)}$ = 113.024; p<0.001 *	$F_{(3,68)}$ = 1.785; p = 0.158
♂ **(Fig. 13C)**	$F_{(1,81)}$ = 47.115; p<0.001 *	$F_{(3,81)}$ = 0.379; p = 0.769
♀ **(Fig. 13D)**	$F_{(1,68)}$ = 39.97; p<0.001 *	$F_{(3,68)}$ = 0.557; p = 0.645
♂ **(Fig. 13E)**	$F_{(1,81)}$ = 54.702; p<0.001 *	$F_{(3,81)}$ = 1.080; p = 0.36
♀ **(Fig. 13F)**	$F_{(1,70)}$ = 180.528; p<0.001 *	$F_{(3,70)}$ = 0.875; p = 0.46
♂ **(Fig. 13G)**	$F_{(1,81)}$ = 128.072; p<0.001 *	$F_{(3,81)}$ = 2.689; p = 0.052 (*)
♀ **(Fig. 13H)**	$F_{(1,70)}$ = 106.146; p<0.001 *	$F_{(3,70)}$ = 0.066; p = 0.978

3.1.3 Minocycline ameliorates depressive-like behaviour exclusively in male HAB rats

Concomitant with an anxious phenotype, HAB rats were repeatedly characterized by enhanced depressive-like behaviour compared to NAB rats. In accordance, in the FST performed on day 22 of treatment with either minocycline (40 mg / kg), escitalopram, or the combination, HAB rats displayed reduced struggling (p<0.001 vs. NAB; Fig. 14A & B) and increased immobility (p<0.001 vs. NAB; Fig. 14C & D) irrespective of sex. Treatment with minocycline alone, but not escitalopram or the combination, was able to reverse this phenotype exclusively in male HAB rats by enhancing struggling (p<0.05 vs. corresponding Veh; Fig. 14A) and decreasing immobility (p<0.05 vs. corresponding Veh; Fig. 14C). Female HAB as well as male and female NAB rats (Fig. 14B - D) did not respond to the treatment.

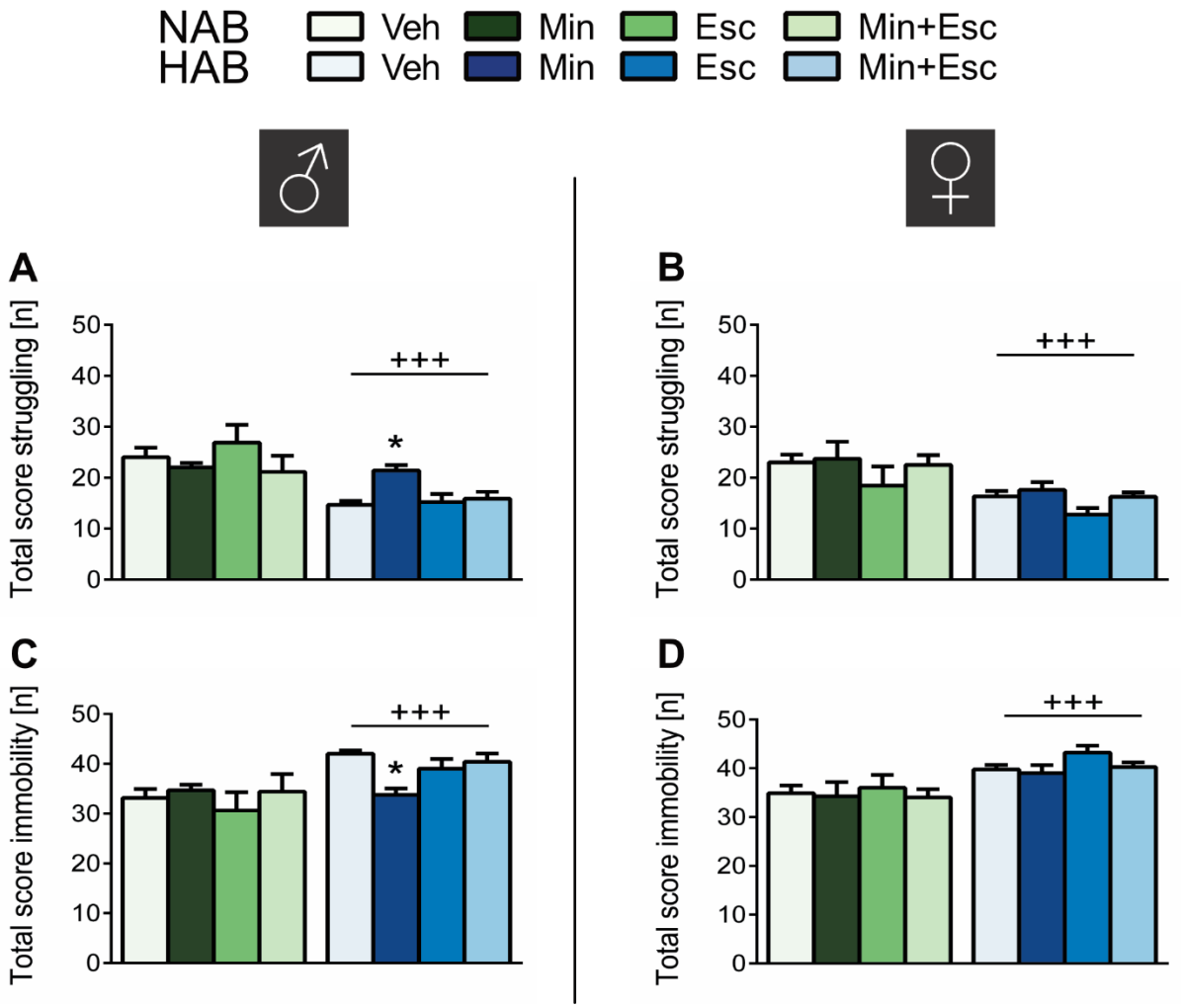


Figure 14. Depressive-like behaviour after treatment of male and female NAB and HAB rats with either vehicle (Veh), minocycline (Min), escitalopram (Esc), or a combination of both (Min+Esc) for 22 days. HAB rats showed an increased sex-independent depressive-like phenotype as indicated by a decreased total score of struggling **(A & B)** and an increased total score immobility **(C & D)**. Min alone ameliorated depressive-like behaviour exclusively in male HAB rats **(A & C)**. Data represents mean + SEM, * $p<0.05$ vs. corresponding Veh, +++ $p<0.001$ vs. NAB. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 8 - 16 per treatment group.

Chronic minocycline rat study, depressive-like behaviour statistics:

sex	Trait effect (HAB vs. NAB rats)	Trait x treatment
♂ **(Fig. 14A)**	$F_{(1,84)} = 27.618$; $p < 0.001$ *	$F_{(3,84)} = 4.082$; $p = 0.009$ *
♀ **(Fig. 14B)**	$F_{(1,67)} = 21.168$; $p < 0.001$ *	$F_{(3,67)} = 0.024$; $p = 0.99$
♂ **(Fig. 14C)**	$F_{(1,84)} = 16.496$; $p < 0.001$ *	$F_{(3,84)} = 3.316$; $p = 0.024$ *
♀ **(Fig. 14D)**	$F_{(1,67)} = 22.978$; $p < 0.001$ *	$F_{(3,67)} = 0.237$; $p = 0.87$

3.1.4 An increased dose of minocycline does not alter behaviour in female HAB rats

In the course of assessing potential sex-dependent differences in effective doses, 80 mg / kg minocycline was administered to male and female HAB rats. Again, all HAB rats showed naturally occurring social preference ($p<0.001$ vs. respective non-social stimulus, significance not indicated; Fig. 15A & B) which was further increased by minocycline ($p<0.05$ vs. Veh). Anxiety-like behaviour in both the LDB and EPM remained unchanged (Fig. 15C - F). Depressive-like behaviour was ameliorated in male HAB rats ($p<0.05$ vs. Veh; Fig. 15G & H), whereas female HAB rats (Fig. 15I & J) did not respond to a higher dose of minocycline. Hence, in all subsequent analyses, samples from the 40 mg / kg minocycline experiments were used.

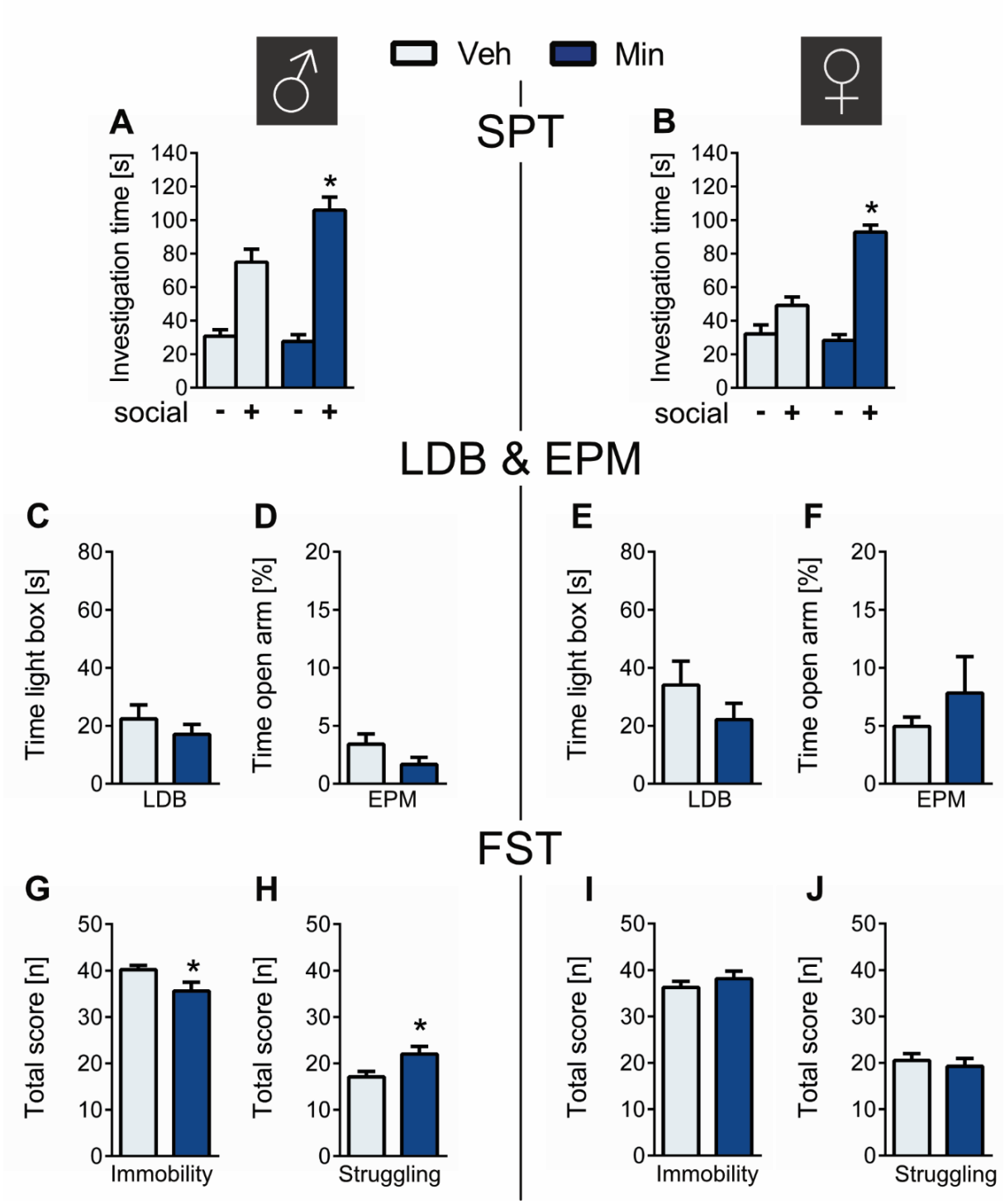


Figure 15. Behavioural outcome after treatment of male and female HAB rats with vehicle (Veh) or minocycline (Min) at a higher dose (80 mg / kg) for 22 days. Both male **(A)** and female **(B)** HAB rats showed natural occurring social preference for a social (+) over a non-social (-) stimulus (significance not indicated) on day 15 of treatment, which was facilitated by Min. Anxiety-like behaviour remained unchanged in both the light-dark box (LDB, day 17; **C & E**) as well as on the elevated plus-maze (EPM, day 19; **D** & **F**). In the FST, Min alleviated depressive-like behaviour in male **(G & H)**, but not female **(I & J)**, HAB rats. Data represents mean + SEM; * $p<0.05$ vs. Veh. One-way ANOVA followed by a *post hoc* test using Bonferroni correction, or Student's T-test; n = 8 - 10 per treatment group.

Chronic minocycline increased dose rat study, behavioural statistics:

sex	Stimulus effect (non-social vs. social stimulus)	Stimulus x treatment
♂ **(Fig. 15A)**	$F_{(1,16)} = 190.714$; $p < 0.001$ *	$F_{(1,16)} = 14.798$; $p = 0.001$ *
♀ **(Fig. 15B)**	$F_{(1,15)} = 97.423$; $p < 0.001$ *	$F_{(1,15)} = 32.958$; $p < 0.001$ *
	Treatment effect	
♂ **(Fig. 15C)**	$T_{(16)} = 0.912$; $p = 0.375$	
♂ **(Fig. 15D)**	$T_{(17)} = 1.581$; $p = 0.132$	
♀ **(Fig. 15E)**	$T_{(15)} = 1.160$; $p = 0.264$	
♀ **(Fig. 15F)**	$T_{(16)} = -0.660$; $p = 0.525$	
♂ **(Fig. 15G)**	$T_{(17)} = 2.273$; $p = 0.036$ *	
♂ **(Fig. 15H)**	$T_{(18)} = -2.427$; $p = 0.026$ *	
♀ **(Fig. 15I)**	$T_{(14)} = -1.032$; $p = 0.32$	
♀ **(Fig. 15J)**	$T_{(14)} = 0.554$; $p = 0.589$	

3.1.5 Minocycline reduces microglia quantity in the PFC exclusively in male HAB rats

The effects of minocycline (40 mg / kg), escitalopram, or the combination on microglia quantity in infralimbic/prelimbic PFC slices were assessed using Iba-1 as a marker of both resting and reactive microglia. In the PFC of male ($p<0.001$ vs. NAB; Fig. 16A) and female ($p<0.01$ vs. NAB; Fig. 16B) HAB rats, overall lower counts of microglia cells were detected in comparison to the respective NAB brains. A treatment effect was seen exclusively in male HAB rats with *post hoc* analysis revealing reduced microglial numbers after minocycline treatment ($p<0.05$ vs. corresponding Veh; Fig. 16A).

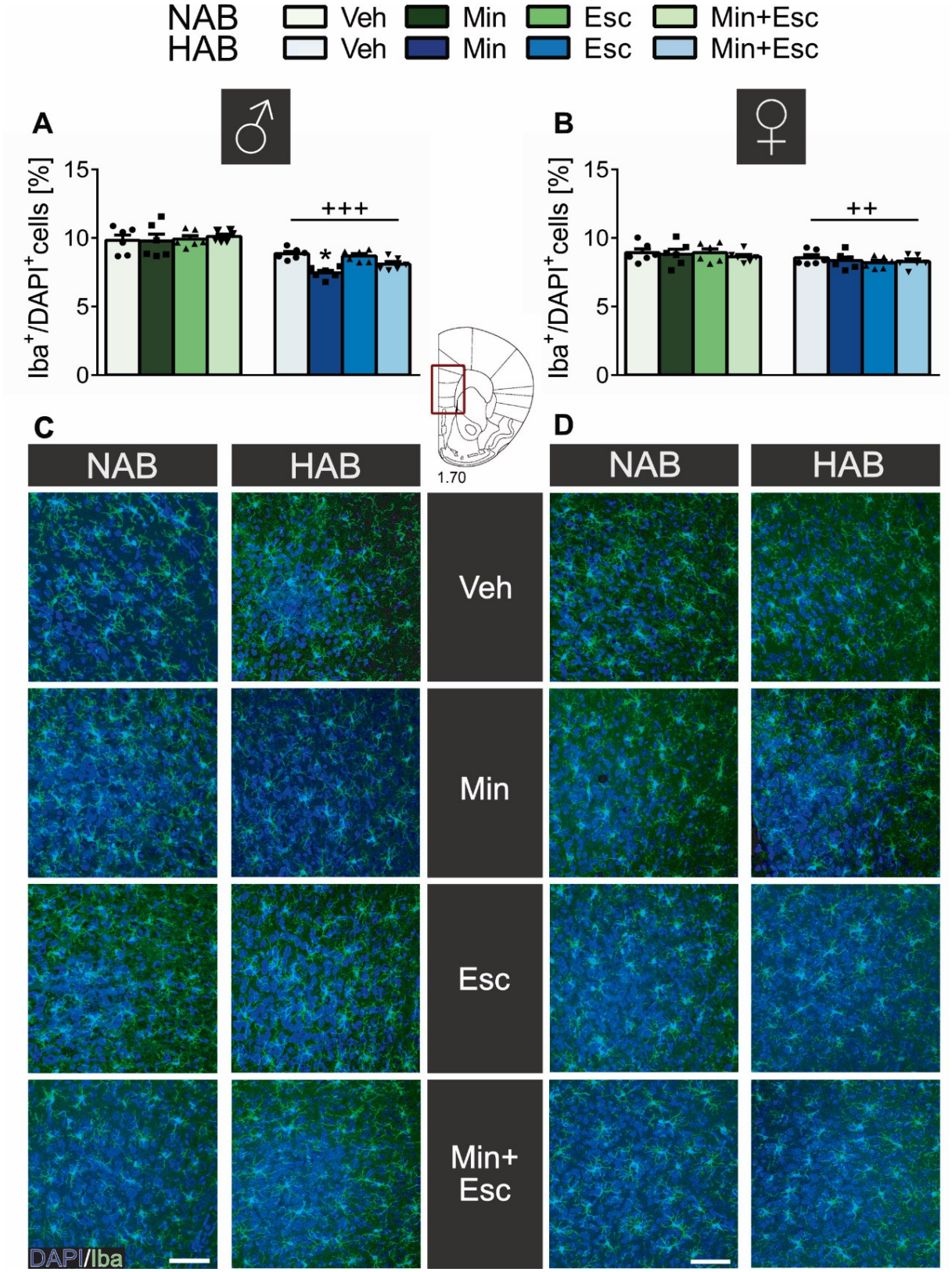


Figure 16. Quantity of Iba+ microglia cells on day 22 of treatment with either vehicle (Veh), minocycline (Min), escitalopram (Esc), or the combination (Min+Esc) in the prefrontal cortex (PFC; infralimbic / prelimbic) of male and female NAB and HAB rats. Both male **(A)** and female **(B)** HAB rats showed reduced microglial numbers in the PFC in general compared to NAB rats. Min alone further reduced microglial numbers in male HAB rats **(A)**. **(C & D)** Representative microphotographs of the analysed PFC with Iba+ microglia (green) and DAPI+ (blue) staining. Data represents mean + SEM; * p<0.05 vs. corresponding Veh

group; ++ p<0.01, +++ p<0.001 vs. NAB. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 6 per treatment group. Scale bar: 50 µm.

Chronic minocycline rat study, microglia statistics:

sex	Trait effect (HAB vs. NAB rats)	Trait x treatment
♂ **(Fig. 16A)**	$F_{(1,40)}$ = 70.586; p < 0.001 *	$F_{3,40}$ = 2.650; p = 0.062 (*)
♀ **(Fig. 16B)**	$F_{(1,40)}$ = 7.616; p < 0.01 *	$F_{3,40}$ = 0.272; p = 0.845

3.1.6 Minocycline reduces IFN-γ, but not IL-12p40, concentrations in male rats

As only male HAB rats responded to minocycline treatment regarding depressive-like behaviour and microglial numbers, subsequent analyses of peripheral pro-inflammatory cytokine concentrations and microbiome composition were restricted to minocycline-treated male HAB vs. NAB rats. Plasma concentrations of both IFN-γ (Fig. 17A) and IL-12p40 (Fig. 17B) were comparable between male HAB and NAB rats. Minocycline reduced IFN-γ levels in male HAB and NAB rats (p<0.05 vs. corresponding Veh; Fig. 17A), whereas IL-12p40 levels were not significantly changed (Fig. 17B).

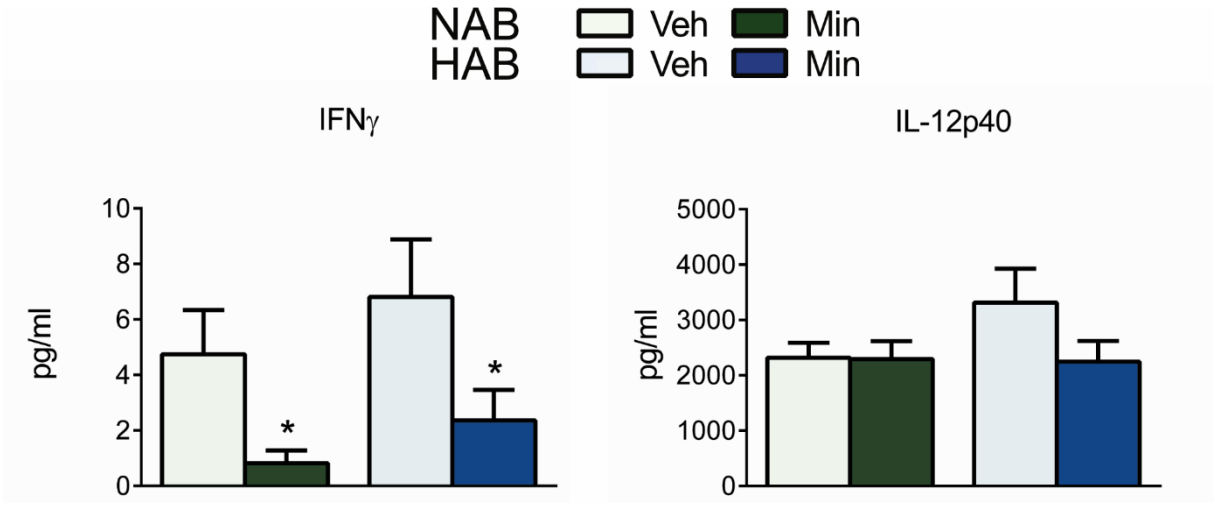


Figure 17. Cytokine concentrations in plasma of male HAB and NAB rats on day 22 of vehicle (Veh) or minocycline (Min) treatment. Min reduced interferon (IFN)-γ concentrations in both HAB and NAB rats **(A)**, while interleukin (IL)-12p40 levels were not affected **(B)**. Data represents mean + SEM, * p<0.05 vs. corresponding Veh group. Mann-Whitney U test; n = 11 – 16 per treatment group.

Chronic minocycline rat study, peripheral cytokine statistics:

Parameter	NAB	HAB
IFN-γ (Fig. 17A)	U = 71.000; p = 0.032 *	U = 67.000; p = 0.022 *
IL-12p40 (Fig. 17B)	U = 115.000; p = 0.642	U = 102.000; p = 0.35

3.2 Influence of minocycline on microbiota in the caecum

Host microbiota is known to control maturation and function of microglia and to modulate many aspects of behaviour (Erny *et al*, 2015). To assess (i) breeding line-dependent differences as well as (ii) minocycline-induced alterations in the intestinal microbiome accompanying the observed differences and changes in depressive-like behaviour and microglial density, the bacterial composition in cecal contents of male HAB and NAB rats was analysed using 16S-rRNA-based deep sequencing.

3.2.1 Minocycline is detectable in both liver and fecal boli

As prerequisite for microbial analysis, the distribution and absorption of minocycline in both liver and fecal boli after 22 days of treatment were analysed by HPLC. Minocycline was detectable in fecal boli and the liver of both male and female rats in comparable concentrations irrespective of trait anxiety (Table 2). This correlated with liquid (i.e. minocycline) intake on the last treatment day (data not shown).

Table 2. Minocycline concentration in liver and fecal boli of male and female rats after 22 days of vehicle (Veh) or minocycline (Min) treatment. Data depicts mean concentration values of pooled HAB and NAB rats per sex; n (liver) = 13 (♀) and 36 (♂), n (fecal boli) = 6; n.a. = not assessed.

Sex	treatment	Liver [µg / mg]	Fecal boli [µg / mg]
♂	Veh	0.00 ± 0.00	0.00 ± 0.00
♂	Min	1.05 ± 0.08	1.26 ± 0.11
♀	Veh	0.00 ± 0.00	n.a.
♀	Min	1.12 ± 0.1	n.a.

3.2.2 Gut microbiota composition differs between male HAB and NAB rats and is altered by minocycline

The overall diversity of the cecal microbiota in vehicle-treated rats, as assessed by principal coordinates analysis of Bray-Curtis distances, disclosed significant differences between HAB and NAB rats ($p = 0.012$ vs. NAB; Fig. 18B). No differences were detected regarding bacterial copy numbers and diversity between the two lines (Fig. 18A, C & D). A 22-day treatment with minocycline significantly reduced the bacterial copy numbers ($p<0.01$ vs. Veh; Fig. 18A) as well as the microbial richness, as represented by OTU counts ($p<0.001$ vs. Veh; Fig. 18C), in male HAB and NAB rats. Interestingly, in samples of HAB rats – as opposed to NAB rats ($p<0.05$) – the mean Shannon index, integrating richness and evenness of individual taxa to an estimate of bacterial diversity, was only marginally altered by minocycline treatment (Fig. 18D). Comparing the overall diversity between minocycline- and vehicle-treated rats by principal coordinates analysis of Bray-Curtis distances, four distinct clusters corresponding to the two treatment conditions in HAB and NAB rats each were detected (Fig. 18B).

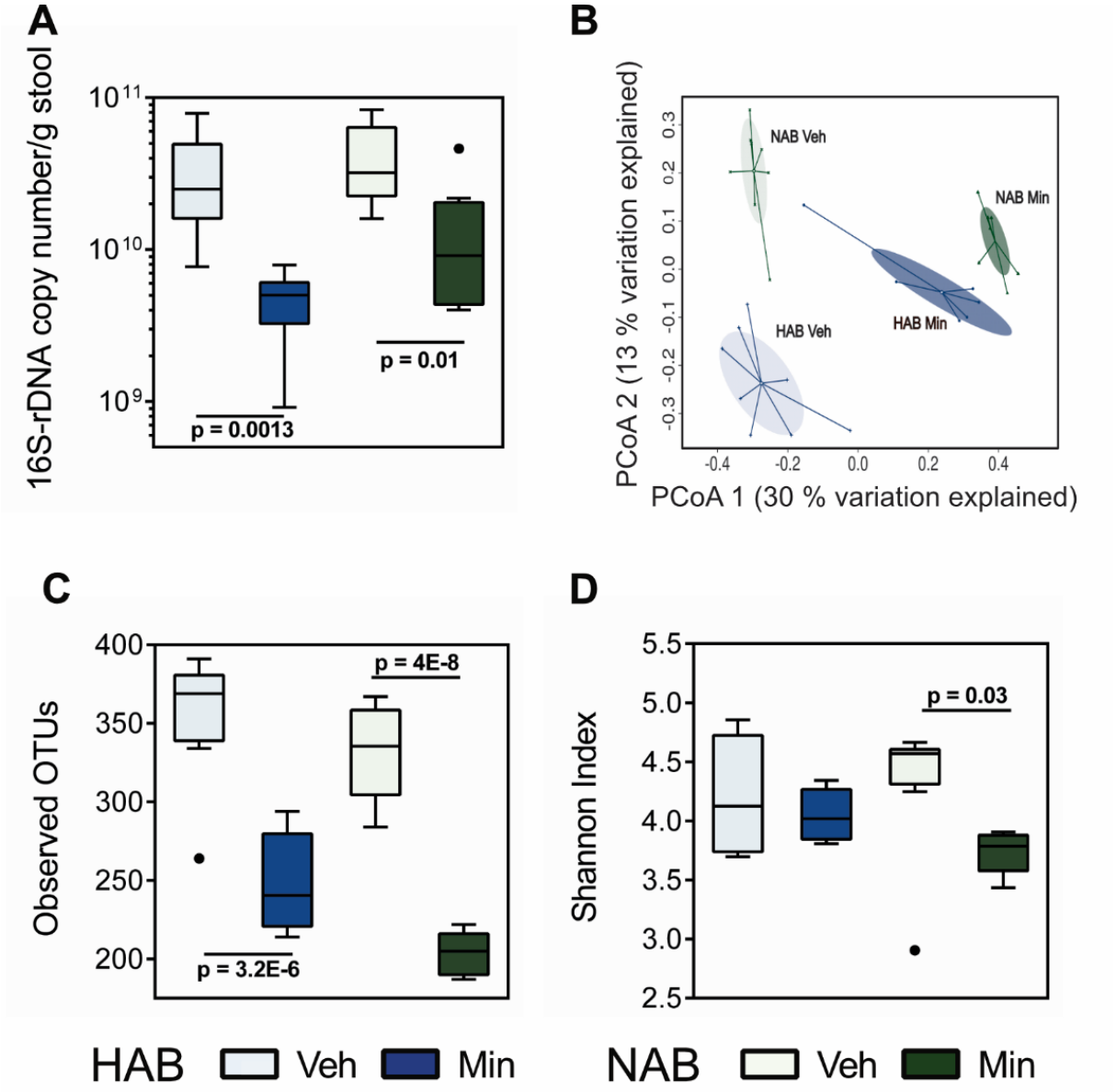


Figure 18. Cecal microbiota composition and profiling after 22 days of vehicle (Veh) or minocycline (Min) treatment. Male HAB and NAB rats showed comparable numbers of bacterial 16S-rRNA gene copies **(A)** and bacterial richness indicated by operational taxonomic units (OTU) numbers **(C)** that were both decreased by Min. An unchanged Shannon Index in HAB, but not NAB, rats suggested increased equitability throughout the taxa present within the HAB microbiome **(D)**. B-diversity of bacterial communities prevalent in cecal contents, as illustrated by principal coordinates analysis (PCoA) of Bray – Curtis distances with the first two coordinates plotted, showed four distinct clusters corresponding to all treatment groups **(B)**. Box plots depict the median and interquartile range, whiskers represent minimum and maximum values, and outliers indicate values more than 1.5 times of upper / lower quartile. Kruskal-Wallis test followed by Dunn's multiple comparison test; n= 6 – 8 per treatment group.

3.2.3 Minocycline increases 3-OH-butyrate concentration and abundance of butyrate producer

Deeper taxonomic analysis of the microbiome composition revealed striking differences between HAB and NAB rats regarding the highly prevalent *Lachnospiraceae* family of the *Clostridiales* order ($p<0.05$ vs. NAB; Fig. 19A). Minocycline treatment elevated the lower frequency in HAB rats ($p<0.05$ vs. Veh) to the level of NAB rats that — in terms of this family — were not affected by treatment. Further, a relative increase in the abundance of the *Clostridiales* Family XIII in both lines was observed after minocycline ($p<0.01$ vs. Veh; Fig. 19B). Significantly augmented levels of 3-OH-butyrate after minocycline treatment were found in both lines that were even higher in HAB than in NAB rats ($p<0.05$ vs. Veh; Fig. 19C). Spearman's rank analysis disclosed positive correlations between 3-OH-butyrate levels and abundance of the two mentioned Clostridiales families in individual HAB rats ($p<0.001$; Fig. 19D & F). In NAB rats, minocycline-induced augmentation of the Family XIII population, but not of Lachnospiraceae, positively correlated with 3-OH-butyrate levels ($p<0.001$; Fig. 19E & G).

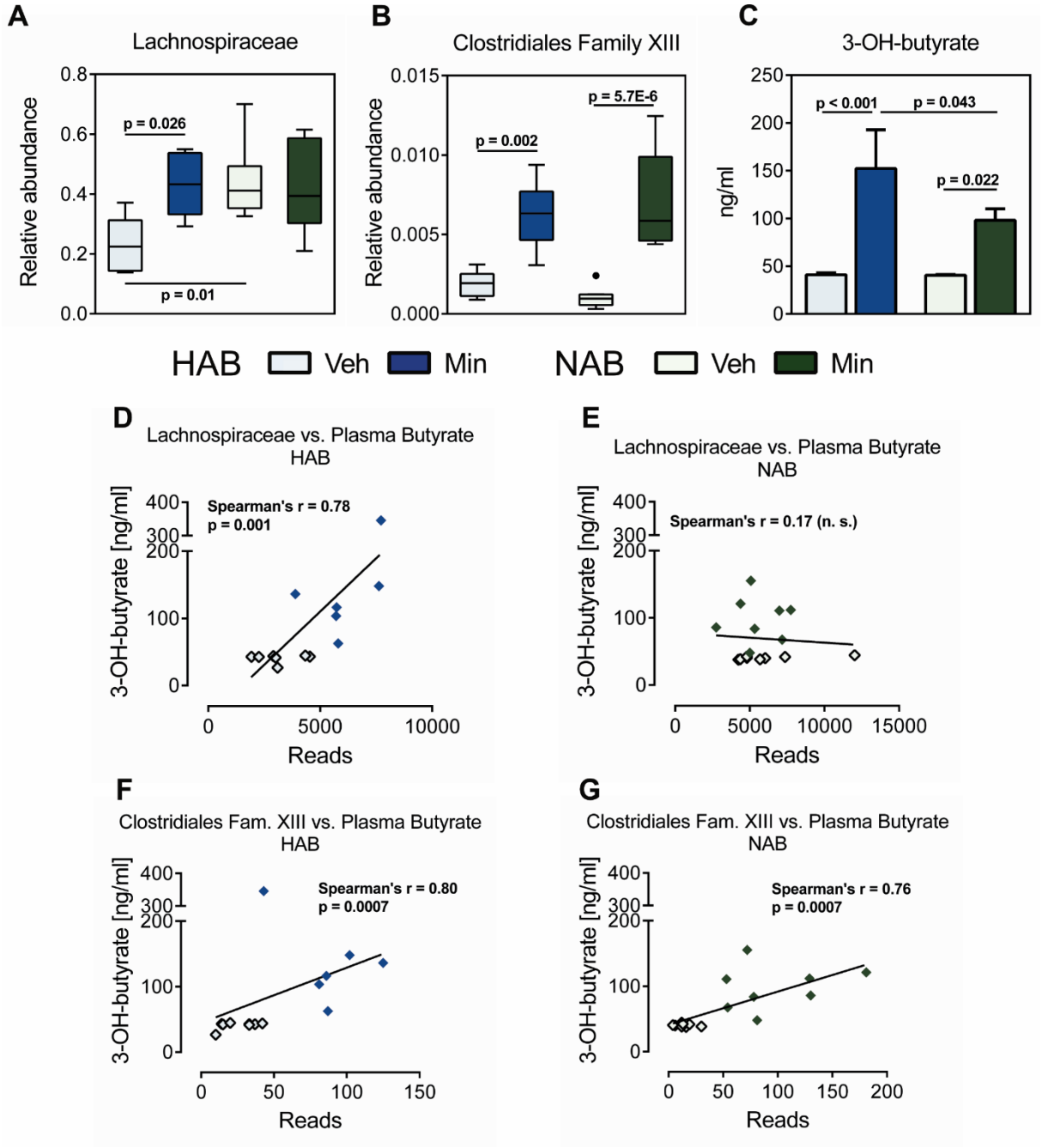


Figure 19. Relative abundance of butyrate-producing bacteria and plasma 3-OH-butyrate levels after 22 days of vehicle (Veh) or minocycline (Min) treatment in male HAB and NAB rats. Regarding Clostridiales families, HAB rats showed a reduced abundance of Lachnospiraceae that was increased to NAB level by Min **(A)**. Both breeding lines demonstrated comparable levels of Clostridiales Family XIII abundance that increased after Min treatment **(B)**. These changes were accompanied by increased plasma 3-OH-butyrate levels with an even higher increase in HAB compared to NAB rats **(C)**. In HAB rats, plasma 3-OH-butyrate levels correlated positively with both Clostridiales families **(D & F)** whereas in NAB rats, a positive correlation was only observed with Family XIII **(E & G)**. Box plots represent interquartile range with horizontal lines indicating the median of values. Outliers indicate values more than 1.5 times of upper/lower quartile, and whiskers show minimum and maximum of remaining values; ANOVA with a subsequent Tukey's analysis. Bars represent mean + SEM; Two-way ANOVA followed by a *post hoc* test using Bonferroni correction. Open symbols indicate Veh-treated, coloured symbols Min-treated rats in correlations; Spearman's correlation; n = 6-8 per treatment group.

3.3 Effects of minocycline on chronic psychosocial stress

A second approach to analyse the underlying pathophysiological mechanisms of diseases is environmental manipulation, like chronic stress. Minocycline has been proven effective in stress-induced depressive-like behaviour and numerous studies propose it as a treatment option for anxiety-like behaviour (Arakawa *et al*, 2012; Levkovitz *et al*, 2015; Wang *et al*, 2017, 2018; Wong *et al*, 2016). However, the current evidence was only provided in models of stress-induced comorbid anxiety- and depressive-like behaviour. The CSC model, which induces only anxiety-like behaviour, represents a unique opportunity to unravel a behaviour-specific mode of action for minocycline. As the department moved to a new building in 2014, it was essential to provide evidence for a functional CSC in the new facility, i.e. verify previously reported stress-induced behavioural and physiological alterations (Langgartner *et al*, 2015), before treatment-specific effects could be evaluated. Further, the effects of acute, as well as subchronic, minocycline treatment on CSC-induced physiological and behavioural parameters were assessed.

3.3.1 Validation of the CSC paradigm

3.3.1.1 CSC exposure induces anxiety-like behaviour

On day 19 of CSC exposure, CSC mice spent less time in the light box compared to SHC mice, which is indicative for increased anxiety-like behaviour ($p<0.05$ vs. SHC; Fig. 20A). Locomotor activity, reflected by the distance travelled, did not differ between the groups rendering activity-dependent behavioural changes unlikely (Fig. 20B). This anxious phenotype was partly confirmed on the following day on the EPM. Here, CSC entered the open arms less frequently ($p<0.05$ vs. SHC; Fig. 20D), though the percentage of open arm time was unchanged (Fig. 20C).

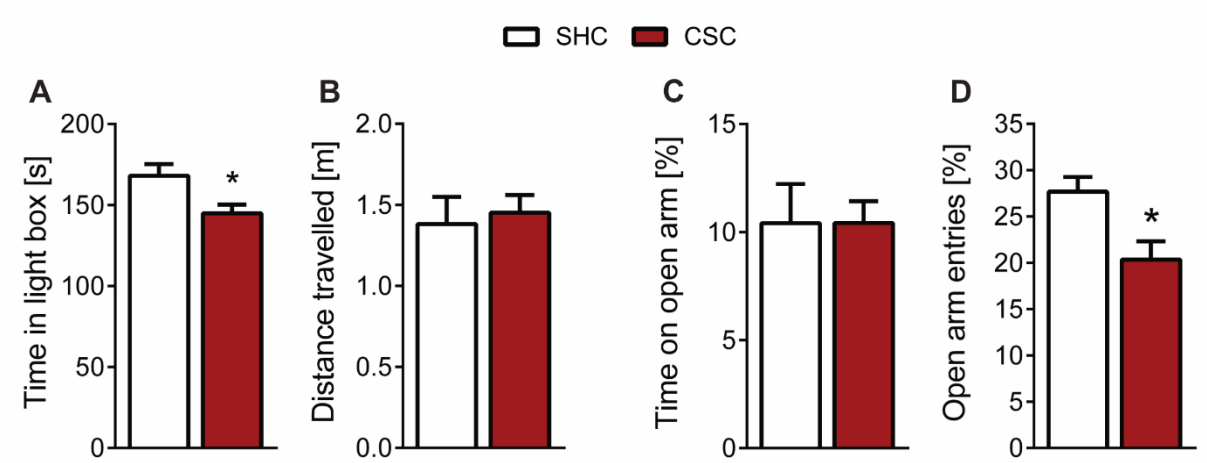


Figure 20. Effects of CSC on anxiety-like behaviour and locomotor activity. After 19 consecutive days of CSC exposure, all mice were tested in the light-dark box. CSC mice showed reduced time in the light box **(A)** but unaltered locomotor activity **(B)** compared to SHC mice. Tested on the elevated plus-maze on the following day, the percentage of time spent on the open arms remained comparable between CSC and

SHC mice **(C),** however CSC mice entered the open arm at a lower percentage **(D)**. Data represents mean + SEM; * p<0.05 vs. SHC. Student's t-test; n = 14-17 per group.

Validation CSC study behavioural statistics:

Parameter	Stress effect (SHC vs. CSC)
Time light box (Fig. 20A)	$T_{(28)}$ = 2.555; p = 0.016 *
Distance travelled (Fig. 20B)	$T_{(28)}$ = -0.056; p = 0.955
% time on open arm (Fig. 20C)	$T_{(29)}$ = 0.672; p = 0.507
% open arm entries (Fig. 20D)	$T_{(29)}$ = 2.745; p = 0.01 *

3.3.1.2 CSC exposure alters physiological parameters

To further validate the CSC paradigm in the new facility, body weight gain as well as adrenal and thymus weight were analysed in comparison to SHC mice immediately after EPM. CSC exposure did not alter body weight gain (p = 0.086 vs. SHC; Fig. 21A) but increased absolute adrenal weight (p<0.01 vs. SHC; Fig. 21B) and decreased absolute thymus weight (p<0.001 vs. SHC; Fig. 21C).

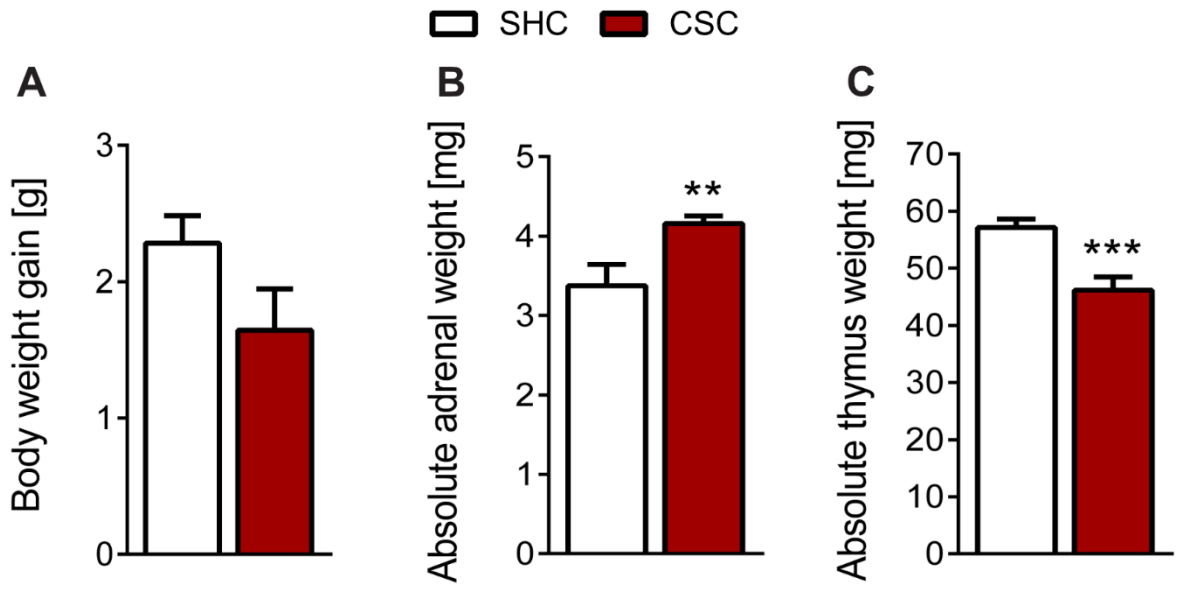


Figure 21. Effects of 20 consecutive days of CSC exposure on physiological parameters. CSC mice appeared to gain less body weight during CSC exposure **(A)** together with an increased absolute adrenal weight (sum of all adrenals; **B**) and a decreased absolute thymus weight **(C)**. Data represents mean + SEM; ** p<0.01, *** p<0.001 vs. SHC. Student's t-test; n = 14-17 per group.

Validation CSC study physiological statistics:

Parameter	Stress effect (SHC vs. CSC)
Body weight gain (Fig. 21A)	$T_{(31)} = 1.770$; $p = 0.086$
Absolute adrenal weight (Fig. 21B)	$T_{(27)} = -3.493$; $p = 0.002$ *
Absolute thymus weight (Fig. 21C)	$T_{(22)} = 4.116$; $p < 0.001$ *

3.3.2 Effects of acute and subchronic minocycline treatment on CSC-induced behavioural and physiological parameters

As CSC mice showed the previously reported CSC-induced physiological and behavioural alterations, the capability of acute (1 h) and subchronic (8 days) minocycline treatment to reverse these alterations was evaluated. Thus, on day 20, mice received a single i.p. injection of 80 mg / kg minocycline 1 h prior to LDB. Starting from day 21, mice were treated for additional 7 days of subchronic treatment *via* the drinking water.

3.3.2.1 Acute minocycline treatment does not alter CSC-induced anxiety-like behaviour

CSC exposure for 20 consecutive days reliably increased innate anxiety-related behaviour compared to SHC mice in the LDB, while an i.p. injection of 80 mg / kg minocycline one hour prior to behavioural testing did not affect behaviour ($p<0.01$ vs. SHC; Fig. 22A). Further, CSC mice showed unchanged locomotor activity compared to SHC mice, indicated by the distance travelled, independent of treatment (Fig. 22B). SHC mice in general did not respond to acute minocycline treatment in any behavioural parameter assessed.

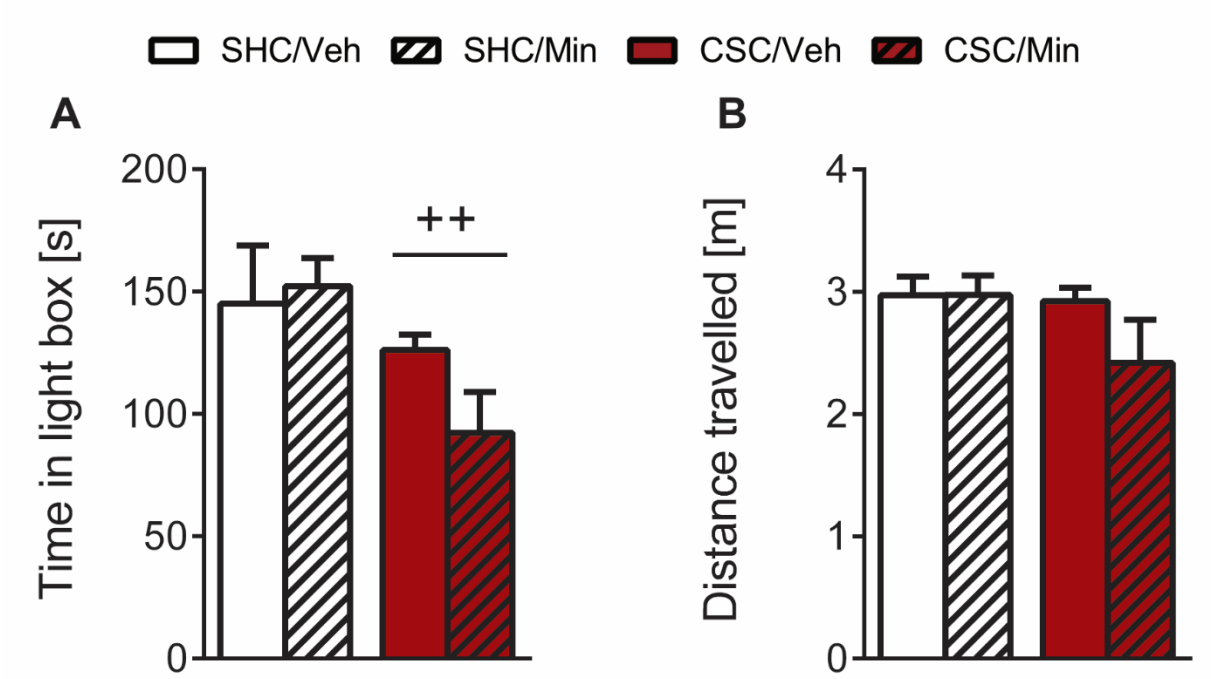


Figure 22. Anxiety-like behaviour and locomotor activity after 20 consecutive days of CSC exposure and acute treatment with either vehicle (Veh) minocycline (Min). CSC mice showed the expected stress-induced increase in anxiety-like behaviour, reflected by decreased time in the light box **(A)**, and comparable distance travelled **(B)** independent of Min treatment. Data represents mean + SEM; ++ $p<0.01$ vs. SHC. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 12-14 per treatment group.

Acute minocycline CSC study behavioural statistics:

Parameter	Stress effect (SHC vs. CSC)	Stress x treatment
Time light box (Fig. 22A)	$F_{(1,44)} = 8.553$; $p<0.01$ *	$F_{(1,44)} = 3.293$; $p = 0.076$
Distance travelled (Fig. 22B)	$F_{(1,44)} = 2.346$; $p = 0.133$	$F_{(1,44)} = 1.641$; $p = 0.207$

3.3.2.2 Subchronic minocycline treatment does not improve CSC-induced anxiety-like behaviour

The acute treatment on day 20 of CSC was continued for 7 days in the respective groups with minocycline administration in the drinking water to investigate potential beneficial effects of a subchronic minocycline treatment. Drinking behaviour of SHC and CSC mice (Fig. 23) did not differ over time within the respective groups. CSC mice drank significantly more on day 2 and day 4 of the subchronic treatment ($p<0.05$ vs. SHC), while minocycline treatment did not affect fluid intake. The drug concentration was calculated daily according to the body weight and drunk volume. Therefore, the treatment regimen of minocycline was assumed stable and mice received the appropriate dose of minocycline. Behavioural testing on day 6 (day

27 of experiment) and day 7 (day 28 of experiment) did not affect drinking behaviour (indicated by arrows in Fig. 23).

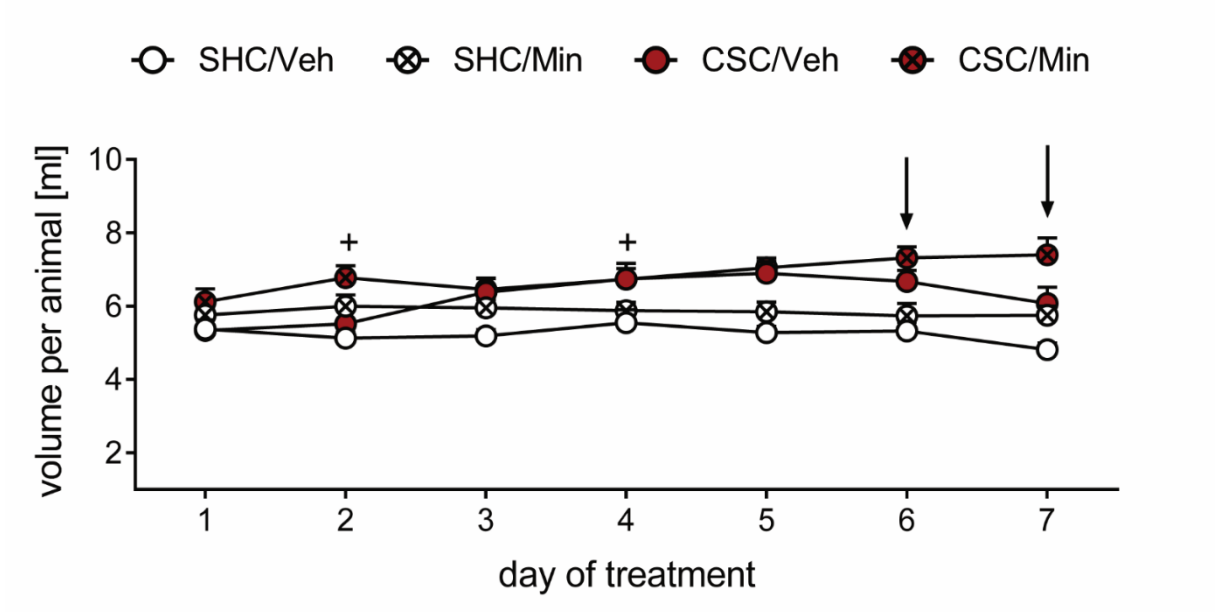


Figure 23. Drinking volume of SHC and CSC mice treated with either vehicle (Veh) or minocycline (Min) for 7 additional days after acute minocycline injection. The fluid intake over 7 days after acute Min injection (experimental day 21 to 28) remained stable within each group. CSC mice drank more than SHC mice on days 2 and 4, while Min treatment and behavioural testing on day 6 and 7 did not affect drinking behaviour. Data represents mean + SEM; + $p<0.05$ vs. SHC. Two-way ANOVA for repeated measures followed by a *post hoc* test using Bonferroni correction; n = 11-14 per treatment group.

Subchronic minocycline CSC study drinking volume statistics:

Parameter	Stimulus effect (day 1 – 7)	Stress effect (SHC vs. CSC)	Stimulus x treatment
Drinking volume (Fig. 23)	$F_{(6,282)} = 0.695$; p = 0.654	$F_{(1,47)} = 1.978$; p = 0.166	$F_{(6,282)} = 0.379$; p = 0.892

The increased anxiety-like behaviour observed in the LDB on day 20 was persistent in the OF on day 27. Here, CSC mice spent less time in the inner zone of the OF ($p<0.01$ vs. SHC; Fig. 24A) with comparable levels of locomotor activity between SHC and CSC mice (Fig. 24B). Subchronic minocycline treatment was not able to reverse this anxious phenotype of affect SHC mice. Neither CSC exposure nor subchronic minocycline treatment in SHC and CSC mice affected depressive-like behaviour in the FST on day 28 as reflected by the time spent immobile (Fig. 24C).

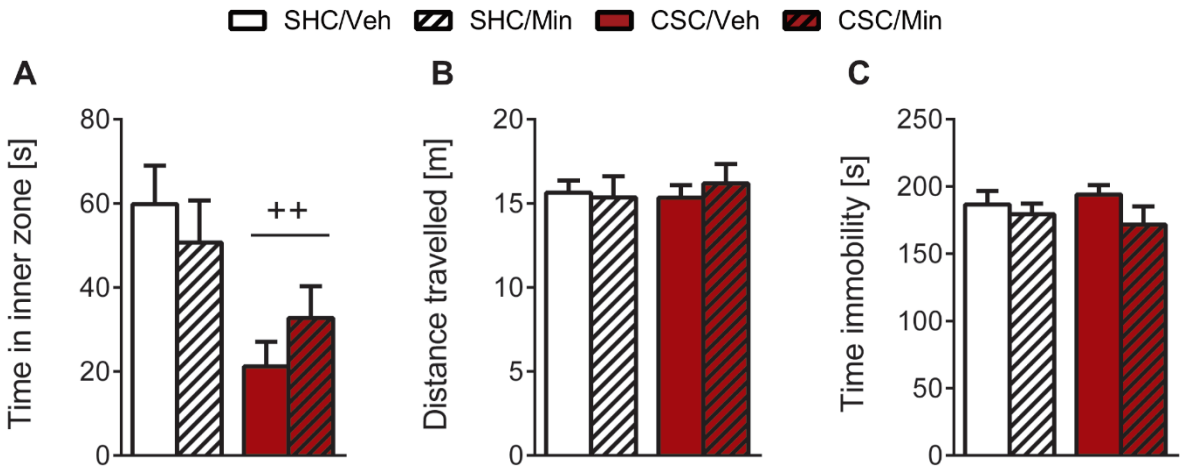


Figure 24. Anxiety- and depressive-like behaviour after 8 days of vehicle (Veh) or minocycline (Min) treatment following 20 days of CSC. Anxiety-like behaviour was elevated in CSC mice compared to SHC mice, as demonstrated by a reduced time in the inner zone of the OF, independent of Min treatment **(A)**, whereas locomotor activity was similar between SHC and CSC mice **(B)**. SHC and CSC mice showed similar times of immobility in the FST that were not affected by Min treatment **(C)**. Data represents mean + SEM; ++ p<0.01 vs. SHC. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 12-14 per treatment group.

Subchronic minocycline CSC study behavioural statistics:

Parameter	Stress effect (SHC vs. CSC)	Stress x treatment
Time immobility (Fig. 24A)	$F_{(1,44)}$ = 0.000; p = 0.985	$F_{(1,44)}$ = 0.601; p = 0.422
Time inner zone (Fig. 24B)	$F_{(1,44)}$ = 10.154; p<0.01 *	$F_{(1,44)}$ = 1.398; p = 0.243
Distance travelled (Fig. 24C)	$F_{(1,44)}$ = 0.508; p = 0.48	$F_{(1,44)}$ = 1.062; p = 0.308

3.3.2.3 Subchronic minocycline treatment does not reverse CSC-induced physiological and neuroendocrine alterations

Besides behavioural alterations, physiological and neuroendocrine consequences of CSC exposure were assessed after 8 days of minocycline treatment. CSC mice gained significantly more weight after stressor termination compared to SHC mice which was unaffected by subchronic minocycline treatment (p<0.001 vs. SHC; Fig. 25A). Further, CSC exposure enhanced absolute spleen (p<0.001 vs. SHC; Fig. 25B) and adrenal (p<0.001 vs. SHC; Fig. 25C) weight irrespective of minocycline.

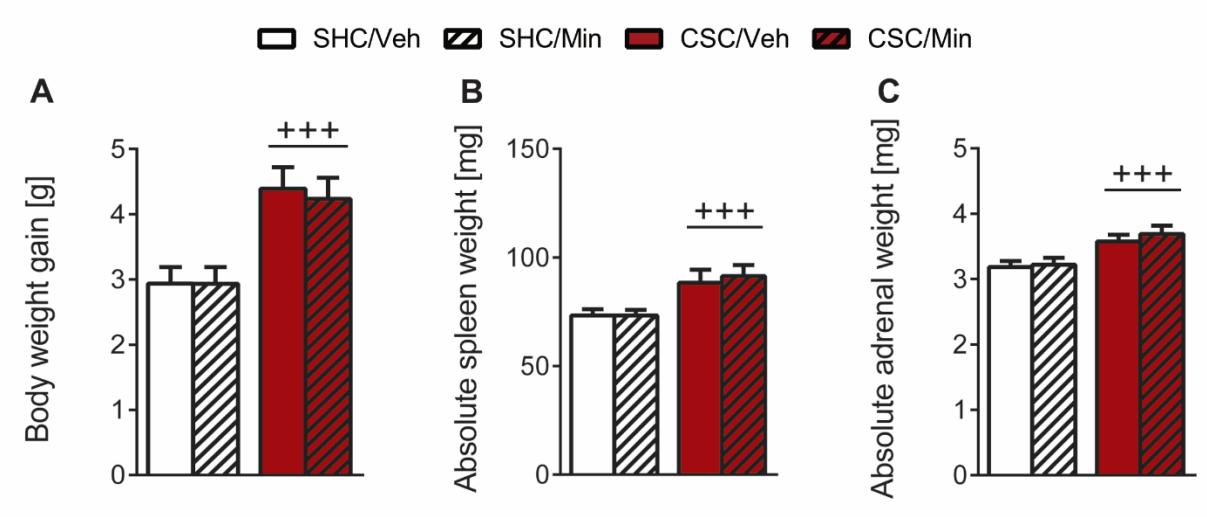


Figure 25. CSC-induced physiological parameters following 20 days of CSC and subchronic (8 days) minocycline treatment. 8 days after stressor termination, CSC mice showed increased body weight gain compared to SHC mice **(A)**. This was accompanied by increased absolute spleen **(B)** and absolute adrenal (sum left and right adrenal) weight **(C).** Data represents mean + SEM; +++ p<0.001 vs. SHC. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 12-14 per treatment group.

Subchronic minocycline CSC study physiological statistics:

Parameter	Stress effect (SHC vs. CSC)	Stress x treatment
Body weight gain (Fig. 25A)	$F_{(1,47)}$ = 10.915; p<0.01 *	$F_{(1,47)}$ = 0.394: p = 0.533
Absolute spleen weight (Fig. 25B)	$F_{(1,46)}$ = 15.649; p<0.001 *	$F_{(1,46)}$ = 0.138; p = 0.712
Absolute adrenal weight (Fig. 25C)	$F_{(1,46)}$ = 16.286; p<0.001 *	$F_{(1,46)}$ = 0.139; p = 0.711

Eight days after stressor termination and minocycline administration, plasma ACTH (Fig. 26A) and CORT levels (Fig. 26B) remained similar between CSC and SHC mice, irrespective of minocycline treatment. *In vitro* stimulation of adrenal explants revealed an increased CORT secretion in response to ACTH compared to basal (saline) stimulation in both SHC and CSC mice (p<0.001 vs. basal; significance not indicated). Analysing adrenal functionality using a two-way ANOVA as separate statistics, ACTH-stimulated adrenal CORT secretion was significantly reduced in CSC compared to SHC mice (p<0.01 vs. SHC; Fig. 26C). This effect was also visible in basal stimulated adrenal explants (p<0.05 vs. SHC) and indicative for reduced adrenal sensitivity. A treatment effect was only seen in adrenal explants of SHC mice showing reduced basal CORT secretion after subchronic minocycline treatment (p<0.05 vs. corresponding SHC/Veh; Fig. 26C).

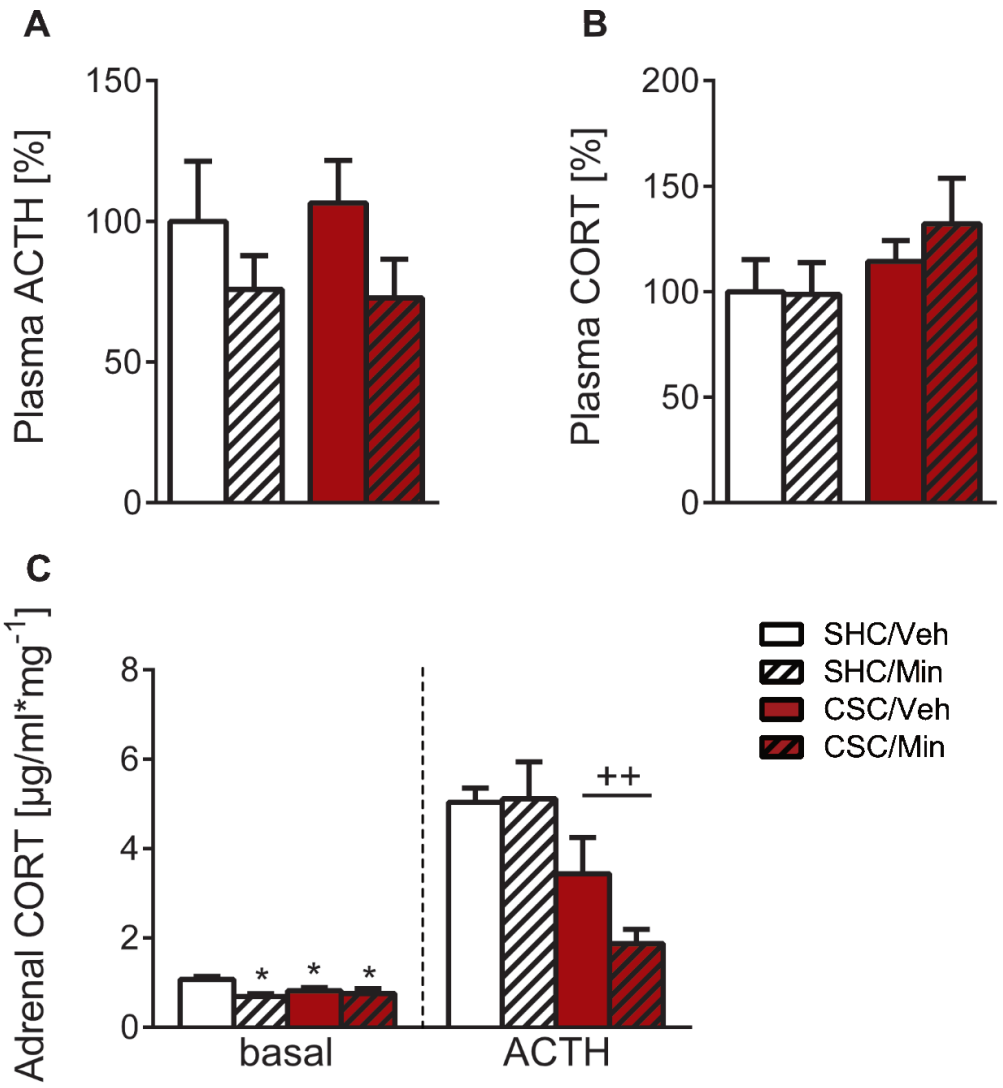


Figure 26. CSC-induced alterations in HPA axis functionality after 8 days of minocycline (Min) treatment. Relative plasma ACTH **(A)** and CORT **(B)** levels were unchanged by CSC and Min treatment. *In vitro* ACTH stimulation of adrenal explants induced CORT secretion compared to basal (saline) stimulation in both SHC and CSC mice that was diminished by CSC exposure. Min reduced CORT secretion after basal stimulation in SHC mice **(C)**. Data represents mean + SEM; * $p<0.05$ vs. corresponding SHC/Veh, ++ $p<0.01$ vs. SHC. Two-way ANOVA followed by a *post hoc* test using Bonferroni correction; n = 6-14 per treatment group.

Subchronic minocycline CSC study neuroendocrine statistics:

Parameter		Stress effect (SHC vs. CSC)	Stress x treatment
Plasma ACTH (Fig. 26A)		$F_{(1,42)} = 0.000$; $p = 0.994$	$F_{(1,42)} = 0.165$; $p = 0.686$
Plasma CORT (Fig. 26B)		$F_{(1,46)} = 1.335$; $p = 0.254$	$F_{(1,46)} = 0.045$; $p = 0.833$
	Stimulation	**Stress effect (SHC vs. CSC)**	**Stress x treatment**
Adrenal CORT (Fig. 26C)	**basal**	$F_{(1,20)} = 1.485$; $p = 0.237$	$F_{(1,20)} = 4.470$; $p = 0.047$ *
	ACTH	$F_{(1,20)} = 15.079$; $p = 0.001$*	$F_{(1,20)} = 1.723$; $p = 0.204$

Discussion

The Discussion includes sections taken, and adapted, from one first author publication: Anna K. Schmidtner, David A. Slattery, Joachim Gläsner, Andreas Hiergeist, Katharina Gryksa, Victoria Malik, Julian Hellmann-Regen, Isabella Heuser, Thomas C. Baghai, André Gessner, Rainer Rupprecht, Barbara Di Benedetto, Inga D. Neumann. **Minocycline alters behavior, microglia and the gut microbiome in a trait-anxiety-dependent manner.** *(In preparation)*

In the present thesis, I was able to advance the understanding of two novel targets for the treatment of depression, namely the inflammatory system and the gut microbiome, and extend our knowledge regarding the potential use of minocycline in the treatment of psychiatric disorders. I could show that chronic minocycline was able to increase social preference in male and female HAB rats and reverse innate depressive-, but not anxiety-like, behaviour exclusively in male HAB rats. In addition, HAB rats showed reduced microglia quantity in the prelimbic and infralimbic PFC independent of sex that was further reduced by minocycline in male HAB rats. Male HAB and NAB rats showed a distinct difference in β-diversity of the microbiome composition. Treatment with minocycline "normalized" the microbiome composition in HAB rats with corresponding changes in the metabolome, providing additional evidence for a role of the microbiome in psychiatric disorders. I was also able to recapitulate the lack of effect of minocycline on anxiety-related behaviour in a second model. In detail, acute and subchronic treatment with minocycline did not reverse CSC-induced behavioural, physiological, and neuroendocrine maladaptations. These results extend the HAB model of depression to a model of inflammation- and gut microbiome- associated processes involved in depressive-like behaviour and its treatment. Moreover, they provide evidence for minocycline as a novel treatment option, but urge for caution in terms of application conditions. In detail, it shows sex- and trait-specific effects on behaviour, microglia, and the microbiome in HAB and NAB rats and a lack of effect in stress-induced behavioural and physiological alterations. Thus, minocycline seems to require certain conditions to elicit a treatment response and further research is warranted to delineate its specifics and detailed mode of action as a prerequisite for clinical application in psychiatric disorders.

4.1 Minocycline alters behaviour, microglia and the gut microbiome in a trait-dependent manner

Minocycline has received growing attention during the last years due to its promising results in clinical studies (reviewed in Rosenblat and McIntyre, 2017). These results are supported by a number of preclinical studies, demonstrating antidepressant properties of minocycline in male rodents. In detail, minocycline ameliorates depressive-like behaviour in naïve rats in the FST (Molina-Hernández *et al*, 2008b, 2008a) as well as in the learned helplessness paradigm (Arakawa *et al*, 2012) or in the olfactory bulbectomy model of depression (Burke *et al*, 2014). A similar antidepressant effect of minocycline is achieved when depressive-like behaviour is induced by an inflammatory challenge with either LPS (Henry *et al*, 2008; O'Connor *et al*, 2009b) or IFN-α (Zheng *et al*, 2015) in mice. In chronically stressed rodents, minocycline acts antidepressant in the FST and sucrose preference in rats (Wang *et al*, 2017, 2018) and in the FST, TST, and sucrose preference in mice (Kreisel *et al*, 2014; Tong *et al*, 2017; Wong *et al*, 2016). Interestingly, these preclinical results of an antidepressant effect are mainly achieved following chronic

stress or an inflammatory challenge, but not in the context of an innate behavioural alteration or under baseline conditions. In addition, a sex-specificity of minocycline has been proposed in rats (Posillico *et al*, 2015) and mice (Chen *et al*, 2018) in the context of pain perception. Thus, I aimed to extend this knowledge to an innate animal model of high anxiety- and depressive-like behaviour that is reflective of the clinical high comorbidity between MDD and anxiety disorders and the sex difference found in most mood disorders. As the recent evidence suggests anxiolytic and pro-social effect of minocycline after acute or chronic stress, or an immune challenge, in male rodents (Henry *et al*, 2008; Kreisel *et al*, 2014; Levkovitz *et al*, 2015; Majidi-Zolbanin *et al*, 2016; Zhu *et al*, 2014b), I included additional tests to specify the effects of minocycline on social and anxiety-like behaviour. Escitalopram is currently viewed as the gold standard of available antidepressants (Kasper *et al*, 2006; Santarsieri and Schwartz, 2015). Like minocycline, escitalopram acts anxiolytic and antidepressant in male rats after chronic stress (Lin *et al*, 2016; Montgomery *et al*, 2001; Sánchez *et al*, 2003). Therefore, it was included as positive control and, corresponding to clinical treatment regimen, to determine the capacity of minocycline as an adjunctive agent.

4.1.1 Minocycline affects social and / or depressive-like behaviour of male and female HAB, but not NAB, rats

All rats displayed natural occurring social preference in the SPT, independent of sex or trait. In male and female HAB rats, minocycline facilitated social approach without changing social behaviour in NAB rats. Fittingly, previous studies show that a 3-day minocycline administration reverses LPS-induced social avoidance in male rats (Zhu *et al*, 2014b). Chronic minocycline treatment ameliorates social avoidance after chronic stress in male mice (Kreisel *et al*, 2014) and after LPS administration in male rats (Zhu *et al*, 2014c, 2014a) and mice (Henry *et al*, 2008). However, HAB rats generally do not show impairments in social interaction. The elevated social preference, therefore, may rather reflect an alleviation of depressive-like behaviour (Krishnan *et al*, 2007; Krishnan and Nestler, 2008) than an improvement in social approach *per se*, supporting an antidepressant effect of minocycline. Importantly, in the aforementioned studies minocycline facilitates social preference only in rodents that were previously affected. Similar to the results obtained in the present thesis, control animals do not respond to minocycline treatment. Escitalopram, and the combinatory treatment, did not alter social behaviour in male and female HAB and NAB rats. In line, citalopram treatment ameliorates deficits in social behaviour in FSL rats but not in Wistar rats that show normal social behaviour (Overstreet *et al*, 2004). These studies indicate that both minocycline and escitalopram might require an abnormal precondition for an effect on social behaviour. The combinatory treatment abolished the effect of minocycline on social behaviour, indicating an inhibitory effect of SSRIs on minocycline function (see below).

Furthermore, the high anxiety phenotype of HAB compared to NAB rats was confirmed in males and females and remained unchanged after minocycline treatment in both lines and sexes. Escitalopram decreased anxiety-like behaviour in male NAB rats while the combinatory treatment did not affect behaviour. A role of minocycline in anxiety-like behaviour was previously proposed in male rodent models of cardiac arrest / cardiopulmonary resuscitation (Fan *et al*, 2006; Neigh *et al*, 2009), mild traumatic brain injury (Kovesdi *et al*, 2012), a genetic model of ASD (Shigemori *et al*, 2015), or acute stress (Levkovitz *et al*, 2015; Soczynska *et al*, 2012). In addition, minocycline reverses anxiety-like behaviour induced by a neonatal inflammatory challenge in male mice (Majidi-Zolbanin *et al*, 2016). Treatment of control animals with minocycline does not alter anxiety-like behaviour in the aforementioned studies, proposing, similar as in social behaviour, an absent anxiolytic effect of minocycline in NAB rats that did not show abnormal behaviour. These studies also suggest that anxiety-like behaviour in HAB rats is manifested *via* a minocycline-independent mechanism. However, an acute icv NPS infusion can attenuate anxiety-like behaviour male HAB rats (Slattery *et al*, 2015) and chronic icv oxytocin infusion in male and female HAB rats alleviates anxiety-like behaviour in females, but not in males (Slattery and Neumann, 2010). Furthermore, diminishing the SNP-induced AVP overexpression in HAB rats with a AVP antagonist (Wigger *et al*, 2004) or infusion of a CRH 1 receptor antagonist to counteract CRH overexpression (Keck *et al*, 2001b) acted anxiolytic, suggesting alternative objectives in anxiety-like behaviour of HAB rats. Moreover, the antidepressant effect of minocycline was seen after 22 days. A longer treatment duration could be necessary to ameliorate anxiety-like behaviour in HAB rats, especially as a prolonged treatment of 8 weeks is needed in the context of paroxetine (Jochum *et al*, 2007). The same might be true for an anxiolytic effect of escitalopram, an SSRI like paroxetine, since the results for anxiety-related behaviour in HAB rats are obtained after 17 (LDB) and 19 (EPM) days of treatment. A required longer treatment with SSRIs in HAB rats might be attributable to a dysregulated 5-HT signalling. In support, male HAB rats show an increased SERT binding and reduced 5-HT_{1A} receptor expression in the hippocampus and raphe nucleus. This induces an increased 5-HT clearance from the synaptic cleft (Keck *et al*, 2005) and proposes an impaired 5-HT transmission that might contribute to the delayed response to antidepressants. As a bidirectional communication between the HPA axis and the 5-HT system is known (Dinan, 1996; Leonard, 2005; Linthorst *et al*, 2002; Ressler and Nemeroff, 2000), this impaired 5-HT signalling might be caused by the hyper-reactive HPA axis in HAB rats (Landgraf *et al*, 1999). Indeed, 8 weeks of paroxetine not only improves behaviour but normalizes the HPA axis response to stress (Keck *et al*, 2003) and the SERT binding in the hippocampus (Keck *et al*, 2005). Further, escitalopram decreased anxiety-like behaviour in male NAB rats. Fittingly, an anxiolytic effect of escitalopram under basal and chronic stress conditions has been shown before in male rats (Lin *et al*, 2016; Sánchez *et al*, 2003).

The present results also extend the antidepressant effect of minocycline (see Introduction chapter 1.2.3) to rats with high levels of innate anxiety- and depressive-like behaviour, but normal social behaviour, that

were otherwise unchallenged. While there are different behavioural tests available for the assessment of antidepressant-like activity, the FST is by far the most commonly used test and viewed as the gold standard (Anyan and Amir, 2017). It is largely based on predictive validity, though, as evidence from rodents studies show that antidepressant substances reduce immobility (Cryan *et al*, 2005). Thus, the FST is critically discussed in its interpretation of "immobility" as representation of "despair" and struggling as the opposite, meaning the absence of a depressive-like phenotype. Immobility can easily be influenced by locomotor activity, which may lead to false positive or negative findings. Male and female HAB rats show reduced locomotor activity in the LDB and OF (Liebsch *et al*, 1998b; Ohl *et al*, 2001), which is not causally related to the anxiety phenotype (Landgraf and Wigger, 2002). In the present study, male HAB rats showed reduced locomotion that was unchanged by minocycline in the LDB, deeming activity-dependent effects of minocycline in the FST rather unlikely (De Kloet and Molendijk, 2016; Slattery and Cryan, 2014). Moreover, in a controversial discussion the FST has recently been suggested as a measurement of anxiety-like behaviour, more precisely an inverse relationship between the performance in the FST and on the EPM in male rats (Anyan and Amir, 2017). In more detail, the more immobility those rats show in the FST, the lower anxiety measures are found on the EPM (Anyan and Amir, 2017; Estanislau *et al*, 2011). Nevertheless, the present results strongly imply an antidepressant-like and facilitating effect on active stress coping of minocycline. The sustained high anxiety and depressive phenotype of HAB rats was affected by minocycline in the SPAT and FST, but not in the more specific anxiety tests EPM and LDB, contradicting the theory of an anxiety measurement.

In addition, though two studies demonstrate an antidepressant effect of minocycline on naïve male animals (Molina-Hernández *et al*, 2008a, 2008b), the majority of all studies shows no effect of minocycline on control animals. In line, in the present thesis male and female NAB rats did not react to minocycline treatment, strengthening the hypothesis that minocycline requires pathological behaviour to be effective. This hypothesis is supported by subgroup analyses of a recent meta-analysis including 22 preclinical studies that reveals an antidepressant effect of minocycline only in diseased animals (Reis *et al*, 2019). Thus, the present data demonstrates a fast antidepressant effect of minocycline. Interestingly, an influence of minocycline on female rodents is rarely tested. Three studies could show that minocycline abolished inflammatory neuropathic pain (Chen *et al*, 2018) and reversed morphine-induced analgesia (Posillico *et al*, 2015) and arthritis-induced pain (Fernandez-Zafra *et al*, 2018), but only in male mice. Similarly, in the present thesis minocycline did not ameliorate depressive-like behaviour in female HAB rats, underlining the proposed sex difference in its effectiveness. Although the exact reason for this difference remains unknown, some factors might contribute to or underlie the lacking effect in female HAB rats. First, a pathological dexamethasone / CRH test is observed in male, but not in female HAB rats (Keck *et al*, 2002). Thus, a HPA axis functionality-restoring effect of minocycline as underlying mechanism could account for the sex difference seen in HAB rats. Similarly, minocycline reversed CUMS-induced

elevated plasma CORT levels (Zhang *et al*, 2019). In addition, male and female rats also show a strong difference in immune functioning and response (Klein and Flanagan, 2016; see chapter 1.4.2), which might contribute to the sex-dependent effect of minocycline. A frequently mentioned factor influencing behavioural differences between sexes are hormonal fluctuations in oestrogen. Clinical studies provide evidence that a decline in oestradiol in women is associated with increased rates of MDD, while in female rodents, treatment with oestradiol exerts antidepressant-like effects (Bernardi *et al*, 1989; Harlow *et al*, 2003; López-Rubalcava *et al*, 2012; Parry, 2008; Walf *et al*, 2004; Walf and Frye, 2010). Therefore, a – at least partial – dysregulation in oestrogen signalling in female HAB rats might contribute to their behavioural phenotype. As a cycle activity was observed in the majority female rats, though, a complete lack of oestrogen signalling is deemed rather unlikely; however, a partial impairment might also explain the reduced breeding success (personal observation) seen in those animals. If the oestrus cycle generally affects behaviour in female HAB rats remains to be studied, especially as it was recently proposed that female oestrus variability is not greater than intrinsic variability in males (Beery, 2018). Further, if a dysregulation of oestrogen signalling is responsible for the lacking effect of minocycline in female HAB rats has to be evaluated in the future. Moreover, a sex dependence in minocycline metabolism as cause for the behavioural difference was considered. Minocycline is generally not metabolized by the body but subject to biotransformation in the liver. The microbiologically inactive metabolites are then excreted *via* the urine and faeces (Jonas and Cunha, 1982; Saivin and Houin, 1988). Therefore, a sex difference in behaviour and microglial density in female HAB rats due to a different minocycline metabolism seems unlikely. Besides, although no difference on antibiotic effectiveness of minocycline between males and females is reported so far, the behavioural effect might require a higher dose in female rats than in males or a longer treatment duration. In support, sex-dependent sensitivity in antidepressant treatment is reported. In rats, the anxiolytic-like effect of diazepam as well as the antidepressant effect of desipramine is found in males while females do not respond to the treatment (Simpson *et al*, 2012), proposing a sensitivity difference between male and female HAB rats as underlying mechanism for the sex difference in the behavioural response to the treatment. To explain the behavioural outcome and microglia in female HAB rats, though, a detailed knowledge about the exact mechanism of minocycline is required. Nevertheless, as female HAB rats did not respond to minocycline treatment, it is tempting to speculate that in female HAB rats, depressive-like behaviour might be mediated by another system than in male HAB rats.

Further, the given behavioural results imply a mechanism for the antidepressant action of minocycline. Minocycline treatment increased struggling and decreased immobility (Molina-Hernández *et al*, 2008b; Wang *et al*, 2018). Previous studies, using the same protocol for the FST as here, show that swimming behaviour is increased in response to SSRI administration, while struggling behaviour is increased after administration of antidepressants that selectively target catecholaminergic signalling. As in the present

thesis minocycline also increased struggling behaviour, but not swimming, it might rather target the catecholaminergic transmission (Cryan *et al*, 2002; Detke *et al*, 1995; Lucki, 1997; Slattery and Cryan, 2014). In line, minocycline does not restore elevated levels of tryptophan or 5-HT in the brain of male mice after LPS injection (O'Connor *et al*, 2009b). In the learned helplessness paradigm, minocycline failed to reverse the elevated 5-HT turnover in the orbitofrontal cortex, pointing towards a lack of effect on 5-HT signalling (Arakawa *et al*, 2012). In turn, minocycline restores DA levels after E. coli infection (Mansour *et al*, 2018) and prevents nigrostriatal dopaminergic degeneration in a mouse model of Parkinson's disease (Du *et al*, 2001). Thus, together with the present results showing increased struggling, these data indicate a catecholaminergic mechanism of action. A stronger effect on catecholamines like DA might also contribute to the sex difference seen in HAB rats. Female rats have a higher DA uptake und greater DA release in the striatum and PFC than male rats (Duchesne *et al*, 2009; Walker *et al*, 2000). As minocycline affect rather catecholamine levels, a high DA level in female rats from the beginning might prevent a beneficial effect. Thus, measurement of DA levels in male compared to female HAB rats might give some indication about a potential mechanism.

In addition, in the present thesis male and female HAB and NAB rats did not respond to escitalopram treatment. Previously, repetitive transcranial magnetic stimulation (Keck *et al*, 2001a) as well as transient inactivation of the infralimbic cortex (Slattery *et al*, 2011) had an antidepressant effect in male HAB rats. Further, chronic citalopram treatment of 8 weeks is necessary to ameliorate the depressive-like phenotype in male HAB rats (Keck *et al*, 2003). In turn, 4-week treatment with escitalopram (Montgomery *et al*, 2001; Yilmaz *et al*, 2011) or 5 weeks with citalopram (Rygula *et al*, 2006) ameliorates chronic stress-induced depressive-like behaviour in male rats. In contrast, unstressed rats do not show behavioural alterations in those studies in response to citalopram or escitalopram. The SSRI fluoxetine is likewise not able to increase struggling behaviour in unstressed male rats (Molina-Hernández *et al*, 2008a) In the present thesis, an amelioration of the depressive-like phenotype was not present in male and female NAB rats. The current study applied a dose of 10 mg / kg escitalopram based on previous publications showing a behavioural effect (Burke, 2002; Jayatissa *et al*, 2006; Papp and Sánchez, 2001; Yilmaz *et al*, 2011). However, an 8-week treatment regimen with 10 mg / kg citalopram was required previously to reverse depressive-like behaviour in male HAB rats (Keck *et al*, 2003). Therefore, the present lack of escitalopram effects on HAB rats are probably due to the fact that escitalopram was applied for only 3 weeks. Also, only one dose of escitalopram was tested, a higher dose might be able to affect depressive-like already at this point. Moreover, it is assumed that the treatment effect of antidepressants is not primarily caused by the immediate increase in monoamines as it takes weeks of treatment for a mood-enhancing effect (Krishnan and Nestler, 2008) but *via* secondary changes in molecular and cellular neuroplasticity (Nestler *et al*, 2002; Pittenger and Duman, 2008). Thus, a longer treatment period with escitalopram might be necessary to improve the treatment-resistant behavioural phenotype of HAB rats. Minocycline was effective during

this timeframe, though, demonstrating a faster and stronger effect than escitalopram that is potentially mediated due to faster or direct secondary changes.

Importantly, the combinatory treatment abolished the antidepressant effect of minocycline in male HAB rats. When minocycline is given in humans in combination with several conventional antidepressants like SSRIs or tricyclics, mood stabilizers and antipsychotics, an overall improvement in MDD symptoms can be observed (Dean *et al*, 2017; Husain *et al*, 2017; Rosenblat and McIntyre, 2017). However, two studies analysing data from the large-scale real-world human study "sequenced treatment alternatives to relieve depression" (STAR*D), concentrating exclusively on the SSRI citalopram, reveal that administration of citalopram in combination with a non-steroidal anti-inflammatory drug reduces the probability of remission (Gallagher *et al*, 2012; Warner-Schmidt *et al*, 2011). In this thesis, augmentation of minocycline with escitalopram abolished the antidepressant effect of minocycline. Interestingly, in rats a combination of minocycline with glutamate antagonists or the tricyclic desipramine, which is a relatively selective noradrenaline reuptake inhibitor, improves depressive-like behaviour (Molina-Hernández *et al*, 2008b, 2008a). However, and similar to the obtained results in the present experiment, when combined with the SSRI fluoxetine either systemically (Molina-Hernández *et al*, 2008a) or locally into the nucleus accumbens (Molina-Hernández *et al*, 2008b), the antidepressant effect of minocycline is abolished. Citalopram or fluoxetine reduce the density of cell positive for tyrosine hydroxylase, the limiting enzyme for DA biosynthesis, in the substantia nigra (MacGillivray *et al*, 2011). This mechanism might counteract the previously mentioned beneficial effects of minocycline on DA signalling. Further, this strengthens the hypothesis of a catecholaminergic mechanism for minocycline and strongly suggests that minocycline does not synergize with the mechanism responsible for the antidepressant effect of SSRIs. Nevertheless, though minocycline and escitalopram do not synergize in their action in this experiment, minocycline does not necessarily target the monoaminergic mode of action, but might affect substance-specific secondary mechanisms or elicit local pharmacokinetic interactions. Of note, an influence of repeated testing (Bouwknecht *et al*, 2004; Cryan and Holmes, 2005; Holmes *et al*, 2001; McIlwain *et al*, 2001) on minocycline-induced behavioural improvement was considered. To counteract this phenomenon, the order of tests for the present thesis was chosen from the least stressful (SPAT) to the most stressful test (FST) and all rats were granted a day of rest between two behavioural tests to avoid stress- or experience-induced bias.

As the behavioural effect of minocycline was restricted to male HAB rats at this dose (40 mg / kg), anxiety-like behavioural was generally not affected, and female HAB rats only showed facilitated social prefence, a dose-dependent effect of minocycline was considered. Most studies apply minocycline in a dose ranging from 10 to 100 mg / kg (Yong *et al*, 2004). The dose of 40 mg / kg in the first experiment was chosen based on previous studies demonstrating a physiological and behavioural effect in both male rats and mice without affecting locomotor activity (Chen *et al*, 2009; Hinwood *et al*, 2012, 2013; Kreisel *et al*, 2014;

Saeedi Saravi *et al*, 2016a; Tong *et al*, 2017; Xu *et al*, 2017). Even a high dose of minocycline, up to 160 mg / kg, does not affect locomotor activity in rats but shows beneficial behavioural effects (Amorim *et al*, 2017; Festoff *et al*, 2006; Saeedi Saravi *et al*, 2016b). Thus, a higher dose of 80 mg / kg minocycline was administered exclusively to male and female HAB rats. Interestingly, the higher dose led to identical results as obtained with 40 mg / kg, namely an increase in social preference in both sexes and alleviated depressive-like behaviour only in male HAB rats, while anxiety-like behaviour and locomotor activity remained unchanged. Dose-response studies of acute or repeated minocycline indicate the same treatment response to 40, 80, and 160 mg / kg minocycline on depressive-like behaviour (Molina-Hernández *et al*, 2008a; Saeedi Saravi *et al*, 2016b). Further, studies using the highest doses of minocycline administer it only acutely (Burke *et al*, 2014; Chen *et al*, 2009; Molina-Hernández *et al*, 2008a) or subchronically (Du *et al*, 2001; Saeedi Saravi *et al*, 2016a) in both male rats and mice. A higher dose than 80 mg / kg minocycline for a longer time might eventually exert beneficial effects in female HAB rats. However, a higher dose for a longer duration to elicit a treatment response in female HAB rats might also lead to negative side effects or locomotor changes. Nevertheless, a beneficial effect cannot be excluded.

4.1.2 Minocycline reduces microglial quantity specifically in male HAB rats

Given the lack of success and delayed onset of action of current antidepressants, research turned towards other systems as potential treatment. Since 1991, the inflammatory hypothesis of depression was developed and extended throughout the last years, studies focus on the analysis of immune functions as a role in psychiatric disorders. Various groups find dysregulations in peripheral cytokine concentrations and, importantly, abnormalities in microglia numbers and activation in the brain in both clinical and preclinical studies (Dantzer *et al*, 2011; Maes, 1995; Smith, 1991; Soczynska *et al*, 2012; Yirmiya *et al*, 2015). Most studies linking neuroinflammation to MDD show that increased microglial activation is associated with mood disorders (Dheen *et al*, 2007; Yirmiya *et al*, 2015). Minocycline is known to intervene in microglial functioning by dampening microglial activity and proliferation, and, thereby, potentially executing its antidepressant effects (see Introduction chapter 1.2.3). Therefore, microglial quantity in male and female HAB rats in comparison to NAB rats were assessed after 22 days of minocycline, escitalopram, or combinatory treatment as potential mechanism contributing to the behaviour phenotype of HAB rats. The PFC is a brain region highly implicated in MDD. Deep brain stimulation of Brodmann Area 25 (infralimbic PFC) in MDD patients normalizes its hyper-functioning and ameliorates symptoms of MDD (Mayberg *et al*, 2005), corresponding to an antidepressant effect of a transient inactivation of the PFC in rats (Slattery *et al*, 2011). Patients suffering from MDD also show reduced grey-matter volume and glial density in the PFC (Krishnan and Nestler, 2008; Rajkowska, 2003; Rajkowska *et al*, 1999), correlating with the severity of symptoms (Carlson *et al*, 2015). Similarly, in a model of

depression, neonatal clomipramine, male rats show a reduced PFC volume and increased depressive-like behaviour that is both reversed by intracranial self-stimulation (Chakraborty *et al*, 2019). Microglial activation in response to stress is also found in the PFC (Hinwood *et al*, 2012, 2013; Tynan *et al*, 2010) and in the olfactory bulbectomy model of depression, microglial inhibition in the PFC acted antidepressant (Burke *et al*, 2014). Therefore, the analysis was performed in the prelimbic and infralimbic prefrontal cortex of male and female HAB and NAB rats.

HAB rats showed reduced microglial numbers in the infralimbic and prelimbic PFC compared to NAB rats independent of treatment and corresponding to their behavioural phenotype. Recently, a dynamic and bi-directional alteration in microglial status rather than an upregulation in microglial function has been hypothesized to underlie depressive-like behaviour. In this study in male rats, chronic stress leads to an initial short-term upregulation of microglial quantity followed by increased microglial apoptosis and a permanent decline in microglial numbers in both the hippocampus and PFC (Kreisel *et al*, 2014). This effect was recapitulated in male mice (Gong *et al*, 2018; Tong *et al*, 2017; Zhu *et al*, 2018) and female rats (Bollinger *et al*, 2017). Additionally, repeated social defeat during adolescence leads to reduced microglial numbers but with a strongly activated profile in the PFC of adult male mice (Rodríguez-Arias *et al*, 2018) and repeated CORT injection, mimicking a repeated stress response, inhibits microglia proliferation in the hippocampus (Wennström *et al*, 2006). Therefore, the lower microglial density in the PFC of HAB rats might be caused by their permanent stressed stage, as expressed by the hyper-reactive HPA axis (Keck *et al*, 2002; Landgraf *et al*, 1999), inducing a constant microglial activation and, thus, apoptosis. Interestingly, an abnormal glial activity and immune function is reported in two other rat models of depressive-like behaviour. Female Wistar Kyoto rats show increased Iba-1 immunoreactivity in the hippocampus but normal levels in the amygdala which is indicative for a higher microglial activity (Mileva *et al*, 2017). In male Wistar Kyoto rats, in the infralimbic cortex an astrocytic deficit is linked to the behavioural phenotype (Gosselin *et al*, 2009) as well as a potential attenuation in microglia expression in response to stress (Sherwin *et al*, 2014). Unfortunately, the authors of the latter paper did not specify whether male or female rats were used. A reduced hippocampal volume and neuronal numbers together with increased astrocyte immunoreactivity in the hippocampus were found in male FSL rats (Gómez-Galán *et al*, 2012; Kaae *et al*, 2012), the latter a phenomenon that is also observed in reactive astrocytes (Eddleston and Mucke, 1993). If the resident microglia in the PFC of HAB rats are continuously and stronger activated than microglia of NAB rats or have impaired functioning with increased apoptosis remains to be studied, though. Considering the long-term effects of social defeat during adolescence (Rodríguez-Arias *et al*, 2018) and the age-independent behavioural and neuroendocrine phenotype of HAB rats, the reduced microglial numbers in the PFC might occur already at a very early stage and induce a long-lasting microglial dysfunction that contributes to the HAB phenotype. Therefore, the analysis of microglial activation stages

and dynamics in HAB rats already from postnatal until an adult age might reveal a mechanism underlying the behavioural phenotype in HAB rats.

Interestingly, minocycline further reduced microglial density exclusively in male HAB rats, an effect that was attenuated by escitalopram co-administration and paralleled the antidepressant behavioural outcome. Likewise, minocycline reduced chronic stress-induced activation of microglia in the infralimbic and prelimbic PFC (Hinwood *et al*, 2012) in male rats. Minocycline prevents microglial proliferation and a subsequent reduction in microglial density after chronic stress in male rats (Gong *et al*, 2018; Kreisel *et al*, 2014; Tikka *et al*, 2001; Tong *et al*, 2017). Thus, the minocycline-induced decline in microglial density in male HAB rats might be caused by inhibited microglial proliferation, but not apoptosis, in the PFC of male HAB rats. Thereby, the dysregulated inflammatory phenotype in HAB rats might be ablated and alleviate depressive-like behaviour in HAB rats. Similar to the reduction in microglial numbers, a transient inactivation of the infralimbic cortex of male HAB rats had antidepressant effects (Slattery *et al*, 2011). Importantly, in the aforementioned studies, rats that were not stressed but treated with minocycline did not show changes in microglial density. A related condition was observed in behaviour when minocycline did not affect social or depressive-like behaviour in rodents that were not challenged beforehand. Consequently, the present results together with the available literature indicate that pathological behaviour is connected to altered microglia activity and minocycline requires not only behavioural aberrations but also an inflammatory state for an effective treatment. In support, in a recent clinical study minocycline failed to alleviate symptoms of schizophrenia, presumably due to a lack of inflammatory activation in the patients (Deakin *et al*, 2018).

Microglial activation and dysregulation can also occur *via* activation from peripheral immune components that cross the BBB (reviewed in Langgartner *et al*, 2019). Essential for the maintenance of BBB integrity is coverage of blood vessels with astrocytes; a state that can be compromised by an inflammatory state (Malik and Di Benedetto, 2018). Male HAB rats show a reduced astrocyte coverage of blood vessels in the brain (Di Benedetto *et al*, 2016), indicating an impaired BBB integrity. During CNS inflammation, stress, or increased BBB permeability, macrophages can infiltrate the brain and enhance inflammatory processes (Wohleb *et al*, 2011; Yin *et al*, 2017). Recently, minocycline has been shown to restore BBB permeability in male rats (Soczynska *et al*, 2012; Yang *et al*, 2015a, 2015b). Together with its anti-inflammatory effects, minocycline might restore the potentially impaired BBB permeability in male HAB rats and thereby reduce the number of macrophages in the brain. This is of importance, as Iba-1, used as a marker for microglia, is also expressed and detectable in macrophages (Imai and Kohsaka, 2002). Therefore, a prevented macrophage infiltration into the brain might contribute to the reduced microglial number in the PFC of male HAB rats after minocycline treatment. In order to validate these hypotheses, a more detailed analysis

of the immune system in HAB rats is necessary. An alternative marker like the microglia-specific transmembrane protein 119 will distinguish between infiltrating macrophages and resident microglia. On a translational level, reduced glial density is found in the amygdala (Bowley *et al*, 2002), anterior cingulate cortex (Cotter *et al*, 2001a), Brodmann's area 24 (anterior cingulate cortex; Öngür *et al*, 1998), and the orbitofrontal cortex (Rajkowska *et al*, 1999) of patients that suffer from MDD (for review see Cotter *et al*, 2001b). Further, reduction in grey-matter volume negatively correlates with the severity of MDD symptoms in men (Carlson *et al*, 2015). The decline in microglia seen especially in male HAB compared to male NAB rats hence might – at least in part – explain the known volume reductions. Although the underlying mechanisms of microglial dynamics need to be revealed in more detail, the present results substantiate the hypothesis of an impaired microglial homeostasis and functioning as underlying mechanism for depressive-like behaviour. A more specific manipulation of microglia will be necessary to study their influence on behaviour in HAB rats. Recently, the application of a colony-stimulating factor 1 receptor antagonist was shown to eliminate about 99 % of all microglia in the brain (Elmore *et al*, 2014). As this method does not inhibit but eliminate microglia in the whole brain, though, their ablation is neither reversible nor region-specific inactivation. Alternatively, microglial activation or inhibition can be induced by LPS and IFN-γ or IL-4 and IL-13, respectively (Subramaniam and Federoff, 2017), enabling in a region-specific manipulation of cellular activity. However, these substances activate the whole immune system of the brain. To achieve a cell-specific manipulation of microglia, a cell-targeted manipulation, like chemogenetic silencing or activation of microglia, is necessary.

The minocycline-induced diminished microglial density in the PFC of male HAB rats was abolished in combination with escitalopram. Interestingly, the SSRIs citalopram and fluoxetine increase cytokine concentrations in the frontal cortex of male mice (Warner-Schmidt *et al*, 2011) and escitalopram is proposed to activate microglia (MacGillivray *et al*, 2011). The lack of behavioural effect of escitalopram treatment and the abolished effect of minocycline in the combinatory treatment of the present thesis might be accounted for by this pro-inflammatory effect of escitalopram. Escitalopram alone also did not affect microglial density in male or female HAB rats, probably due to the presence of an inflammatory status *per se* in HAB rats. Microglia of female HAB rats did also not react to the provided treatment. A genetic component as underlying mechanism for the gender mismatch in MDD has to be considered in male vs. female HAB rats. A recent gene expression study provides a comprehensive characterization of transcriptional profiles of men and women suffering from MDD in comparison to a mouse model of depression. Here, transcriptional changes in six different brain regions, like the ventromedial PFC and the orbitofrontal cortex, only marginally overlap between women and men. In men, gene modules regulating catecholamine metabolism, while in women rather the inflammatory response and synaptic transmission is upregulated. Similarly, chronically stressed male and female mice exhibit a transcriptional overlap of only 20 – 25 % between sexes in response to stress (Labonté *et al*, 2017), suggesting in both organisms a

profound sex difference in transcriptional and therefore physiological responses, especially regarding inflammation and catecholamines. In general, both the innate and adaptive immune system show a distinctly different response pattern in male and female rodents as well as humans with a higher immune response in females in the majority of studies (for review see Klein and Flanagan, 2016; Pitychoutis and Papadopoulou-Daifoti, 2010). Regarding central immune responses, male and female rats appear to have comparable microglial densities in various brain regions. Interestingly, female rats show an increased ratio of primed to ramified microglial morphology in the medial PFC after chronic stress compared to male rats (Bollinger *et al*, 2016, 2017), indicating a stronger microglial activation. Additionally, early perturbations in microglial development show profound effects on adult female, but not male, microglia (Thion *et al*, 2018) that might cause the higher immune activation. A similar phenomenon could be present HAB rats. An even higher immune activation in female HAB rats, together with the modulatory role of microglia in behaviour, neuronal structure, and plasticity (see chapter 1.2), might account for the lacking effect of minocycline on microglia and depressive-like behaviour at the studied time point and dose. Since female HAB rats showed facilitated social preference but not depressive-like behaviour in response to minocycline, accompanying microglial changes might still occur but in another brain region (e.g. amygdala).

4.1.3 Minocycline alters the gut microbiome composition and dampens peripheral immune functioning

The multidirectional communication network within the MGB axis modulates not only on behaviour but also microglia proliferation and maturation (Erny *et al*, 2015) and – in case of a gut microbiota dysbiosis – can result in neuroinflammation mediated by chronic microglial activation (Kim and de La Serre, 2018). As an antibiotic, minocycline modulates – beside the inflammatory system – the microbial composition of the gut (Wong *et al*, 2016), supporting the robust link between microbiome and behaviour known as the microbiome-gut-brain axis (see introduction chapter 1.3.2). Therefore, the gut microbiome and one of its major metabolites, 3-OH-butyrate, as well as peripheral cytokine concentrations of HAB and NAB rats were analysed to unravel potential modulations in the microbiome-gut-brain axis that might contribute to the behavioural phenotype. As the microbiome analysis is extensive, the evaluation of the obtained microbiome data of general phyla was focused on butyrate-producing bacteria. Further, as only male HAB rats responded regarding depressive-like behaviour and microglial density in the PFC, analyses were restricted to male HAB vs. NAB rats. Male HAB and NAB rats originating from the same breeding facility showed comparable total bacterial numbers in their cecal content. However, profound differences were found in global gut microbiota composition, reflecting the robust phenotypic differences between the two breeding lines. Interestingly, in chronic stress-induced (Wong *et al*, 2016) or OB-induced depressive-like

behaviour in mice (Park *et al*, 2013) and in patients suffering from MDD (Jiang *et al*, 2015; Kelly *et al*, 2016; Peter *et al*, 2018; Zheng *et al*, 2016) a marked shift in microbial composition was observed. Transplantation of MDD patient fecal microbiota into male rats or mice induced the same depressive-like phenotype and even physiological features, like a dysregulated tryptophan metabolism, compared to rodents colonized with "healthy microbiota" from control individuals (Kelly *et al*, 2016; Zheng *et al*, 2016), underlining the crucial role of gut microbiota in health and disease. The reported visible shift in microbiome composition similar in humans and rodents also might serve as a prerequisite for clinical studies.

As expected, 3 weeks of minocycline treatment significantly reduced bacterial richness analogous in both lines and resulted in yet persisting, but diminished, group differences. A decline of gut bacterial diversity represents a frequent side effect of antibiotic exposure that was only observed within NAB microbiomes, yet not within HAB rats. Since the calculated Shannon diversity index integrates richness and evenness of taxa, this implies a more even distribution of species in HAB rats following treatment. In healthy humans, 22 days of minocycline treatment induced a shift in microbiome composition, which could be shown by a reduced Shannon diversity index even after a 1-week (Zaura *et al*, 2015). This suggests a partial equalization of the microbiota between HAB and NAB rats that might contribute to the observed antidepressant effect of minocycline in HAB rats. Importantly, in both humans and rats, a comparable shift in microbial composition in MDD and rats with high depressive-like behaviour as well as a comparable reaction of the microbiome to minocycline is observed. Thus, current research indicates that an alteration in specific aspects of gut microbiota might underlie the depressive-like phenotype and, together with the obtained results in this thesis, discloses a crucial prerequisite for a translational approach and facilitate the implementation of preclinical results into clinical practice.

A detailed taxonomic analysis of the microbiome composition between HAB and NAB rats revealed distinct differences in bacterial families. HAB rats showed reduced levels of Lachnospiraceae in comparison to NAB rats, which is in line with two recent studies demonstrating that underrepresentation of the Lachnospiraceae family in human fecal microbiota correlated with depression (Naseribafrouei *et al*, 2014; Peter *et al*, 2018). Minocycline treatment expanded the Lachnospiraceae abundance of HAB rats to the level of NAB rats. A similar dynamic change in Lachnospiraceae was found in a model of chronic restraint stress-induced depressive-like behaviour. Minocycline treatment was able to reverse the behavioural phenotype and increased Lachnospiraceae abundance in microbiome samples (Wong *et al*, 2016). Further, minocycline increased the relative abundance of the Clostridiales Family XIII in both HAB and NAB rats. Both Lachnospiraceae and Clostridiales Family XIII accommodate a large part of butyrate-producing bacterial genera (Ueki *et al*, 2018). Correspondingly, both HAB and NAB rats show an increase in plasma 3-OH-butyrate levels that was higher in HAB than in NAB rats. Plasma 3-OH-butyrate levels positively correlated with both bacterial families in HAB rats, while in NAB rats only a positive correlation

between 3-OH-butyrate and the Clostridiales Family XIII was detectable, proposing a rather Lachnospiraceae-mediated antidepressant effect. Butyrate itself is crucial in promoting colonic homeostasis and both butyrate and 3-OH-butyrate are known for its anti-inflammatory effects on the peripheral and central immune system (see Introduction chapter 1.3.3). In the periphery, butyrate inhibits macrophage and T cell activity (reviewed in Kim *et al*, 2014). In line, after minocycline treatment a decreased plasma IFN-γ concentration in both HAB and NAB rats was observed. IFN-γ is produced by IL-12-activated T helper type 1 cells and though there is no significant difference or even a trend in IL-12 concentrations between HAB and NAB rats after minocycline treatment, minocycline seemed to reduce IL-12 levels. Thus, minocycline might exert its peripheral anti-inflammatory effects by inhibiting T cell activation (reviewed in Garrido-Mesa *et al*, 2013; Giuliani *et al*, 2005) together with butyrate and decreasing IFN-γ concentrations in the plasma, supporting a regulatory role of both minocycline and butyrate on peripheral T cells and inflammation. The gut microbiome has also profound impact on the appropriate development of microglia (Erny *et al*, 2015; Thion *et al*, 2018). An involvement of the central immune system remains to be studied, however an anti-inflammatory effect of butyrate on microglia has been reported in terms of inhibition of microglial activation (Fu *et al*, 2015) and induction of microglial apoptosis (Chen *et al*, 2007). Butyrate-mediated microglial apoptosis might therefore contribute to the reduced microglial numbers in the PFC of male HAB rats seen in this study. Recently, 3-OH-butyrate and butyrate have shown antidepressant-like effects in both rats (Wei *et al*, 2015) and mice (Resende *et al*, 2013; Valvassori *et al*, 2015). These findings point towards a crucial role of bacteria-produced butyrate as a mediator of behaviour and microglia alterations in male HAB rats.

A microbiome dysbiosis in HAB rats might have long-lasting and severe effects on microglia in female than in male HAB rats, thereby preventing a beneficial effect of minocycline on microglia and depressive-like behaviour. Moreover, the gut microbiota has profound influence on BBB permeability (Braniste *et al*, 2014). A dysbiosis in gut microbial composition can impair gut barrier function and increase bacterial infiltration into the mucosal layer and eventually into systemic circulation (Abdel-Haq *et al*, 2018; Kelly *et al*, 2015b). Increased circulation, and a subsequent immune response, can compromise the BBB and cause a heightened microglial activation and a pro-inflammatory status in the brain (Riazi *et al*, 2008), causing the behavioural and microglial phenotype seen in HAB rats. In support, certain bacterial strains not only activate microglia but induce microglial apoptosis (Lehnardt *et al*, 2007), which might contribute to the decreased microglial numbers in HAB rats. In GF mice, an increased BBB permeability could be rescued by colonization with butyrate-producing bacteria or butyrate administration (Braniste *et al*, 2014; Kelly *et al*, 2015b), underlining the regulatory role of microbiota in BBB homeostasis. It is therefore tempting to speculate that in HAB rats an increased BBB permeability, potentially caused by gut microbial dysbiosis, induces the behavioural and microglial phenotype. The minocycline-mediated increase in butyrate levels might exert beneficial effects on HAB gut barrier and BBB function and thus on behaviour and microglia.

4.1.4 Conclusion and outlook of chronic minocycline treatment in rats

In summary, the present results provide evidence that the high depressive- and anxiety-like phenotype of HAB rats is accompanied by reduced microglial numbers in the infralimbic/prelimbic PFC and a distinct shift of the gut-bacterial composition. This emphasizes that HAB rats are a valid model for inflammation- and microbiome-associated depressive-like behaviour. Additionally, it points towards a complex interplay between microbiota/microbial metabolites and the immune system determining behaviour in HAB rats, as proposed by the MGB axis. Chronic minocycline treatment, but not escitalopram or the augmentation strategy, reversed depressive-like behaviour in a sex- and trait-specific manner, proposing HAB rats as model for treatment-resistant depression and minocycline as potential treatment. The behavioural results were accompanied by decreased microglial numbers in the PFC. Minocycline also changed the microbial composition markedly by increasing Lachnospiraceae abundance to NAB levels, indicating a "normalization" of the HAB microbiome corresponding to the behavioural and microglial alterations. Thus, modulation of the MGB axis reverses behavioural aberrations and thus represents a valid novel treatment target in psychiatric disorders. The observed changes in the metabolite 3-OH-butyrate likely contribute to the phenotypical effects. Although the attributable contribution of minocycline and butyrate to the anti-inflammatory and behavioural effect in this study remains to be identified, they strengthen the multimodal effects of minocycline. Importantly, the observed results in microglia and depressive-like behaviour were restricted to male HAB rats, underlining a sex-dependent effect of minocycline. This sex-dependence might be caused be several mechanisms like a differentially regulated HPA axis and differences in immune reactivity. Therefore, the anti-inflammatory effect of minocycline might be the driving force in HAB rats that leads to its beneficial effects, whereas a different immune regulation in female HAB rats might prevent this. In addition, as the microbiome composition of females was not studied in the present thesis, the impact of minocycline on female gut microbiota and consequential changes in composition and / or metabolome has to be considered as an underlying mechanism for the lack of effect in female HAB rats.

In the course of clinical application, the present results indicate that promoting an anti-inflammatory state by control of microglial activation and/or modulation of the gut microbiome and metabolome, as shown after prolonged minocycline treatment, offers promising potential as a therapeutic strategy in treatment-resistant MDD. However, the present results together with the currently available literature recommend a cautious consideration as to whether minocycline is suitable in humans. The augmentation with SSRIs like escitalopram seems ineffective regarding both behaviour and microglia. Combining minocycline with SNRIs or SDRIs might elicit a faster and stronger treatment response. Moreover, a pro-inflammatory profile might be necessary for minocycline to elicit a treatment effect and the distinct sex difference in depressive-like behaviour of HAB rats strongly discourages the application in women. Indeed, a recent clinical study with minocycline failed to alleviate symptoms of schizophrenia, presumably due to a lack of

inflammatory activation in the patients (Deakin *et al*, 2018), which may explain some of the recent high profile clinical failures of minocycline. It might therefore be recommended to include the use of inflammatory biomarkers to identify the subpopulation of patients responding to immune-targeted treatment.

Nevertheless, the present study was designed to detect an early response (3 weeks) to minocycline and did not mimic a clinical antidepressant treatment. A longer treatment duration or a higher dose might reveal different effects. Besides, some limitations of the executed experiments and analyses have to be considered. The present results are of exploratory and descriptive nature, and a causality between the observed effects has to be shown yet. Therefore, future studies are needed to identify the mechanism underlying the distinct effects of minocycline in the complex interplay of gut microbiome, the immune system, and behaviour. In particular, fecal transplantation and administration of microbiome metabolites, especially butyrate, in HAB rats might give direct evidence for an involvement of gut microbiota and the peripheral immune system. Further, a more comprehensive analysis of microglia activation and function, potentially *via* morphological changes or the expression pattern of activation, proliferation, and apoptosis marker, will provide evidence for a more mechanistical background. Following this, a specific activation and inhibition of microglia will reveal a more detailed view on their effects in HAB rats. Beyond a systemic effect of minocycline, its detailed intracellular mechanisms remain elusive. Several intracellular signalling cascades, like IDO signalling, were proposed as underlying mechanism of action for minocycline. Overall, these approaches should provide a causal link between the microbiota-gut-brain axis and behaviour in depression and a valid foundation for translation into clinical practice.

4.2 Minocycline does not reverse CSC-induced behavioural, physiological and neuroendocrine consequences

In the course of specifying the effects of minocycline on symptoms of psychiatric disorders, an alternative model to the genetically inherited anxiety- and depressive-like behaviour of HAB rats was employed. As chronic stress is a major risk factor for psychiatric disorders (see Introduction chapter 1.4), the CSC paradigm was chosen. The CSC paradigm has been developed at the University of Regensburg (Reber *et al*, 2007) and has been successfully established at the University of Ulm (Reber *et al*, 2016b). It is well characterized for its diverse effects on behavioural, immunological, and physiological parameters (reviewed in Langgartner *et al*, 2015; Reber *et al*, 2007; Reber and Neumann, 2008a; Uschold-Schmidt *et al*, 2012). Among those, increased anxiety-like behaviour shown in several behavioural tests and elevated adrenal, but decreased thymus, weight (Langgartner *et al*, 2018a) are the most prominent and reliable biomarkers. As minocycline has shown beneficial effects on behavioural consequences of chronic stress,

an amelioration of CSC-induced symptoms seems probable. Importantly and in contrast to HAB rats, CSC mice do not show changes in depressive-like behaviour. Therefore, it offers the opportunity to identify minocycline-induced effects specifically on anxiety-like behaviour that were not achieved in the comorbid phenotype of HAB rats. However, in 2014 the department moved into a novel facility and hence, experiments are now conducted in novel laboratories. An *a priori* re-establishment of the paradigm was hence crucial in order to determine potential minocycline-induced changes in the CSC paradigm. Thus, I aimed to re-establish the CSC paradigm in the laboratory facilities to ensure valid experimental results on later manipulations. After confirming an established CSC, I analysed the effects of 80 mg / kg minocycline applied acute and subchronically following 20 days of chronic psychosocial stress. In the course of this, the influence of acute minocycline on anxiety-like behaviour and the impact of subchronic minocycline treatment on anxiety- and depressive-like behaviour as well as physiological and neuroendocrine parameters were evaluated.

4.2.1 Successful validation of the CSC paradigm

The present thesis demonstrated a successfully established CSC paradigm after the department moved to a novel facility, pronounced by increased anxiety-like behaviour in the LDB and on the EPM and increased adrenal, but decreased thymus, weight after 20 consecutive days of CSC. In line, previous studies showed a robust increase in anxiety-like behaviour in the LDB and the EPM after 19 and 20 days of CSC exposure, respectively (Langgartner *et al*, 2015; Reber *et al*, 2007; Reber and Neumann, 2008a). Further, CSC mice showed increased adrenal weight while thymus weight was decreased (Langgartner *et al*, 2018a; Reber *et al*, 2007, 2016b). Therefore, the CSC-induced physiological and behavioural parameters could be induced at the new facility and provide evidence for a valid CSC paradigm.

These results are of importance, as a critical issue in using animal models and interpreting the obtained results is experimental reproducibility between laboratories. False positive or negative results by flawed preclinical studies can create wrong expectations and study designs in clinical and preclinical studies that eventually lead to costly inefficiencies (Freedman *et al*, 2015; Slattery and Cryan, 2014). This is all the more true since about 50 % of all conducted studies seem to be irreproducible (Freedman *et al*, 2015), like the light-enhanced startle test (Walker *et al*, 2003), and minor environmental changes like water composition (Langgartner *et al*, 2017a) can influence the outcome of experiments. In the course of identifying potential beneficial effects of minocycline on CSC-induced maladaptations, a validation and reliable reproduction of the CSC paradigm and its stress biomarkers after moving to the new facility was an essential prerequisite.

4.2.2 8 days of minocycline after stressor termination is not sufficient to alleviate stress-induced physiological and affective symptoms

In addition, the capability of an 8-days minocycline treatment (80 mg / kg) to reverse CSC-induced physiological as well as affective disorders was evaluated, as previous studies demonstrate CSC-induced consequences lasting for this period. Immediately after 20 days of CSC, the time spent in the light box of the LDB was reduced in CSC mice concomitant with decreased locomotor activity. 8 days after stressor termination, CSC mice persistently showed increased anxiety-like behaviour in the OF / NOR test but comparable locomotion and time spent immobile in the FST as SHC mice. Further, CSC mice had an increased spleen weight and gained significantly more weight than SHC mice. A CSC-induced increase in adrenal was sustained after 8 days, paralleled by reduced adrenal sensitivity after *in vitro* stimulation and unchanged plasma ACTH and CORT levels in comparison to SHC mice. In line with the present results, a long-term presence of several CSC-induced physiological, immunological, and behavioural symptoms was postulated previously. Thus, 8 days after stressor termination, CSC mice still express the highly anxious phenotype accompanied by reduced locomotor activity during the dark phase. Body weight changes during the CSC paradigm remain variable. However, after stressor termination an increased body weight gain is observed. These symptoms are concomitant with a long-term elevated release of pro-inflammatory cytokines from *in vitro* stimulated mesenteric lymph nodes. Additionally, a CSC-induced dysregulation of the HPA axis is robustly reported on the level of the adrenal gland and plasma ACTH and CORT responses (Langgartner *et al*, 2015; Reber *et al*, 2008; Slattery *et al*, 2012). Although plasma morning ACTH and CORT level in general remain unchanged in the CSC paradigm, an acute mild heterotypic stressor on the last day of CSC is able to enhance plasma CORT levels (Uschold-Schmidt *et al*, 2012) while a strong heterotypic stressor like the FST elevates both plasma ACTH and CORT levels (Füchsl *et al*, 2013). Though a FST was performed on the last day in the present experiment, plasma CORT and ACTH concentrations remained unchanged between SHC and CSC mice. A delay between the performed FST and sample collection is crucial to detect changes as the release of CORT into the plasma is earliest seen after 15-30 min (de Kloet *et al*, 2005). The latter was not considered in the present experiment, explaining the lack of increased plasma concentrations of ACTH and CORT in the current experiment.

Interestingly, 8 days of minocycline were not able to alleviate increased anxiety-like behaviour, increased spleen weights, and HPA axis maladaptations in CSC mice in contrast to all expectations. These were based on multiple studies demonstrating that the CSC paradigm increases peripheral immune activation in terms of elevated plasma cytokine levels and splenomegaly with reduced *in vitro* GC sensitivity. In detail, 19 days of CSC reduces regulatory T cell counts and increases T cell effector function in peripheral lymph nodes that secrete elevated concentrations of pro-inflammatory cytokines upon stimulation (Schmidt *et al*, 2010a). CSC mice further develop spontaneous colitis and impaired colonic barrier function, accompanied

by more colonic macrophages, dendritic cells and T cells, and increased plasma cytokine concentrations (Langgartner *et al*, 2018b; Reber *et al*, 2007, 2011). Another consequence of the CSC paradigm is an increased spleen weight and a GC resistance of splenocytes, an effect dependent on bite wounds inflicted during CSC (Foertsch *et al*, 2017). Interestingly, chronic treatment with a mixture of antibiotics before and during the CSC procedure is able to prevent the development of colitis and an increased inflammatory response (Reber *et al*, 2011). In addition, CSC exposure leads to a robust shift in gut microbiota composition that is attenuated by administration of the immunomodulatory bacteria *mycobacterium vaccae* (Reber *et al*, 2016b), while transplantation of SHC faeces into CSC mice has mild stress-protective effects (Langgartner *et al*, 2018c). These studies demonstrate the role of peripheral immune activation and gut microbiota in the CSC-induced phenotype and the necessity of a balance between inflammatory and immunoregulatory microbial input that determines the stress response. Fittingly, minocycline is known to act anti-inflammatory in the periphery (reviewed in Garrido-Mesa *et al*, 2013; Giuliani *et al*, 2005), modulate gut microbiome composition (Wong *et al*, 2016), and effectively attenuate inflammation-induced anxiety- and depressive-like behaviour (see Introduction chapter 1.2.3). An acute application of minocycline reverses acute stress-induced anxiety-like behaviour in rats (1 h prior to testing; Levkovitz *et al*, 2015), while a subchronic treatment of 4 days ameliorates mild traumatic brain injury-induced anxiety (Kovesdi *et al*, 2012). In mice, anxiety-like behaviour is reversed after cardiac arrest and prevented in repeated social defeat stress with 5 (Neigh *et al*, 2009) or 8 (McKim *et al*, 2018) days of minocycline treatment, respectively. Of note, though minocycline also attenuates stress-induced behavioural changes, the influence of minocycline on HPA axis reactivity has been rarely studied so far. Two studies in rats demonstrate a beneficial effect in this context, showing that minocycline prevents increased HPA axis activity caused by a neonatal immune challenge (Majidi-Zolbanin *et al*, 2016) and chronic stress-induced elevated CORT levels (Zhang *et al*, 2019). Thus, considering that minocycline has anti-inflammatory, antibiotic, and beneficial effects on chronic stress-induced behaviour, in contrast to the obtained results, an amelioration of CSC-mediated behavioural and physiological symptoms was expected to occur after 8 days of treatment. Since HAB rats in the present thesis showed an innate pro-inflammatory phenotype and pathological behaviour, and responded to minocycline treatment, a treatment effect in the CSC should have been visible. Nevertheless, in line with the obtained results of this experiment a recent study displayed unsuccessful acute minocycline treatment in anxiety- and depressive-like behaviour in mice (Vogt *et al*, 2015). Chronic minocycline treatment likewise failed to ameliorate chronic stress-induced depressive-like behaviour in mice (Kreisel *et al*, 2014). Therefore, the present thesis indicates that the anxious phenotype of CSC mice might not solely be mediated by the inflammatory system but also potentially by an inflammatory-independent mechanism. Additionally, an antidepressant effect of minocycline was not achieved in this experiment. However, as CSC mice generally do not show enhanced depressive-like behaviour (Slattery *et al*, 2012), a lack of effect might be contributed to a missing

pathological state in CSC mice. The same assumption can be utilized for the lacking effect of minocycline on plasma GC concentrations.

The exact underlying mechanism for a failed amelioration of CSC-induced symptoms by minocycline remains to be studied, though some assumptions can be made. First, the dose applied in this experiment was chosen, as previously mentioned (see chapter 4.1.1), based on literature demonstrating a behavioural effect of minocycline at a broad range and should therefore not account for the observed behavioural effect. As locomotor activity was unchanged, it seems also rather unlikely that a possible effect was concealed by an overdose. Although all mice were single-housed and single-housing is known to induce anxiety- and depressive-like behaviour in rats (Wang *et al*, 2017), previous studies showed that for male mice group housing is stressful *per se* (Singewald *et al*, 2009). A counteracting mechanism of isolation-induced enhanced anxiety-like behaviour in CSC mice appears, therefore, implausible. Hence, it can be concluded that a treatment of 8 days might not be sufficient to reverse CSC-induced symptoms but a longer duration might yield beneficial effects of minocycline.

4.2.3 Conclusion and Outlook of CSC studies

In summary, the CSC paradigm was successfully validated in the new facility at the University of Regensburg and its long-term effects were confirmed. Further, the present thesis recapitulates a lack of effect of minocycline on anxiety-like behaviour, HPA axis maladaptations and increased spleen weight in a chronic psychosocial stress model in contrast to previous expectations. However, at this point a conclusion about the effectiveness of minocycline in the context of CSC-induced behavioural, physiological, and immunological alterations should be drawn carefully. Under the applied experimental conditions, minocycline was not effective but to qualify a statement concerning a general hypothesis, additional studies have to be performed. It has to be considered that minocycline was applied only for 8 days and thus a longer treatment duration might yield a different outcome. Beside an increased treatment duration, previous studies indicate another approach. The anxiolytic effect of minocycline has been shown before, albeit minocycline was able to prevent the development of high anxiety, but not reverse it (Kreisel *et al*, 2014; Majidi-Zolbanin *et al*, 2016; Zhang *et al*, 2019). As mentioned above, both treatment with *mycobacterium vaccae* and a mixture of antibiotics ameliorated symptoms when applied prior to CSC exposure (Reber *et al*, 2011, 2016b). These studies indicate that rather a prevention than a reversal of symptoms might be efficient. Therefore, the time point of application might be crucial and minocycline treatment before CSC exposure might be able to prevent stress-induced effects. To specify the influence of minocycline on CSC-induced maladaptations, the immune system has to be considered. Evaluation of peripheral and central immunological parameters like microglia and cytokine concentrations might reveal alternative explanations for a failed treatment success and simultaneously offer a new mode of action for

minocycline effects. In addition, as the CSC is known to induce spontaneous colitis that might contribute to an altered treatment response. Finally, it is crucial to factor in the bactericidal effects of minocycline. Future studies to unravel minocycline-induced microbiome and metabolome changes will give some indication about potential mechanisms underlying the behavioural and physiological phenotype as well as the treatment response of CSC mice. Nevertheless, although deemed rather unlikely due to the anti-inflammatory and anxiolytic effects of minocycline, it cannot be excluded that minocycline in not beneficial in the context of CSC.

4.2.4 Overall conclusion

In conclusion, the present thesis reveals a specific effect pattern of minocycline and further outlines the conditions of its effectiveness. Concerning behaviour, minocycline ameliorated depressive-like behaviour in male, but not female, HAB rats. In the first instance, this demonstrates, to my knowledge, for the first time a sex-dependent effect of minocycline on depressive-like behaviour. In addition, in NAB rats or CSC mice, no antidepressant effect was visible. Those behavioural results indicate, in line with a recent meta-analysis (Reis *et al*, 2019), that minocycline requires an alteration in depressive-like behaviour *a priori* to the treatment, as both CSC mice and NAB rats do not show a depressive phenotype. Interestingly, it remains unknown why CSC mice do not express a depressive-like phenotype. Unravelling this mechanism might give some indication about the actual target of minocycline. Moreover, in both the innate and the stress-induced model of anxiety-like behaviour, minocycline was not able to ameliorate the phenotype. The anxiolytic effect of minocycline is shown only after traumatic injury, acute stress, or a direct inflammatory challenge (Fan *et al*, 2006; Kovesdi *et al*, 2012; Levkovitz *et al*, 2015; Majidi-Zolbanin *et al*, 2016; Neigh *et al*, 2009; Soczynska *et al*, 2012). These models include an acute and strong traumatic impact on the animal that likely induces an acute inflammatory response. In the CSC model, stress is applied in a chronic fashion and the anxiety-like behaviour in HAB rats is independent of age. Likewise, the CSC induces chronic inflammation and the inflammatory phenotype in HAB rats develops presumably already during early development. This leads to the assumption that minocycline shows no or only delayed effects on anxiety-like behaviour that might be dependent on an acute inflammatory activation. Hence, minocycline does not only show a trait- and sex-dependence but also demands specific preconditions. Thus, with the present results I could advance the current knowledge about minocycline towards a more fine-tuned outline of the prerequisites that have to be considered to elicit a behavioural response. The anti-inflammatory effect of minocycline is postulated as underlying mechanism for its impact on behaviour. In male HAB rats, a pro-inflammatory phenotype was expressed by reduced microglial density in the infralimbic and prelimbic PFC that might underlie the antidepressant effects of minocycline. However, CSC mice do not respond to minocycline treatment despite a high inflammatory state.

Therefore, the present results extend the presumed sole effect of minocycline on inflammation to a multimodal effect that is not restricted to its anti-inflammatory mechanism. In addition, a pathological dexamethasone suppression test is found in male HAB rats, while female HAB rats and CSC mice do not show a pathological negative feedback loop of the HPA axis. It is tempting to speculate that a mechanism of minocycline is the restoration of HPA axis functionality and might therefore be effective in male HAB rats, but not female HAB rats or CSC mice.

Considering these results, I could extend the mechanistic background of minocycline and provide a more defined picture for its effectiveness, potentially serving as a prerequisite for a translation into clinical application. Thus, a future translation of minocycline treatment into clinical application for psychiatric disorders requires a careful consideration of the patient's circumstances, i.e. sex, inflammatory state, or potential cause of the disease. This emphasizes that minocycline rather represents medication for personal tailored treatment of psychiatric disorders than an antidepressant for a broad spectrum of patients. Nevertheless, a modulation of the complex interplay of the MGB axis reveals promising potential in the treatment of psychiatric disorders.

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Abbreviations

3-OH-butyrate	β-hydroxybutyrate
5-HT	5-hydroxytryptamine, serotonin
ACTH	adrenocorticotropic hormone
ASD	autism spectrum disorder
AVP	vasopressin
BBB	blood brain barrier
BL6	C57BL/6N
CD	cluster of differentiation molecule
CNS	central nervous system
CORT	corticosterone
CRH	corticotropin-releasing hormone
CRP	C-reactive protein
CSC	chronic subordinate colony housing
CSF	cerebrospinal fluid
CUMS	chronic unpredictable mild stress
DA	dopamine
DSM	Diagnostic Statistical Manual for Mental disorders
EDTA	ethylendiamintetraacetic Acid
ELISA	enzyme-linked immunosorbent assay

EPM	elevated plus-maze
Esc	escitalopram
FSL	Flinders Sensitive Line
FST	forced-swim test
GABA	γ-aminobutyric acid
GAD	Generalized Anxiety disorder
GC	glucocorticoid
GF	germ-free
GIT	gastrointestinal tract
HAB	high anxiety-related behaviour
HPA	hypothalamic-pituitary-adrenal
HPLC	high-pressure liquid chromatography
IBD	inflammatory bowel disease
Iba-1	ionized calcium-binding adapter molecule 1
icv	intracerebroventricular
IFN	interferon
IL	interleukin
i.p.	intraperitoneal
KO	knockout

LAB	low anxiety-related behaviour
LDB	light-dark box
LPS	lipopolysaccharide
MDD	Major Depressive Disorder
MGB	microbiota-gut-brain
Min	minocycline
mRNA	messenger RNA
NAB	rats non-selected for anxiety-like behaviour
NLR	NOD-like receptor
OF / NOR	open field / novel object recognition
OTU	operational taxonomic unit
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCoA	principal coordinates analysis
PFC	prefrontal cortex
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitatice PCR
rpm	revolutions per minute
RT	room temperature

SAD	social anxiety disorders
SB	sodium butyrate
SCFA	short-chain fatty acid
SDRI	selective dopamine reuptake inhibitor
SERT	serotonin transporter
SHC	single-housed control
SNP	single nucleotide polymorphism
SNRI	serotonin and noradrenaline reuptake inhibitor
SPAT	social preference / avoidance test
SSRI	selective serotonin reuptake inhibitor
TLR	toll-like receptor
TNF	tumour necrosis factor
TST	tail-suspension test
Veh	vehicle
vs.	versus
YLD	Years lived with disability

Curriculum vitae and publications

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Publications

Schmidtner AK, Masis-Calvo M, de Moura Oliveira VE, Grossmann C, de Jong TR, Neumann ID (2018). Animal models of social stress: the dark side of social interactions. *Stress* **21**: 417-32

Schmidtner AK, Slattery DA, Gläsner J, Hiergeist A, Gryksa K, Malik VA, Hellmann-Regen J, Heuser I, Baghai TC, Gessner A, Rupprecht R, Di Benedetto B, Neumann ID. Minocycline alters behavior, microglia and the gut microbiome in a trait anxiety-dependent manner. (submitted)

Schmidtner AK, Gryksa K, Reber SO, Slattery DA, Neumann ID. Minocycline does not reverse chronic psychosocial stress-induced physiological and behavioural alterations in mice. (in prep)

Gryksa K, **Schmidtner AK,** Masis-Calvo M, Meyer M, Havasi A, Rodriguez-Villagra OA, Murgatroyd C, Jurek B, Neumann ID. Update of behavioural, genetic, and physiological parameters of rats selectively bred for high- and low- anxiety related behaviour. (in prep)

Schmidtner AK, Malik VA, Di Benedetto B, Slattery DA, Neumann ID (2017). Sex-dependent attenuation of deficits in behaviour and microglia in high anxiety-related behaviour rats by minocycline. *European Neuropsychopharmacology* **27:**S29–S30.

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Team CSC – forever

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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Ort, Datum Anna Schmidtner