Diplomarbeit

Effects of dextran sulphate sodium-induced colitis and kappa opioid antagonism on stress induced Iba-1 expression within the murine limbic system

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Graz, am 25.09.2019 *Veit Kramer eh*

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Zusammenfassung

Psychologischer Stress wird mit einer großen Bandbreite an Erkrankungen, wie Angststörungen und Depression aber auch Diabetes und kardiovaskulären Erkrankungen, wie Herzinfarkt und Schlaganfall, in Zusammenhang gebracht. Angesicht der Tatsache, dass eine große Anzahl an Todesfällen weltweit auf Krankheiten zurückzuführen ist, welche mit Stress in Verbindung stehen, kommt der Entwicklung neuer Medikamente zur Linderung einiger oder aller negativer Symptome von Stress eine große Bedeutung zu, in der Hoffnung die frühzeitige, stressbedingte Mortalität signifikant zu reduzieren.
In dieser Studie wurden die Effekte eines Kappa-Opioid-Rezeptor (KOR) Antagonisten, Norbinaltorphimine (norBNI), und einer durch Dextran-Sulphate Sodium (DSS) induzierten Kolitis, auf die zerebrale Mikroglia von C57BL/6N Mäusen im Rahmen eines Wasservermeidungsstresses (WAS) untersucht. Insgesamt neun relevante Regionen (ROI) wurden für die Untersuchung der Mikroglia ausgewählt: der Gyrus cinguli (CC), die Region 1 des Cornu Ammonis (CA1), die Region 3 des Cornu Ammonis (CA3), die mediale Amygdala (MeA), die Substantia nigra (SN), der Gyrus dentatus (DG), der Cortex infralimbicus (ILC), der laterale Hypothalamus (LH) und der Nucleus paraventricularis des Hypothalamus (PVH). Um die Aktivität der mikroglialen Zellen im Gehirn sichtbar zu machen, wurde eine Immunhistochemie (IHC) mit Ionized calcium binding adaptor Molecule 1 (Iba-1), welches ein Marker für die mikrogliale Aktivierung ist, durchgeführt. Evaluiert wurde die mikrogliale Aktivierung indem die Fläche aller Iba-1 positiven Zellen innerhalb eines 300x300 µm großen Auszählfeldes gemessen wurde. Die einzige Ausnahme zu dieser Methode waren der PVH und der DG, bei denen es sich um klar begrenzte, anatomische Strukturen handelt und welche deshalb zu Gänze ausgemessen wurden. norBNI führte zu einer signifikanten Reduktion der Iba-1 exprimierenden Zellen innerhalb des ILC. Diese Veränderungen sind wahrscheinlich auf die Tatsache zurückzuführen, dass es sich dabei um eine Region handelt, die als erstes in die Stressreaktion involviert und daher besonders sensibel für norBNI vermittelte Veränderungen der mikroglialen Aktivierung ist.

Abstract

Psychological stress is associated with a variety of different mental and health disorders such as anxiety, depression but also diabetes and cardiovascular diseases like ischemic heart disease and stroke. Given that a large number of deaths worldwide are attributable to diseases linked to stress, research in drug development to alleviate some or all of the negative symptoms of stress stands a high hope of reducing the number of premature deaths worldwide by a significant extent. In this present study, the effects of the kappa-opioid-receptor (KOR) antagonist, norbinaltorphimine (norBNI) and dextran-sulphate sodium (DSS) induced colitis on the cerebral microglia in C57BL/6N mice were examined under conditions of water-avoidance-stress. Microglia was observed in nine regions of interest (ROI) in the brain: the cingulate cortex (CC), region 1 of the cornu ammonis (CA1), region 3 of the cornu ammonis (CA3), the medial amygdala (MeA), the substantia nigra (SN), the dentate gyrus (DG), the infralimbic cortex (ILC), the lateral hypothalamus (LH) and the paraventricular hypothalamic nucleus (PVH). To visualise the activity of brain microglial cells, immunohistochemistry (IHC) for Ionized calcium-binding adaptor Molecule 1 (Iba-1), a marker of microglial activation was performed and evaluated by measuring the area of Iba-1 positive cells within a 300x300 µm counting area except for the PVH and DG, where all the cells within this clearly defined brain areas were included. norBNI led to a significant reduction in the number of Iba-1 expressing cells in the ILC. These changes are likely attributable to the fact that this region is amongst the first to be involved in the stress response and therefore is the first to show the effects of stress as could be seen in other studies involving norBNI.

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Table of abbreviations

AMC – Amoeboid Microglial Cell

ANS – Autonomic Nervous System

AVP – Arginin-Vasopressin

BBB – Blood Brain Barrier

BNST – Bed Nucleus of the Stria Terminalis

CA1 – Region 1 of the Cornu Ammonis

CA3 – Region 3 of the Cornu Ammonis

CC – Cingulate Cortex

CeA – Central Amygdala

C-Fos – A Marker for Neuronal Activation

CNS – Central Nervous System

CN X. – Vagus Nerve

CRH – Corticotropine-Releasing Hormone

DG – Dentate Gyrus

DSS – Dextran Sulphate-Sodium

EC – Entorhinal Cortex

ENS – Enteric Nervous System

GCR – Glucocorticoid Receptor

HPA-axis – Hypothalamus-Pituitary-Adrenal-Axis

Iba-1 – Ionized Calcium-binding adaptor Molecule 1

IGF – Insulin Like Growth Factor

IHC – Immunohistochemistry

ILC – Infralimbic Cortex

KOR – κ-Opioid Receptor

LH – Lateral Hypothalamus

MC – Microglial Cell

MeA – Medial Amygdala

MHC – Major Histocompatibility Complex

mPFC – Medial Prefrontal Cortex

norBNI – Norbinaltorphimine

NTS – Nucleus of the Solitary Tract

PAG – Periaqueductal Grey

PBN – Parabrachial Nuclei

PBS – Phosphate-Buffered Saline

PC – Papez Circuit

PD – Parkinson's Disease

PLC – Prelimbic Cortex

PNS – Parasympathetic Nervous System

PP – Processive Pathway

PVH – Paraventricular Nucleus of the Hypothalamus

RMC – Ramified Microglial Cell

ROI – Region of Interest

RS – Restraint Stress

SN – Substantia Nigra

SNS – Sympathetic Nervous System

SP – Systemic Pathway

TGF-β – Transforming Growth Factor-β

TLR – Toll-like Receptors

TNF-α – Tumor Necrosis Factor-α

VEH – Control Vehicle

WAS – Water Avoidance Stress

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1 Introduction

1.1 Stress, regulator of homeostasis

There have been many attempts to categorise and define stress. Of the many different approaches none has, as of yet, emerged as universally accepted by the scientific community. Many definitions focus more on the psychological aspect of stress whereas others try to define it mainly through the physiological changes that occur in the body. So maybe the pragmatic way is to define stress as the process that occurs during activation of the stress systems. There is a certain underlying consensus, that there is no universally accepted concept of stress and therefore it should not come as a surprise that more recent publications do not try to establish a new, all-encompassing theory of stress. Accordingly, most authors restrain themselves to those aspects of stress which are part of their respective empirical work.

So one could say that stress is the inner and outer condition of an individual, that threatens homeostasis in such a way that it activates the implementation of reactions to regain homeostasis. Within this construct those reactions run the risk of overshooting, which ultimately lead to damage to the organism itself. Therefore, it is imperative to have a very intricate network of feedback and feedforward mechanisms that tightly control for the outcome in order to avoid said damage. We also know that there is stress that can be "good", so called eustress, and yet prolonged phases of stress are usually associated with stress that turns out to be counterproductive to the body's attempt to regain homeostasis. This is what is called distress (Selye, 1975). At this point it should be noted that stress does not need to be an actual threat to the body's homeostasis. It is enough to perceive something as stressful to activate the stress system (Dedovic et al., 2009). Knowing the underlying mechanisms of stress is essential for the development and research into new medications which alleviate some or even all of the negative effects that stress has on the body.

In principle, the physiological reactions to stress can be separated into two different communication systems. First there is the immediate and very swift neuronal response and second there is the slower but usually more prolonged, endocrine response (Godoy et al., 2018).

1.1.1 Processive and systemic stress

In modern language the word stress is most often used to describe psychologically taxing situations. From the perspective of the body and brain however there is a distinction to be made between different kinds of stress. As shown by the work of J.P. Herman and W.E. Cullinan (Herman, Cullinan, 1997) different kinds of stressors are relayed by different parts of the brain and therefore processed differently before they converge again leading to a response in the paraventricular hypothalamic nucleus (PVH).

They propose the term ‘processive’ for stress that needs to be processed and interpreted to have an effect (positive or negative) on the PVH and the term ‘systemic’ for stress that is relayed directly to the neurons of the PVH via mainly brainstem connections (Herman, Cullinan, 1997). Elaborating on this concept I will show here the different pathways that stress takes within the central nervous system (CNS) in order to give a full understanding of the neuronal circuits involved and to help further possible explanations of my observations (Figure 1).

The systemic pathway (SP) is a stress activation system that, unlike the processive pathway (PP), is not in need of cognitive processing in order to stimulate the PVH and therefore produce a stress response. The type of stressor that elicits a stress response in the SP (e.g. haemorrhage, hypotension or respiratory distress) usually calls for an immediate response as it is immanently life-threatening (Herman, Cullinan, 1997). The PP on the other hand uses many different sensory modalities, memory and therefore association, to not only react, like the SP, but also to predict future stressful events and all of the stimuli it reacts to are not immediately life-threatening (Herman, Cullinan, 1997). It is therefore obvious that it also involves various cortical areas. Following I will describe some of the primary participating regions within the PP and how they can influence the SP which will be described later.

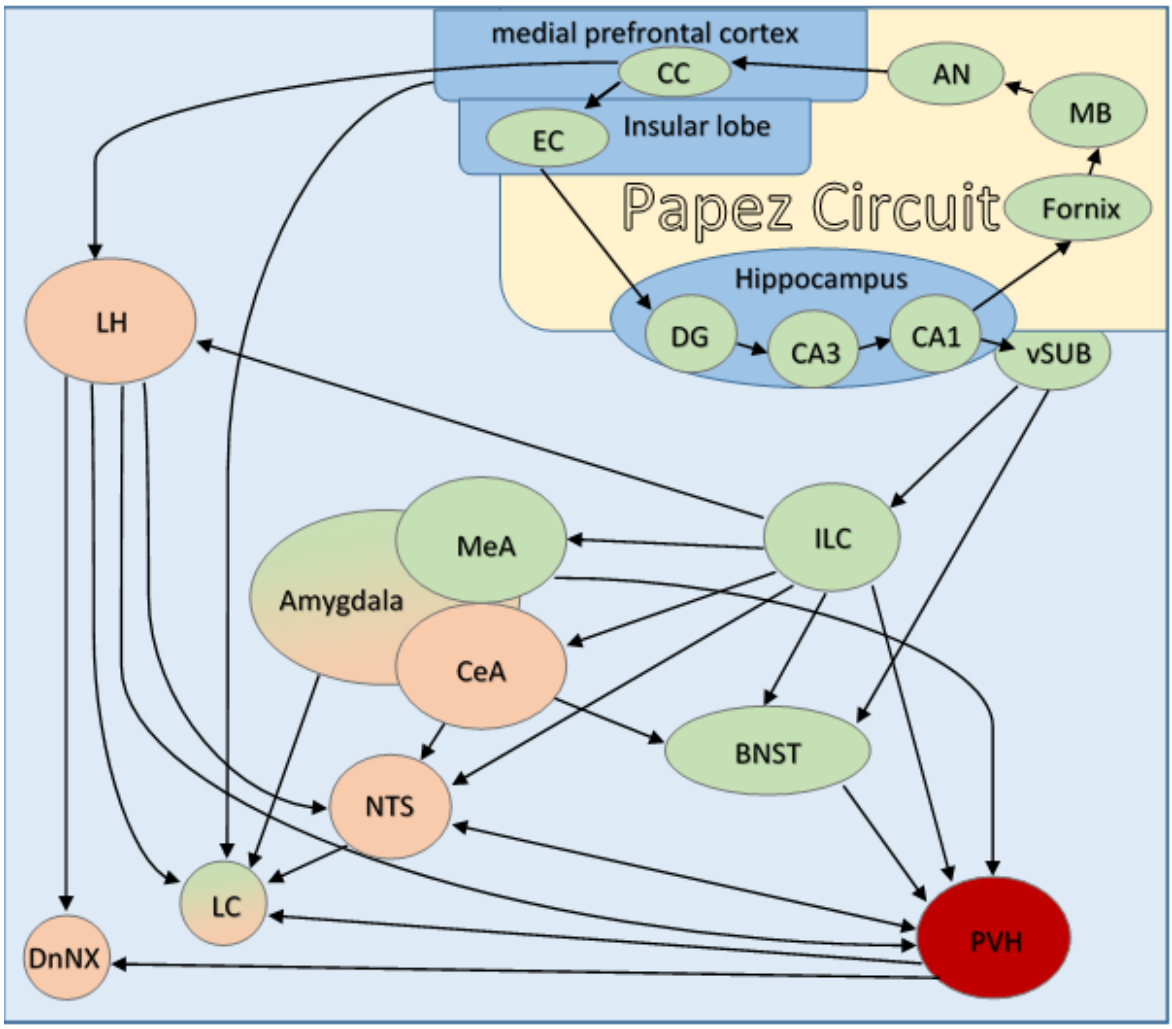

Figure 1. Neuroanatomy of stress: Shown are the main neuroanatomical brain structures involved in systemic (light orange), processive (light green) or systemic and processive (bicolored) stress. Arrows show projecting neuron populations. Areas within or touching the beige field are structures within the Papez circuit (PC). Within the hippocampus the PC includes the trisynaptic loop made up of the dentate gyrus (DG), region 1 of the cornu ammonis (CA1), region 3 of the cornu ammonis (CA3) as well as the ventral subiculum which is not strictly part of the PC but together with the CA1 serves as the main output region of the hippocampus. Further it encompasses the fornix, the mammillary bodies (MB) and the anterior thalamic nuclei (AN). The cingulate cortex (CC) is part of the medial prefrontal cortex and also serves an important role within the PC receiving input from the AN and itself projecting to the entorhinal cortex (EC) which lies within the insular lobe and "closes" the loop by projecting back to the DG. Structures of the PC as well as the infralimbic cortex (ILC) and the medial amygdala (MeA) are all involved in the processive pathway (PP) of stress reaction by either directly projecting to the nucleus paraventricularis (PVH) of the hypothalamus and the locus coeruleus (LC) or indirectly by modifying systemic pathways (SP). The LC sits in a very unique position as it can be activated by systemic as well as processive stressors and directly influences the sympathetic branch of the autonomic nervous system (ANS), just as the lateral hypothalamus (LH) can directly influence visceral functions by projecting to the dorsal nuclei of the nervus vagus (DnNX). Although part of the SP, the bednucleus of the striaterminalis (BNST), the nucleus of the solitary tract (NTS) and the central amygdala (CeA) still receive various projections from areas of the PP.

a. Lateral hypothalamus

The lateral hypothalamus is a key area that connects many different structures within various brain regions. Given its diverse connections the impact of the lateral hypothalamus (LH) might be difficult to assess. Its main input stems from the hippocampus, the medial prefrontal cortex (mPFC) and the central amygdala (CeA) (Ulrich-Lai, Herman, 2009). However, the output regions of the LH are immense. Important for this study (Figure 1) are the nucleus of the solitary tract (NTS), the PVH and the locus coeruleus (LC) through which modulation of stress responses can be achieved as well as the dorsal nucleus of the vagus nerve (CN X.) through which it can directly modify gastrointestinal motility and secretion (J. Li, Hu & de Lecea, 2014).

b. Cingulate cortex

The cingulate cortex (CC) is part of the so called Papez circuit (PC). This circuit (Figure 1) consists of the entorhinal cortex (EC), which projects to the hippocampus formation, which in turn projects through the fornix into the mammillary bodies, which have connections to the anterior thalamic nuclei, which project to the CC, which closes the circuit by projecting back to the EC (Wei et al., 2017). Originally, Papez proposed the PC to be an essential part of emotional control. However, newer studies indicate that it is more closely linked to memory formation (Wei et al., 2017). The anterior CC plays a significant role within this neuronal network and seems to integrate motivational outcomes and action, making it an integral part for reward associated behaviour (Hayden, Platt, 2010).

c. Hippocampus formation

The complex circuitry of the hippocampus is called the trisynaptic loop (Figure 1) and consists of the main input from the EC which projects either directly to the region 1 of the cornu ammonis (CA1) region or indirectly first to the dentate gyrus (DG) which projects to the region 3 of the cornu ammonis (CA3) region and then connects back to the CA1 region, which mainly projects to the subiculum (Behrends, 2010). As already mentioned, it is part of the PC and therefore part of the limbic system.

It is well established that the hippocampus has an inhibitory effect on the PVH (Ulrich-Lai, Herman, 2009). Notably, lesions to the area of the ventral subiculum, where most of the inhibitory neurons that indirectly affect the PVH reside, leads to an increase in stress hormones following psychogenic stress, but not systemic stress, strongly indicating its involvement in the PP (Ulrich-Lai, Herman, 2009). Even though the hippocampus has no direct connections to the brainstem, it can modulate the autonomic tone by mediation

through the infralimbic cortex (ILC), which itself does project to the NTS (Ulrich-Lai, Herman, 2009).

Another noteworthy region in this area is the DG. It is one of the few brain regions that retains the ability of neurogenesis throughout the adult life, which seems to be an important mechanism for hippocampus related brain function (Toda, Gage, 2018). The ability of neurogenesis also seems to play a vital role for stress modulation by the hippocampus, since chronic stress impairs the hippocampal ability to modulate downstream regions of the stress response, notably the bed nucleus of the stria terminalis (BNST) and the LH in turn limiting their inhibitory effect on the PVH (Surget et al., 2011).

d. Amygdala

Functions of the amygdala are manifold. They include amongst many others, influences of memory processing, conditioned fear response and reactions to respiratory distress (Davis, 1992). For the scope of the present study we need to focus on the medial amygdala (MeA) and the CeA. Within the MeA especially high neuronal activity can be observed during swim stress, whereas no response is observed by ether (Herman, Cullinan, 1997), indicating an involvement in the PP but not in the SP. In contrast the CeA seems to be primarily involved in the SP but not directly in the PP (Ulrich-Lai, Herman, 2009). The CeA in particular has very little direct connections to the PVH and the hypothalamus in general, however it seems to communicate with the PVH via indirect connections through the BNST and the NTS (Figure 1).

e. Infralimbic cortex

The ILC is part of the larger mPFC. The ILC takes part in cognitive functions such as conditioned fear, memory formation under stress as well as fear extinction (Berg, Eckardt & Masseck, 2019, Berretta et al., 2005, Koot et al., 2014). Inactivation of the ILC does not affect basal heart rate or blood pressure and inhibits conditioned cardiovascular responses, whereas electrical stimulation of the ILC does increase both, strongly indicating the ILC in a selective, stress-induced cardiovascular regulation (Wood et al., 2019, Ulrich-Lai, Herman, 2009). Its main output regions are the BNST, NTS, and the CeA (Figure 1) through which it can achieve the above mentioned functions (Wood et al., 2019) but it does not have any direct connections with the PVH (Godoy et al., 2018). Because the ILC is part of the mPFC, its effects on stress responses may be hard to asses given that different parts of the mPFC have different functions. As of now, data suggests that the ILC has an antagonistic effect on the prelimbic cortex (PLC), as activation of the ILC seems to have

an anxiolytic effect, whilst activation of the PLC enhances anxiogenic behaviours (Godoy et al., 2018).

f. Substantia nigra

The substantia nigra (SN) is a dopaminergic nucleus of the midbrain, which is of central importance for motor function (in humans, especially at the start of movement), but also for reward functions. It consists of the pars compacta, with a large number of dopaminergic neurons, which are almost black due to neuromelanin produced during dopamine synthesis, and the pars reticulata, which mainly consists of neurons innervated by gamma-amino-butyric acid-containing neurons (Behrends, 2010).

Damage in the SN region can result in a variety of diseases, like Parkinson's disease (PD), impulse control disorders (e.g. ADHD), drug dependence, obesity and others (Y. Zhang et al., 2017).

PD in particular has recently become the focus of research, due to it being the second most common neurodegenerative disease, affecting 2-3% of people over the age of 65 and the SN playing a central role in its development.

But not only age-related degradation processes, which lead to creeping degeneration of neurons, seem to be connected with the SN, mental stress as well leads to disorders in the SN facilitating the loss of dopaminergic neurons, which has been associated with increased suppression of microglial responses (Ong et al., 2017).

Others have shown that injuries and diseases lead to increased microglial activity in this area, both in terms of number and reactivity (Kostuk, Cai & Iacovitti, 2018). Increased microglial activity in turn is associated with oxidative stress, i.e. the excess of free oxygen radicals that leads to the degeneration of dopaminergic neurons (Ong et al., 2017).

However, evidence has also been found that the loss of dopaminergic neurons behind PD is due to inflammatory processes caused by the permanent over-activation of the microglia (Garcia-Dominguez et al., 2018).

1.1.2 HPA-axis

The hypothalamus-pituitary-adrenal-axis (HPA-axis) forms the hormonal part of the two-part stress response system, the other part being the sympathetic nervous system (SNS), which is part of the autonomic nervous system (ANS), which will be discussed later.

Basically, after the CNS has determined that a stressful event has occurred, be it systemic or processive, the HPA-axis takes the function of the messenger who, via release of stress hormones, informs the rest of the body.

The HPA-axis consists of three components, the hypothalamus, the pituitary gland and the adrenal gland. It serves as the convergent system that gets influenced and influences all stress related reactions of the body. The HPA-axis's main input receiver is the PVH as all stress processing areas of the brain ultimately terminate within it or directly activate the SNS via the LC, either activating or suppressing its hormonal release (Godoy et al., 2018). If activated the PVH releases arginin-vasopressin (AVP) through axonal transport directly into the posterior pituitary gland and corticotropin-releasing hormone (CRH) into the hypothalamo-hypophyseal portal system from where it reaches the anterior pituitary gland (Carlson, 2017). The posterior pituitary gland receives a collection of axonal projections from the PVH which release AVP and oxytocin. The APG is a gland which upon stimulation can release a variety of different hormones, important to us being adrenocorticotropin. Adrenocorticotropin is the main effector hormone for the release of glucocorticoid hormones in the adrenal gland, the main stress related one being cortisol. Cortisol is a hugely influential hormone with an enormous variety of different functions in different tissues. Many of its functions can be understood, if looked upon from the viewpoint of it being a stress hormone. It stimulates gluconeogenesis in the liver, in high doses mostly suppresses the immune system, which is a system that uses up a lot of energy and it increases the availability of amino acids amongst many more functions (Katzung, 2018). In this sense it acts as a catabolic hormone to provide the body with all the energy it can mobilise to deal with a potentially life threatening situation.

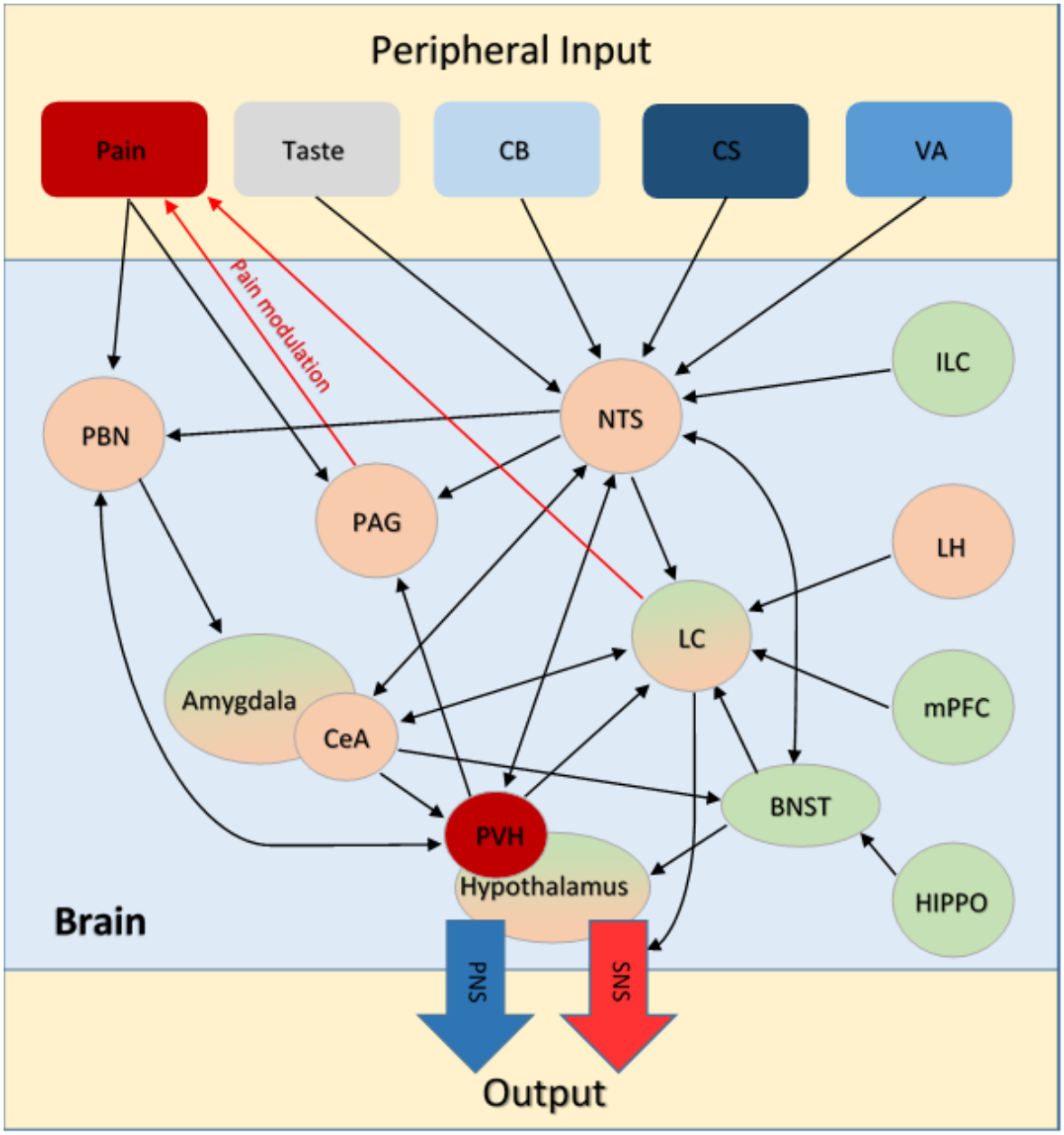

Figure 2: Neuroanatomy of stress. Shown are the main neuroanatomical brain structures involved in systemic (light orange), processive (light green) or systemic and processive (bicolored) stress. Arrows show projecting neuron populations. In the panel to the top is the main peripheral input to the stress pathway: visceral pain from the intestine, taste information from the taste buds, O^2 partial pressure from the carotid body (CB), blood pressure from the carotid sinus (CS), and chemical information conveyed by general visceral afferent (VA) fibres. Overarching structures which can modify the stress output mainly from cortical regions are the infralimbic cortex (ILC), the lateral hypothalamus (LH), the medial prefrontal cortex (mPFC) and the hippocampus (HIPPO), which are shown on the right hand side. The nucleus of the solitary tract (NTS) plays a pivotal role in receiving varied visceral information processing it and relaying it directly to the paraventricular nucleus of the hypothalamus (PVH) tying it tightly to the systemic stress pathway (SP). The PVH in turn projects to two other very important brain stem nuclei, the parabrachial nuclei (PBN) and the periaqueductal grey (PAG), which both are activated by painful stimuli in the viscera, the PBN relaying this information to the PVN, whilst the PAG being able to modulate the pain (red arrow) via the nucleus raphe

magnus through inhibitory interneurons in the spinal cord. Just as the PAG, the LC can also modulate pain perception by means of inhibitory interneurons yet it can also directly influence the sympathetic branch of the autonomic nervous system (ANS), whilst the parasympathetic branch (PNS) can be influenced by the PVN. Lastly the bed nucleus of the stria terminalis (BNST) is a very important integrator of limbic stress information.

As mentioned, CRH is directly released into the blood stream meaning it not only has an effect on the anterior pituitary gland, but also on the body as a whole. There are two receptor types responsible for interaction the CRH-R1 and the CRH-R2 receptor (Ketchesin, Stinnett & Seasholtz, 2017). Since these receptors are also present in the gastrointestinal tract it should come as no surprise that CRH can also influence the gastrointestinal tract. More specifically it increases colonic permeability and colonic hyperalgesia (Santos et al., 1999).

1.1.3 The autonomic nervous system

The ANS is part of the peripheral nervous system and acts by innervating all of the non-voluntary muscles as well as all the glands and the myocardium. It consists of three parts, the SNS, which is commonly referred to as the "fight or flight" -system, the parasympathetic nervous system (PNS) which acts as an antagonist as well as a synergist (Wehrwein, Orer & Barman, 2016) to the SNS and is often referred to as the "rest and digest" –system and the enteric nervous system (ENS), which is comprised of all of the neurons of the digestive system and often referred to as the "gut brain".
As we have seen before the hypothalamus plays a central role in the bodily as well as the mental reactions to stress by means of modulation of the HPA-axis. Yet the hypothalamus also serves as integrative structure for the ANS in that it modifies the SNS and the PNS in their activity according to real and/or perceived threats to homeostasis and through them also the activity of the ENS (Behrends, 2010). It should be noted that the afferent fibres transmitting visceral information to the CNS are not separated like the SNS and PNS efferent fibres are (Behrends, 2010).

As seen in Figure 2, there are three main regions in the brain stem that directly receive visceral information through these general visceral afferent fibres. This information is of different quality and can in turn modulate the SNS and the PNS via the PVH of the hypothalamus:

a. *Nucleus of the solitary tract*

The NTS is in fact a collection of different neuron populations with different specialisations (Conn, 2008). The rostral part of the NTS is mainly concerned with the input of gustatory information from the taste buds, whereas the medial and caudal parts receive inputs from the general visceral afferent pathways via mainly the CN X. (Behrends, 2010, Conn, 2008). This input consists of information from the chemoreceptors of the glomus caroticum, information about blood pressure from the sinus caroticus and general chemical and mechanical information mostly from the viscera (Figure 2). This makes the NTS an important structure since all cardiovascular information from the periphery has to pass through the NTS in order to reach the PVH (Dufloth, Morris & Michelini, 1997).

There are a few noteworthy connections from the NTS such as to the amygdala, the LC, the BNST, the parabrachial nuclei (PBN), the periaqueductal grey (PAG) and direct projections to the PVH (Herman, 2018, Dufloth et al., 1997, Shin, Geerling & Loewy, 2008, Carlson, 2017). Also the PVH contributes a large amount of neuronal projections back to the NTS (Ulrich-Lai, Herman, 2009). Through these connections a swift reaction of the SNS, the PNS as well as the HPA-axis is made possible if, for example a sudden fall of blood pressure should be detected.

b. *Parabrachial nuclei*

The PBN are a subset of different nuclei that receive information about blood pressure and oxygenation from the NTS (Carlson, 2017). Additionally they receive information about visceral pain directly via the spinal cord (Cechetto, Standaert & Saper, 1985). They relay this information to the amygdala, to the hypothalamus, where again a modulation of the SNS and PNS is achieved, and to higher cortical regions of the forebrain where this information can influence behaviour (Behrends, 2010, Palmiter, 2018).

c. *Periaqueductal grey*

The cells of the PAG have a variety of different functions that play a role not only in autonomic reactions during a fight or flight response but also in motivated behaviours (Behrends, 2010, Silva, McNaughton, 2019). The PAG plays a vital role in the suppression of painful stimuli in the spinal cord. Critically, it does this by expressing all three opioid receptor types (μ, κ, δ) (Nobre et al., 2000) which trough activation, of neurons projecting to the raphe nuclei stimulate inhibitory interneurons to the spinal cord to suppress painful messages even before they reach the thalamus (Menant et al., 2016). To achieve this in a meaningful way, the PAG receives input from ascending pain fibres of the spinal cord

(Figure 2) and is modulated by the amygdala, the NTS and the hypothalamus (Menant et al., 2016).

d. Locus coeruleus

The LC, just as the PVH, is a real hub for the brain's stress response and other functions like arousal, the sleep-wake-cycle and attention amongst others (Behrends, 2010). Many nuclei project with their fibres to the LC (Figure 2), like the NTS, the amygdala, the BNST, the LH and cortical areas such as the mPFC (Aston-Jones, Waterhouse, 2016, Schwarz et al., 2015). On the other hand, the LC with its projections reaches a large variety of cortical and subcortical regions. Amongst those is a direct connection to the intermediolateral cell column which mediates the entire sympathetic innervation of the body as well as intense interconnections with the CeA(Godoy et al., 2018). It should be noted that the LC receives fibres from the PVH but does not have a significant number of neurons projecting back to the PVH (Ulrich-Lai, Herman, 2009). The LC is active both in the PP and in the SP as it can be activated by both systemic and processive stressors (Godoy et al., 2018, Ulrich-Lai, Herman, 2009). The LC also takes part in active pain suppression by innervating inhibitory interneurons within the spinal cord, just like the PAG (Behrends, 2010).

e. Bed nucleus of the stria terminalis

The BNST has very dense connections with the PVH (Figure 2) and therefore serves as the primary integrator of limbic e.g. processive stress pathways, since the limbic areas themselves have little to no direct connection to the PVH (Choi et al., 2007).

f. Paraventricular nucleus of the hypothalamus

The PVH is the receiver for almost all stress related responses. The PP as well as the SP converge at this point (Godoy et al., 2018, Ulrich-Lai, Herman, 2009). Just as importantly the PVN connects to a variety of different brainstem and spinal cord nuclei (Figure 2) such as the LC, the PBN, the dorsal nucleus of the CN X., the NTS, and possibly even both the SNS and the PNS branch of the ANS (Ulrich-Lai, Herman, 2009). It therefore does not only regulate stress responses on a hormonal level by activating the release of stress hormones, but also by directly connecting to the relevant ANS structures. Projections to the LC indicate a coordinative function for limbic and autonomic processing (Godoy et al., 2018).

1.1.3.1 The sympathetic nervous system

Within the hypothalamus, specifically within the PVH and the LC, there are special neuron populations projecting directly into the preganglionic SNS (Ulrich-Lai, Herman, 2009, Godoy et al., 2018).

As already mentioned above, the SNS is the body's primary initiator of a "fight or flight" response meaning that an activation leads to catabolic metabolism in order to provide energy for the upcoming encounter. It achieves this in two different ways. On the one hand there is a direct innervation of the to be affected tissues, which translates into accelerated heart rate, bronchial relaxation, decreased gut motility while increasing sphincter tone, sweating and pupillary dilation to name a few and on the other hand there is an indirect effect on the tissues via the initiation of the release of adrenaline and noradrenaline in the adrenal gland, which in turn has inhibitory effects on the HPA-axis (Mravec, 2011). Most of the direct effects on organs are elicited through a combination of different densities of α- and β-adrenoceptors. As part of this process, the body also increases lipolysis and glycogenolysis (Behrends, 2010). Although the SNS acts as an antagonist to the PNS, it is never truly inactive. Rather, it is finely tuned up or down in relation to the PNS just like the PNS is in relation to the SNS, in order to achieve the right balance between the two.

1.1.3.2 The parasysympathetic nervous system

The second part of the ANS is the PNS. Unlike the SNS it only uses direct innervation of the effector organs who express a variety of different muscarinic receptors for different outcomes (Behrends, 2010). As the main goal for the PNS is to help the body regenerate and get into an anabolic state, the effects of the PNS on the organ systems are correlating. It therefore decelerates the heartrate, constricts the bronchi and increases the secretion of mucus, increases gut motility whilst decreasing sphincter tone and leads to a pupillary constriction amongst many others (Wehrwein et al., 2016). This ensures that in times where there is no threat the body can actively regenerate, absorb nutrients and heal to be more resilient for the next encounter. As is obvious from its trivial name, the PNS is tightly interlinked with the ENS as one of its main effector organs.

1.1.3.3 The enteric nervous system

The ENS is often labelled "the second brain" and in fact it has been shown that if the connection between the brain and the gastrointestinal system e.g. the CN X., is severed that the ENS is still able to operate independently of any CNS input (Y. Li, Owyang, 2003).

This independence is possible because the ENS consists of a surprisingly large number of neurons. In humans it is comprised of approximately 200-600 million neurons (Furness et al., 2014), which are divided into two distinct systems. On the one hand there is the submucosal plexus of Meissner. It forms the inner of the two plexus, innervating the smooth muscle cells of the muscularis mucosae and the glandular epithelial cells of the intestinal wall regulating secretion and absorption (Behrends, 2010). On the other hand, there is the myenteric plexus of Auerbach comprising the outer layer. Innervating the smooth muscles of the tunica muscularis it is capable of coordinating independent peristalsis (Behrends, 2010). Given its autonomic nature the ENS has a variety of different receptors to receive chemical, mechanical and nociceptive information which is then directly used to influence its executive functions (Behrends, 2010). Last but not least, the microbiome has an immense effect not only on the ENS but on the organism as a whole. Pretty much all of the effects the microbiome has on the organism are, as of now, subject of research and there are many discoveries yet to be made (Arnold, Roach & Azcarate-Peril, 2016).

1.2 Microglia and its role in the brain

1.2.1 Introduction

Microglia are a type of glial cells that can only be found in the CNS namely the brain and the spinal cord. One of its primary functions, and the one microglia are most commonly associated with, is that of a primary immune response cell in the brain, the reason being that the regular cells of the immune system may not easily enter the CNS through the blood-brain-barrier (BBB). But there are also a variety of other functions microglia can perform, some of which are associated with different morphological forms. A big part of these functions seem to be relevant during the developing phase of the brain but also in myelinisation and synaptic formation processes microglia seems pivotal (Charanjit, Ling, 2012, Hammond et al., 2019).

Stressful life events are associated with an increase in pro-inflammatory cytokine production which is known to induce changes in cortical microglia, even leading to mental disorders such as depression. These changes in microglia seem to be inflammatory in nature and can be observed by looking at the expression of Ionized calcium binding adaptor molecule 1 (Iba-1), a microglia specific marker upregulated through microglial activation (Calcia et al., 2016, Tynan et al., 2010). In the meta analysis of Calcia et al. (Calcia et al., 2016), one study (Tynan et al., 2010) showed a moderate increase of Iba-1

positive cells within the CA-3 region of the hippocampus of rats after 14 days of restraint stress (RS). Also repeated social defeat stress evoked a marked increase in Iba-1 positive cells within the prefrontal cortex (Tynan et al., 2010). Unpredictability of the stressor on the other hand did not lead to a significant change in Iba-1 expression (Kopp, Wick & Herman, 2013). Given these divergent findings, the microglial reaction in different brain regions, the influence of the type and intensity of the stressor, the interaction between microglia and changes within the HPA-axis and the sex of the animals still remain a large field of investigation (Calcia et al., 2016).
In the following paragraph I summarize the functions of microglia in the brain, in order to fully understand the changes I have seen during the current experimental setup.

1.2.2 Microglial classification

Microglia can be classified in many different ways. First discovered in 1919, Pio del Rio-Hortega initially based his classification purely on histological differences. Today it is possible to determine a wide variety of different surface markers as well as look at the genetic expression of genes within the cell. Also the ability to detect many different cytokines within the tissue has greatly enhanced our understanding of microglia and their role within the brain (Hanisch, 2002).

1.2.2.1 Microglial functions

Function is one of the ways by which microglia can be classified. Most of these functions are achieved by amoeboid microglial cells (AMCs).

a) Phagocytosis

The ability of AMCs to remove cellular debris in the brain is one of their better known functions. In hypoxic-ischaemic injuries microglia has been observed in phagocytosis of necrotic and apoptotic cells (Kaur, You, 2000) and AMCs are responsible for removal of intracerebrally injected Escherichia coli (Kaur, Too & Ling, 2004, Fu et al., 2014). This ability of AMCs is of vital importance to the brain since AMCs are the only cells with the ability of phagocytosis native to the brain. The role AMCs play in phagocytosis of apoptotic cells and supernumerary neurons is of detrimental importance to the developing brain (Charanjit, Ling, 2012).

b) Immunology

As mentioned earlier, microglial cells (MCs) are the primary immune cell of the brain. As such they need a variety of different tools to enable them to act as such. The following list

serves only to illustrate the broad variety of immunological functions of MCs and is by no means complete.

i. Pattern recognition receptors

The term pattern recognition receptors is a collective term for many diverse receptors. Their main immunological function is the recognition of proteins and pathogens and to then facilitate an immunological response in form of an increased production of inflammatory mediators, which could also be shown for MCs (Mariani, Kielian, 2009) One of the primary subgroups are the so called toll-like-receptors (TLRs), of which there are up to twelve subtypes which serve as binding sites for mostly, but not exclusively, pathogenic molecules and which, upon activation, initiate the production of proinflammatory cytokines like interleukins (Behrends, 2010).

ii. Complement receptors

The complement system is very strong in defence against microorganisms. Many different complement receptors have been found on MCs (Charanjit, Ling, 2012) as well. Specifically complement activation fragment C5a which for example favours chemotaxis of MCs seems to play an important role in activation and phagocytosis (Gasque et al., 1997).

iii. Cytokine and chemokine secretion

Microglia are known to secrete cytokines and chemokines as a response to an inflammatory environment (Chao et al., 1995, Deng et al., 2009). Interleukin-1 is known for inducing the expression of E-selectin, a protein which helps extravasation of leukocytes through the endothelial wall of blood vessels. Tumor necrosis factor-α (TNF-α) on the other hand stimulates phagocytosis and production of interleukin-1 in macrophages. Both mechanisms could be demonstrated for MCs (Munoz-Fernandez, Fresno, 1998).
As for chemokines, macrophage inflammatory protein-1α and monocytic chemoattractant protein-1 have been shown to modulate the inflammatory reactions of MCs in hypoxic-ischaemic insults in the neonatal rat brain (Cowell et al., 2002).

iv. Antigen representation

Major histocompatibility complex I (MHC I) antigens are needed for T-lymphocyte activation by presenting these antigens to them. The finding that MCs constitutively have MHC I antigens is probably owed to the fact that they are also macrophages (E. A. Ling, Kaur & Wong, 1991). MHC II antigens however are only expressed under pathological conditions such as intracerebral injection of Escherichia coli (Kaur et al., 2004). This is

evidence that MCs can interact with T-helper lymphocytes as part of their immune response.

c) Iron acquisition

Iron is a very important yet often overlooked aspect of brain development and function. Lack of sufficient iron during brain development can lead to persisting developmental deficits even after normal iron levels are obtained, as iron is essential to neurotransmitter release and myelination (Beard, Connor, 2003). In hypoxia of the periventricular white matter in developing rat brain, accumulation of iron exacerbates the damage already done by hypoxia. AMCs in the region show an increased expression of iron regulatory proteins and transferrin receptors, both being linked to iron acquisition and in PD the deposition of iron leads to a selective degeneration of dopaminergic neurons within the SN, with microglia involved in the deposition. (Jiang et al., 2019, Rathnasamy, Ling & Kaur, 2011).

d) Nitric oxide production

Under physiological conditions MCs do not produce or release NO. This ability is reserved for neurons and endothelial cells, which use it as neurotransmitter or vasodilator respectively (Vincent, Tilders & Van Dam, 1998). However, under pathological conditions AMCs can also start to produce NO through the so called inducible NO synthase (You, Kaur, 2000). These locally increased levels of NO have been linked to neuron and oligodendrocyte damage (Bal-Price, Brown, 2001).

e) Growth factors

Maybe one of the most important roles AMCs might have is the production of growth factors. Transforming growth factor-β (TGF-β) is one such example (Nakajima et al., 2007). Generally, TGF-β is constantly present in the brain in low levels. In low doses it serves as an anti-inflammatory agent until there is inflammation whereupon AMCs start to secrete higher levels of TGF-β (Lu et al., 2005).

Insulin like growth factor-1 (IGF) and IGF-2 have many different functions. In the brain they not only promote oligodendrocyte proliferation but also play an important role in myelin synthesis (Lin et al., 2017). It should come as no surprise that AMCs express IGF-1 and IGF-2 and are thus tightly connected to brain development (Kaur et al., 2006).

f) Myelination

Both brain-derived neurotrophic factor (BDNF) and IGF-1 are known to be produced by MCs (Nakajima et al., 2001, Lin et al., 2017). It has been shown that MCs play a vital role in remyelination trough the help of IGF-1 (Lloyd, Davies & Miron, 2017) and BDNF could

be linked to a promyelinating effect on oligodendrocytes (Xiao et al., 2010, Parkhurst et al., 2013).

g) Neurogenesis

In neurogenesis the role of MCs can be generally divided into three actions. Firstly, is the above already mentioned secretion of various proteins like BDNF and IGF-1, which are crucial to normal brain development. Secondly, they seem to support neuronal migration as well as removal of supernumerary synapses (Mosser et al., 2017), and lastly MCs secrete a whole different set of cytokines like leukemia inhibitory factor (Nakanishi et al., 2007) and ciliary neurotrophic factor (Kim, de Vellis, 2005) which play an important role in astrocyte differentiation.

h) Synapses

Thrombospondin is an extracellular matrix protein expressed in early postnatal stages by MCs (Chamak, Dobbertin & Mallat, 1995). It induces synaptogenesis and its lack leads to a radical reduction in the number of synapses (Christopherson et al., 2005).
KARAP/DAP12 is another protein expressed by microglia that plays a role in synaptic function and plasticity (Roumier et al., 2004). Additionally, as mentioned above, MCs have an important role during development of the brain by removal of supernumerary synapses.

i) Vascularisation

The retina as an extension of the brain has been under research for some time now and it was shown that MCs have an influence on the vascular branching density and endothelial cell proliferation within the retina (Biswas et al., 2017). Another recent finding suggests that MCs are important for the repair of vascular damage in the rat brain (Bowyer et al., 2016).

j) Receptor expression.

Microglia express a variety of different receptors such as receptors for glucocorticoids, estrogen, progesterone, TNF-α, substance-P and kappa-opioid-receptors (KOR)'s to name a few (Colonna, Butovsky, 2017, Bollinger, Bergeon Burns & Wellman, 2016, Bellavance, Rivest, 2014, Chao et al., 1996).

1.2.2.2 Microglial morphology

The second way to categorize microglia is by their morphology. This was the original way of categorization and is still useful to distinguish between different morphologic types of microglia.

a) Amoeboid microglia

AMCs are usually regarded as the adolescent form of ramified microglial cells (RMCs). AMCs can be observed in the rodent brain as early as embryonic day 12 (C. C. Wang et al., 1996) and slowly start to transform into their mature form namely RMC. In rodents this process is usually completed at around postnatal day 15-17 (Ashwell, 1991). As is becoming obvious, AMCs are of vital importance for the developing brain. As of now, there is a total of nine different microglial states with a majority of these being present in the early phases of development and a sharp decrease in diversity towards adulthood (Hammond et al., 2019).

b) Ramified microglia

As mentioned, all microglial cells turn into RMCs after the development phase. In literature this stage is often referred to as "resting". It marks a phase of relative steady state in the brain where the number of microglial cells turns out to be at around 12 % of all the cells within the brain, which rises slowly as the brain ages (Lawson, Perry & Gordon, 1992, E. Ling, Leblond, 1973). This state seems to be maintained by replicating resident microglia but in a pathological situation (e.g. damage to the brain or microbial invasion) infiltrating monocytes can enter the brain through the intact BBB and then rapidly differentiate into microglia (Lawson et al., 1992).

The label "resting" is a bit misleading as microglia in this state is, in fact very active (Nimmerjahn, Kirchhoff & Helmchen, 2005). RMCs are constantly moving in a seemingly random way, while staying in their respective areas to scan the environment for abnormalities to react to. To accomplish this task, RMCs have many protrusions (hence the name ramified) jutting out of their soma in all directions. These are constantly deployed and retracted to scan the cells' surroundings (Verkhratsky, Butt, 2013). It should be noted, that RMCs are by no means stuck to this state and that they can readily revert back to a more amoeboid form if need should arise.

1.3 Gut-brain interactions

1.3.1 Ways of communication between gut and brain

The bidirectional relation between gut and brain is now well established and accepted in the scientific community, but the ways of communication are still a matter of ongoing research (H. X. Wang, Wang, 2016, Farzi et al., 2019). The CNS can influence the digestive tract via efferent neurons of the SNS and PNS as well as by neuroendocrine secretions of the adrenal medulla and the adrenal cortex (Farzi et al., 2019). On the other

hand there are five proposed mechanisms by which the gut, and therefore the microbiota within it, can communicate and influence the body as a whole and the brain specifically (H. X. Wang, Wang, 2016).

a) Neuroanatomical pathway

There are two anatomical structures that enables communication between the gut and the brain. On the one hand there is the CN X. and on the other there are direct spinal afferent fibers of the spinal cord (Behrends, 2010). The afferent fibers of the CN X. have their cell bodies in the ganglion nodosum and project directly into the NTS from where other neurons connect to the dorsal nucleus of the CN X., the hypothalamus as well as other regions. The spinal afferent fibers have their cell bodies in the dorsal root ganglia of the spinal cord and terminate within the dorsal horn of the spinal cord from where the information is relayed to the thalamus (H. X. Wang, Wang, 2016, Behrends, 2010, Brierley, Hibberd & Spencer, 2018).

b) Neuroendocrine pathways

The gut is to be considered the largest hormone producing organ in the human body with a wide variety of hormones, neuropeptides and growth factors being produced by it (Rehfeld, 2014). Just like microglia in the brain, the gut as well produces TGF-β, which in the gut serves as a growth factor and promotes differentiation and inflammation. IGF-1 and IGF-2 are also produced by the gut and act there as growth factors much like in the CNS (Rehfeld, 2014, Nakajima et al., 2007, Charanjit, Ling, 2012). Other important hormones that are produced by the gastrointestinal tract include substance P, which stimulates motility, neuropeptide Y, which regulates smooth muscle cell contractility, and dynorphins (Rehfeld, 2014).

c) Interaction between microbiota and the peripheral immune system

A large part of the immune system is located within the gut especially in the colon. There the humoral as well as the adaptive immune system continuously interacts with the microbiota and other ingested proteins (Abbas, Lichtman & Pillai, 2015). How the microbiota influences the immune system is not entirely clear but TLRs probably play a significant part in this communication and even though the brain is for the most part, an immune privileged site, there is accumulating evidence that the peripheral immune system may have a significantly larger impact on the brain than previously expected (Sommer et al., 2015, Miyaoka et al., 2017, H. X. Wang, Wang, 2016, Calcia et al., 2016).

d) Neurotransmitters

There are multiple studies showing that the microbiome not only produces almost all the neurotransmitters that the brain uses as well but also in sometimes significantly larger amounts than the brain itself. Through these an extensive communication with the brain can be achieved (H. X. Wang, Wang, 2016, Lyte, 2014).

e) Intestinal mucosa and blood brain barrier

Stress alters the intestinal mucosa's barrier function in a way that it becomes permeable enough for certain molecules which through TLRs might activate the immune system which in turn could alter the permeability of the BBB (H. X. Wang, Wang, 2016).
So colitis basically is a disruption of the normal gut physiology which can then affect the gut itself, the microbiota and, secondarily, the brain via one or more of the above mentioned.

1.3.2 Dextran sulphate-sodium induced colitis

Dextran sulphate-sodium (DSS) is a well-established paradigm for mimicking inflammatory bowel disease in mice, since it reliably induces colitis (Laroui et al., 2012). It is used as a model for inflammatory bowel disease where the typical symptoms of colitis are usually established at around day 3 and reaches a peak at day 7 during which time colonic bleeding usually occurs (Laroui et al., 2012, Chassaing et al., 2014). The exact mechanism by which DSS induces colitis is not known, but damage to the epithelial layer of the large intestine and hence resulting contact of the underlying tissue with intestinal contents is a contributing factor. The resident intestinal microbiome seems to play an especially important part, given the fact that mice raised under germ-free conditions develop only very mild colitis under DSS and mice raised in different facilities can vary significantly in their susceptibility to the same dosage of DSS (Chassaing et al., 2014).

1.4 Administered drug and target receptors

1.4.1 κ-Opioid-receptors

KORs are one of the four major subtypes of opioid receptors, with the other three being μ-opioid-receptors, δ-opioid-receptors and the nociception opioid peptide receptor (Corbett et al., 2006). Of the KORs there are three proposed subtypes labelled κ_1, κ_2 and κ_3, yet there is no clear evidence how these subtypes relate to each other (Corbett et al., 2006). The natural, endogenous ligands of KORs are the so called dynorphins which are usually non-specific and have more or less affinity to the other opioid receptor types, yet there are a number of synthetic substances selectively acting only on KORs (Schwarzer, 2009).

KORs are implicated in a large variety of different functions. They are G protein-coupled receptors yet the full extent of their functions is still a matter of scientific research (Y. H. Wang et al., 2010). KORs seem to alter and even antagonise the effects of μ-opioid-receptors on pain, reward and memory processes (Pan, 1998) and there have been studies linking KORs to consciousness, mood, anxiety and stress reactions (Land et al., 2008, Xuei et al., 2006, Corbett et al., 2006).
The expression of KORs can be observed in various regions of the brain such as the BNST, amygdala, PAG, PBN, LC, NTS, SN and most notably the PVN where they seem to be involved in the inhibition of the release of AVP and oxytocin (Mansour et al., 1995). There is also good evidence for peripheral KORs, probably within the colon, and with regard to analgesic effects on visceral pain in rat models (Sengupta et al., 1999).

1.4.2 Norbinaltorphimine

norBNI is one of the few selective inhibitors of KORs and was the first to be discovered back in 1987 (Portoghese, Lipkowski & Takemori, 1987). The development of these new drugs met an increasing interest in the possible treatment of stress-induced psychiatric disorders such as depression and anxiety disorders. norBNI has an extraordinary long half-life time, inhibiting KOR-agonists for up to 14 days after intraperitoneal administration, with the maximum effect being reached within 48 hours (Williams et al., 2018, Patkar et al., 2013, Munro et al., 2012). Interestingly norBNI seems to impair the panic reaction in an electrical stimulation test after both, intraperitoneal and intracerebral injection, indicating a central activity of the compound (Maraschin et al., 2017).

1.5 Aims of the thesis

The top ten causes of premature death combined are responsible for 31.2 million deaths each year, which is 54% of all deaths worldwide. Of those top ten causes, at least five (ischaemic heart disease (No 1.), stroke (No. 2), Alzheimer's disease and other dementias (No 5.), trachea, bronchus and lung cancers (No. 6) and diabetes (No. 7)) are directly or indirectly (Kotlega et al., 2016, Chauvet-Gelinier, Bonin, 2017, Surwit, Schneider, 1993, Cohen, Edmondson & Kronish, 2015, Mravec, Horvathova & Padova, 2018) influenced by stress, which means 20.5 million, or roughly 36%, of all deaths each year are caused by diseases at least partially attributable to stress (World Health Organization, 2018).
Accumulating evidence suggests that microglia play a pivotal role in the brain's reaction to stress, especially to chronic stress (Calcia et al., 2016), which is why this study tried to shed some light on this topic.

Specifically, in the current experimental setup, mice were subjected to water avoidance stress (WAS), a psychological stressor, after which Iba-1 expression, a marker of microglial activation, was quantified throughout the cortico-limbic system of the brain. Half of the test subjects were treated with DSS to induce colitis, to evaluate whether the microglial response to psychological stress is altered under conditions of chronic internal, inflammatory stress. In addition, subgroups of mice, as detailed below, received the KOR antagonist norBNI, to investigate a potential role of the opioidergic system under these experimental conditions.

The regions of interest (ROI) in the mouse brain were within the CC, CA1, CA3, the MeA, the SN, the DG, the ILC, the LH as well as the PVH. These regions were chosen because multiple studies have shown them to be integral parts in the brains stress system (Tsigos, Chrousos, 2002, Ziegler, Herman, 2002).

2 Material and methods

2.1 Experimental animals

For this study 32 8-week-old adult male C57BL/6N mice were used. They were obtained from Charles River (Sulzfeld, Germany). The temperature and humidity under which the animals were housed was fixed (21°C and 50% air humidity respectively) as well as the 12-hour day-night-cycle which started at 06:00h (lights on) and ended at 18:00h (lights off). The Federal Ministry of Science and Research of the Republic of Austria (BMWF-66.010/0119-II/3b/2011) approved the experiments and they were conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC). The study was designed in a way that the number of animals as well as their suffering was minimized.

2.2 Experimental protocol

The animals were divided into four groups, each group consisting of 8 animals. Each cage contained two animals, for a total of 16 cages.

After arrival, the animals were habituated to the local animal facility for 2 weeks. After habituation two groups were treated with DSS (2% w/v in tap water) for 7 days to induce mild colitis whereas the other two groups acted as control receiving only normal drinking water (Figure 3). Beginning on day five of the DSS treatment, half of the mice from the DSS and control group received an intraperitoneal injection with norBNI (10 mg/kg), while the other half was injected with normal saline solution.

On day 7 of the treatment protocol, mice were subjected to a WAS session for 30 minutes whilst the disease activity score and the number and form of faecal boli were noted. Following a 90 min stress-free interval the mice were euthanized using a pentobarbital overdose (150 mg/kg body weight, injected intraperitoneally). The brains were collected, frozen in 2-methyl-butan on dry ice and stored at -70°C until analysis.

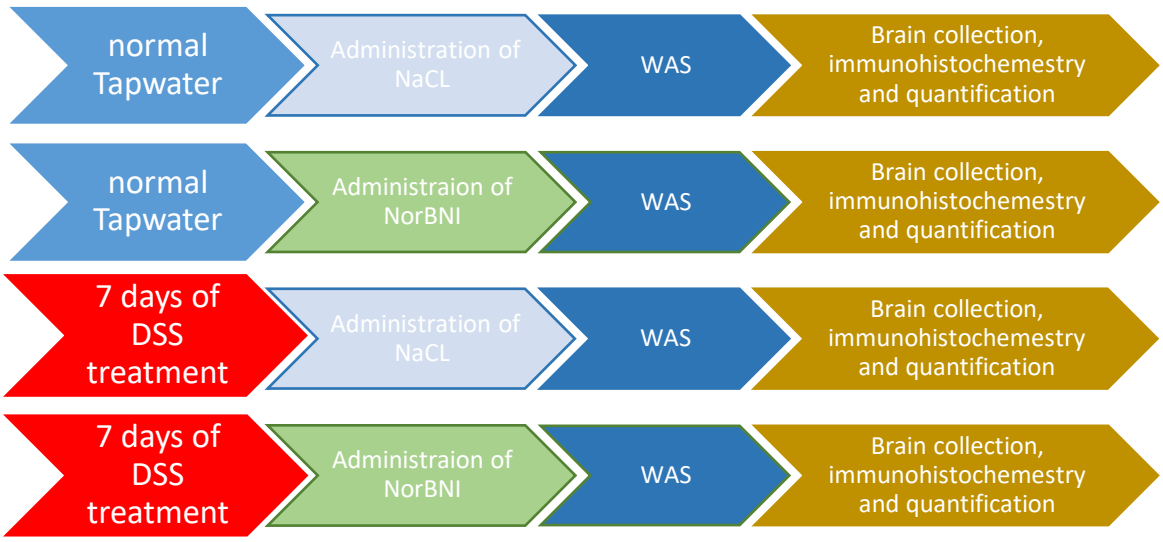

Figure 3. Treatment protocol. 32 C57BL/6N mice were divided into four groups of eight animals each. Mice received tap water or dextran sulphate sodium (DSS, 2%, added to the drinking water) for 7 consecutive days. On day 5 of the DSS treatment protocol, half of DSS- and half of water-treated animals were injected with norbinaltorphimine (norBNI, 10 mg/kg,i.p). Two days post-injection mice were subjected to water avoidance stress (WAS) after which there was a 90 minute stress free interval. Afterwards mice were euthanized using pentobarbital (150 mg/kg body weight, injected i.p.). Brains were collected, frozen in 2-methyl-butan and subsequently stored at -70°C for immunohistochemistry. Cell counting was done using a computerized image analysis system (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada).

2.3 Induction of experimental colitis

To induce colitis in the mice, DSS (molecular weight 36,000–50,000; MP Biomedicals, Illkirch,France) was added to the drinking water to obtain a 2% solution. DSS treatment lasted for seven days. The control mice received normal tap water. To avoid bacterial contamination the water which contained DSS was renewed every other day.

2.4 Administration of norBNI

As mentioned before, on day five of the DSS treatment, norBNI, obtained from Tocris, Abingdon, United Kingdom, was injected intraperitoneally at a dose of 10 ml/kg (Langlois et al., 1997) and a volume of 10 ml/kg to half of the DSS and tap water treated mice. The remaining mice of each group received an intraperitoneal injection of normal saline solution as vehicle (VEH). Two days after injection, mice were submitted to WAS.

2.5 Water avoidance stress

WAS has proven itself to be an adequate stressor for mice (Reichmann, Painsipp & Holzer, 2013, Bonaz, Tache, 1994, Miampamba et al., 2007). For this procedure, the mice were placed on a small platform (6x3x6 cm, length x width x height) that was surrounded by water on all sides and which was raised just 0.5 to 1 cm above the waterline for 30 minutes. This platform was located inside a tank (61x40x22 cm, length x width x height) to contain the water which had a temperature of 25°C. The room was brightly lit (230-250 lux) throughout the whole procedure.

2.6 Iba-1 immunohistochemistry

To visualise the activity of brain microglial cells, I performed immunohistochemistry (IHC) for Iba-1, a marker of microglial activation. In preparation for IHC, fresh frozen brains were individually taken and fixed with Tissue-Tek® O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, Netherlands) on a sample holder within a Microm HM 560 cryostat (Fisher Scientific). The olfactory bulb was removed since it contained no ROI. The rest of the brain was then cut into 20 µm thick coronal slices and every slice was mounted on Superfrost Plusmicroscope slides (Menzel, Braunschweig, Germany). The slides were then stored in a freezer at -20°C until all brains were cut and IHC could commence. For IHC every sixth section was used and adjacent sections were Nissl-stained for easier identification of the ROIs.

For IHC the selected sections were surrounded by a hydrophobic line that was drawn with a fat pen (ImmEdge Pen, Vector Laboratories, Burlingham, California, USA) in order to avoid spilling of the antibody solution. For fixation, the slides were incubated in 4% paraformaldehyde (Sigma-Aldrich, Vienna, Austria) in a 0.1 M phosphate-buffered saline solution (PBS) of pH 7.4 for 10 minutes. Following the incubation the slides were washed three times for 5 min amounting to a total of 15 minutes. The washing was done in washing buffer (WB: 0.1 M PBS with 0.05 % Tween 20) wherein the Tween acted as a facilitator for antibody penetration into the tissue. After washing, the slides were incubated in 0.3% H_2O_2 for another 15 min to block the endogenous peroxidase enzyme activity in the tissue. Again the slides were washed three times for 5 min in WB. Then the sections were incubated for 5 min in10% normal goat serum in antibody diluent solution (AD; 0.1 M PBS containing 0.05% Tween 20 and 1% bovine serum albumin) to block all unspecific binding sites therefore reducing unwanted background staining to a minimum. The first part of the IHC was finalized by incubating the slides with the primary antibody (rabbit

polyclonal anti-Iba-1 antibody, catalogue number: 019-19741,1: 1000, Wako) overnight at 4°C.

The second part of the IHC started on the next day with the slides being washed three times for 5 minutes in WB, after which they were incubated in AD containing the biotinylated secondary antibody (goat anti-rabbit IgG 1:200, Vectastain Elite ABC Kit, Vector Laboratories) at room temperature for 30 min. After finishing another round of washing the slides for three times in WB, they were incubated in avidin-biotin complex (ABC; Vectastain Elite ABC Kit, Vector Laboratories) to enable staining of the primary/secondary antibody complex. To finish the process, the slides were washed three times in WB and subsequently developed in 3,3-diaminobenzidine substrate (DAB substrate kit for peroxidase, Vector Laboratories) for 4 min and then washed three times with distilled water and air-dried overnight. Xylol (100%) was used to clear the slides which were then coverslipped with Entellan (Merck, Darmstadt, Germany). This process results in a brown precipitate in regions with macrophages and brain glia which can be seen and quantified with a light microscope.

2.7 Cell counting and quantification

After immunohistochemical staining, ROIs were examined under a light microscope (Axiophot, Zeiss, Oberkochen, Germany). The microscope was connected with a computerized image analysis system (MCID Basic, version 7.0, Imaging Research Inc., Brock University, St. Catharines, Ontario, Canada) to allow for easier and faster examination. For additional accuracy the investigator was able to manually edit the images to exclude artefacts. To ensure objectivity all slides were coded so that the investigator had no knowledge of the treatment group during investigation. To identify the ROI adjacent sections were Nissl-stained to more easily identify them on a mouse brain stereotaxic atlas by Paxinos and Franklin (Paxinos, Franklin, 2001). To quantify the Iba-1 expression, an intensity-based threshold was defined to include as much specific signal as possible whilst excluding the background staining. In order to quantitate the signal in a given ROI, two or three consecutive sections were examined bilaterally and the mean Iba-1 expression for each ROI was calculated and used for statistical analysis. ROIs within the CC, CA1, CA3, MeA, SN, DG and LH were examined for 3 consecutive sections whereas ROIs within the ILC and PVH were examined for 2 consecutive sections. A 300x300 µm counting area was used to quantitate Iba-1 expression in all regions except for the PVH and DG where all the cells within this clearly defined brain areas were included.

2.8 Statistics

Statistical evaluation of the data was done on SPSS 20 (SPSS Inc. Chicago, IL, USA). For analysing the data, a two-way analysis of variances (ANOVA) was used to account for the main effects treatment (DSS vs. control) and drug (norBNI vs. vehicle). In case of a significant interaction of these 2 factors, the Tukey posthoc test was run and otherwise the main effects were interpreted.

Probability (P) values of $P \leq 0.1$ were regarded as a trend, values $P \leq 0.05$ were regarded as statistically significant and values of $P \leq 0.01$ were regarded as highly significant. All data are presented as means ± SEM, n referring to the number of mice in each group.

3 Results

3.1 Iba-1 expression in the hippocampal area

Within the hippocampus, the CA1 region did not show any change in the expression of Iba-1 due to norBNI or DSS (Figure 4 A-D). Likewise, there was no interaction between the two factors. The same held true for the DG where there was no statistically significant change in Iba-1 expression attributable to norBNI or DSS as well as no interaction between the two factors (Figure 4 E-H).

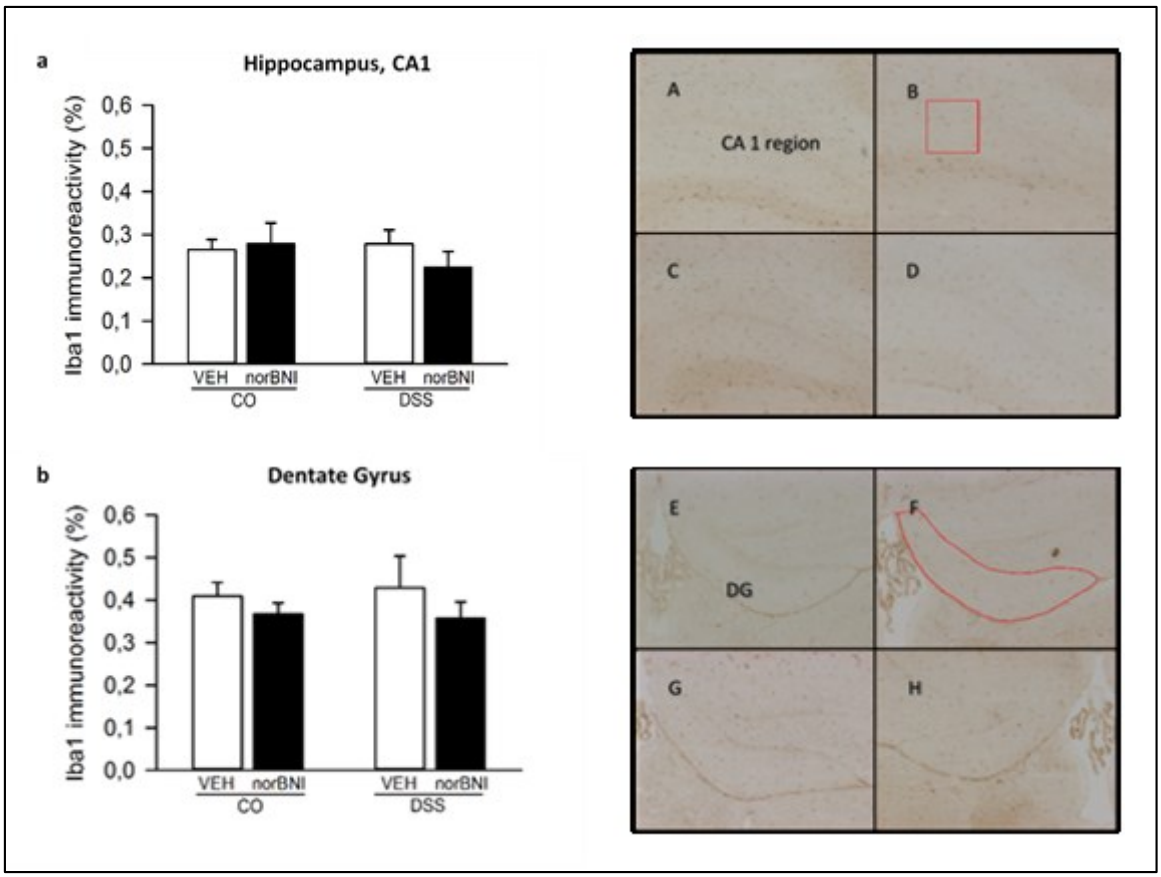

Figure 4. In the dentate gyrus (DG) and the hippocampal region of the cornu ammonis 1 (CA1) neither norbinaltorphimine (norBNI) nor dextran sulphate-sodium (DSS) altered the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells. Graphs a and b on the left show the effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS tretment on Iba-1 immunoreactivity in the indicated regions of interest. The panels on the right show immunohistochemical micrographs of the tap water control group with (A, E) and without (B, F) norBNI treatment. The lower rows show micrographs of the DSS treatment groups with (C, G) and without (D, H) norBNI treatment. Panels B and F show exemplary counting areas. Data are presented as means ± SEM, n = 7-8 / group.

In the CA3 region statistics revealed a significant interaction between DSS and norBNI treatment ($F_{(1,23)} = 5.635$; $P < 0.05$). Post-hoc testing revealed that norBNI tended to decrease Iba1 immunoreactivity within the DSS group ($P = 0.063$), but did not change Iba1

signals in the control group. In addition, compared to norBNI treatment in the control group, Iba1 immunoreactivity in the DSS group was reduced (Figure 5).

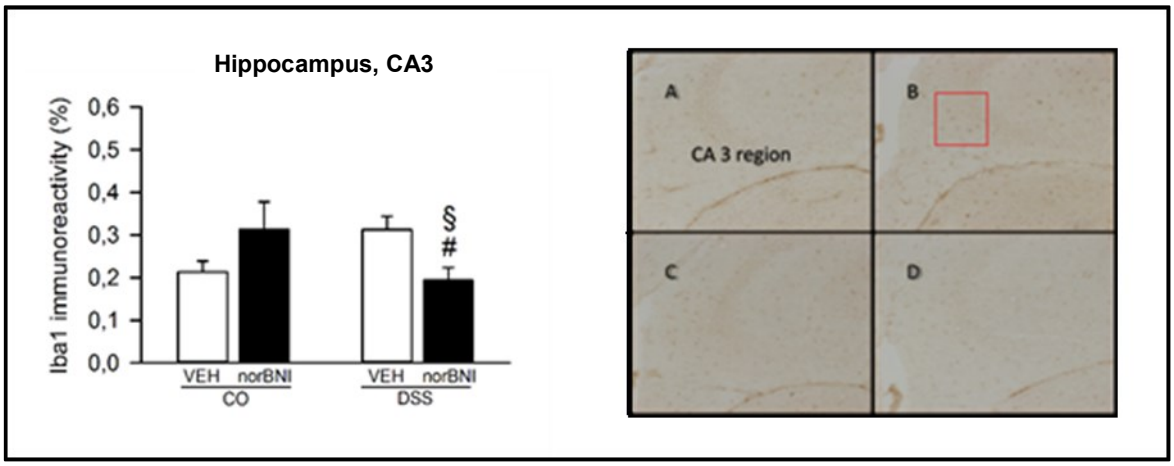

Figure 5. In the hippocampal region of the cornu ammonis 3 (CA3) norbinaltorphimine (norBNI) tended to decrease the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells under dextran sulphate-sodium (DSS) treatment compared to DSS treated controls and compared to norBNI treatment without DSS. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group. #, $p < 0.1$ vs. CO/norBNI; §, $p < 0.1$ vs. DSS/VEH

3.2 Iba-1 expression in the medial amygdala

In the MeA there was no observable influence of DSS or norBNI as well as no interaction between the two factors (Figure 6).

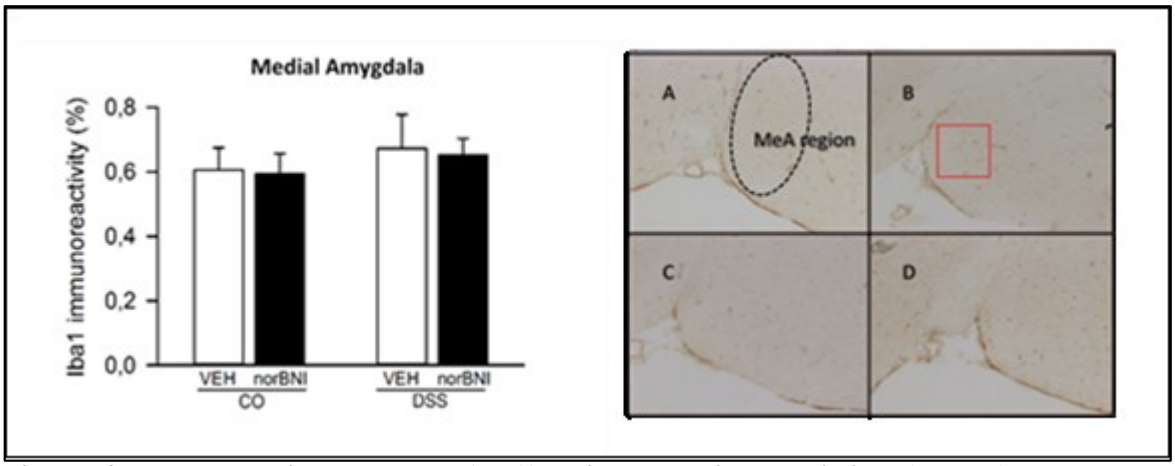

Figure 6. In the medial amygdala (MeA) neither norbinaltorphimine (norBNI) nor dextran sulphate-sodium (DSS) altered the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the

DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data is presented as means ± SEM, n = 7-8 / group.

3.3 Iba-1 expression in the medial prefrontal cortex

In the CC neither norBNI nor DSS had any effect on the number of Iba-1 expressing cells. Also there was no interaction between the two factors (Figure 7).

Within the ILC region norBNI had a statistically significant effect on the expression of Iba-1 in both the control and DSS groups ($F_{(1,21)} = 8.029$, $p < 0.01$). It reduced the expression of Iba-1 in both groups independently of, and without significant interaction with, DSS treatment (Figure 8).

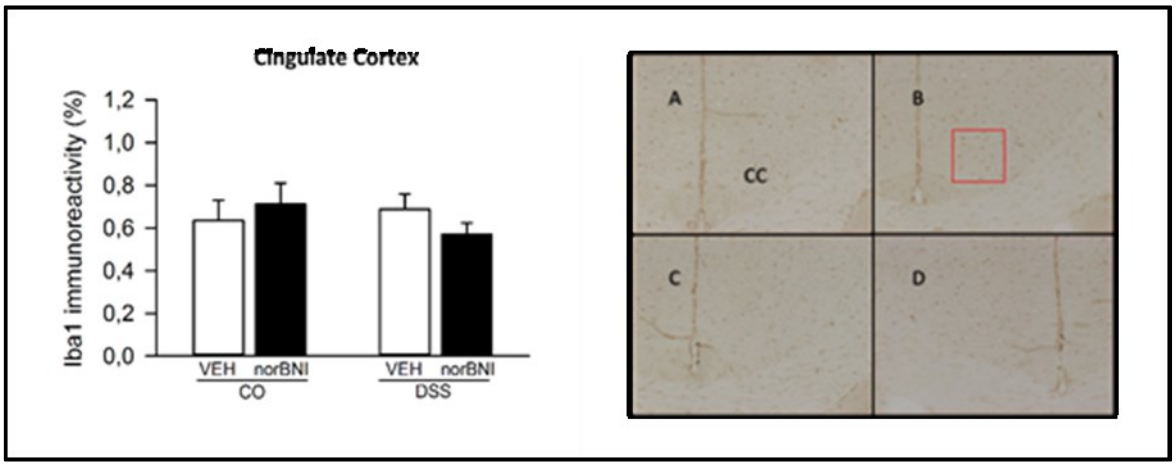

Figure 7. In the cingulate cortex (CC) neither norbinaltorphimine (norBNI) nor dextran sulphate-sodium (DSS) altered the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group.

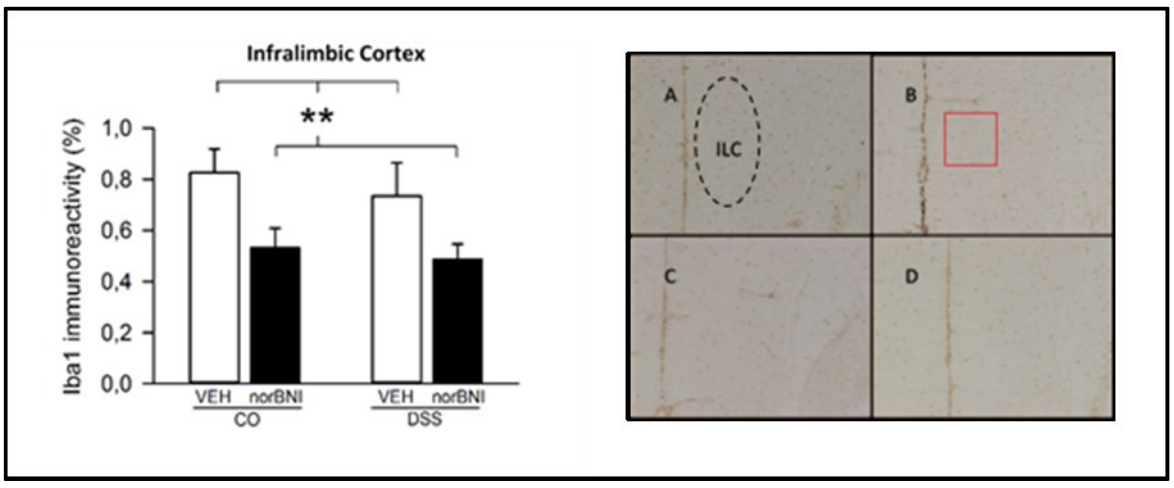

Figure 8. In the infralimbic cortex (ILC) norbinaltorphimine (norBNI) reduced the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells in both the control (CO) and the dextran

sulphate-sodium (DSS) group. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group.**, $p < 0.01$, main effects norBNI vs. VEH groups.

3.4 Iba-1 expression in the hypothalamus

Although not statistically significant, norBNI tended to decrease the expression of Iba-1 in the PVH in both the control and DSS groups ($F_{(1,20)} = 3.572$; $p = 0.073$) (Figure 9), consistent with observations in the ILC (Figure 8). Unlike in the PVH, norBNI and DSS had no influence on the Iba-1 expression in the LH and there was no interaction between the two factors (Figure 10).

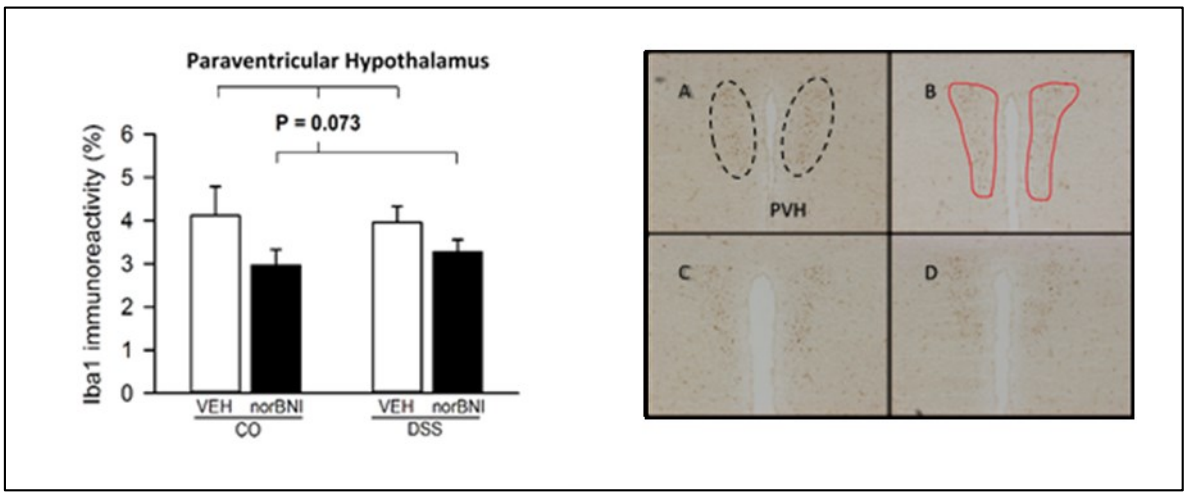

Figure 9. In the paraventricular hypothalamus (PVH) norbinaltorphimine (norBNI) reduced the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells in both the control (CO) and the dextran sulphate-sodium (DSS) group. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group.

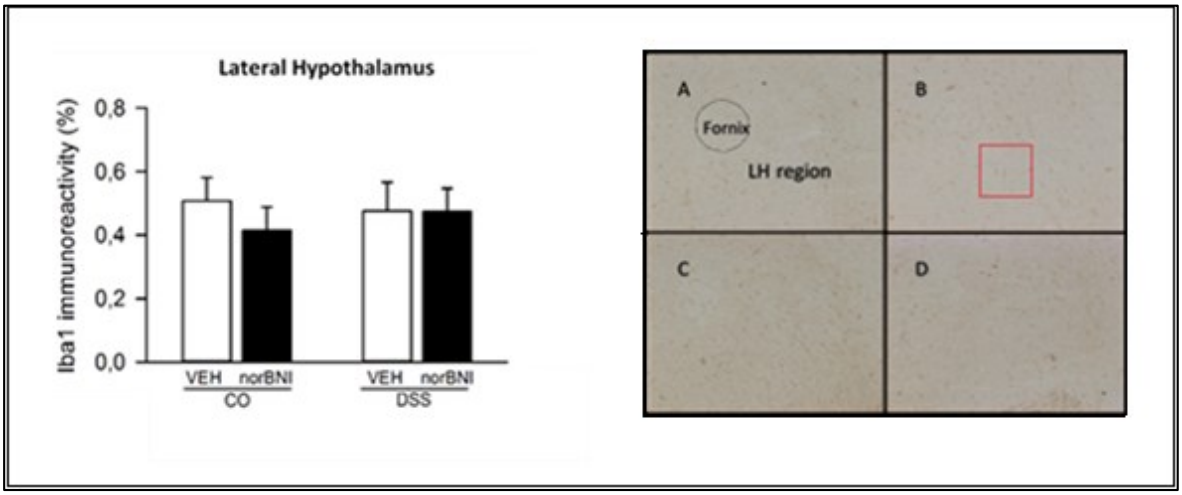

Figure 10. In the lateral hypothalamus (LH) neither norbinaltorphimine (norBNI) nor dextran sulphate-sodium (DSS) altered the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells. The graph on the left shows the effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panels on the right show immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group.

3.5 Iba-1 expression in the substantia nigra

Within the region of the SN there were no perceivable changes in the Iba-1 expression due to DSS or norBNI. Neither were there any interactions between the two factors (Figure 11).

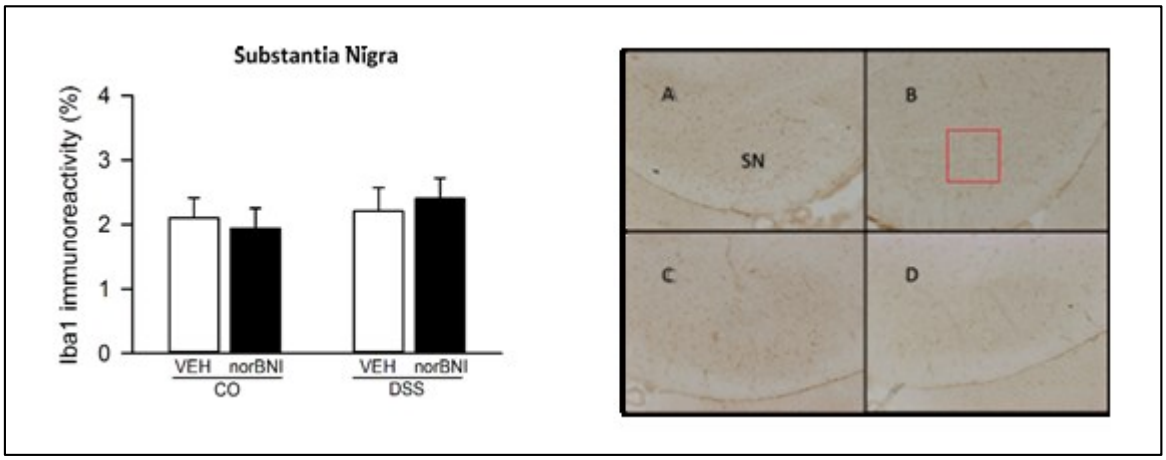

Figure 11. In the substantia nigra (SN) neither norbinaltorphimine (norBNI) nor dextran sulphate-sodium (DSS) altered the number of ionized calcium-binding adapter molecule 1 (Iba-1) expressing cells. The graph on the left shows effects of norBNI relative to vehicle (VEH) in mice without (CO) and with DSS treatment. The panel on the right shows immunohistochemical micrographs of the tap water control group with (A) and without (B) norBNI treatment. The lower row shows micrographs of the DSS treatment group with (C) and without (D) norBNI treatment. Panel B shows an exemplary counting area. Data are presented as means ± SEM, n = 7-8 / group.

4 Discussion

4.1 Influence of stress on microglia

a) A function of time

The influence of stress on microglia is very diverse and varies with brain regions and type of stressor and the results vary between different studies as well (Bollinger et al., 2016). There is an increasing number of studies related to the effects of acute and chronic stress on microglia (Ohgidani et al., 2016, Calcia et al., 2016). Both types of stress were found to induce microglial activation, yet there is controversy regarding the reason responsible for the different outcomes following acute stress, sometimes yielding significant changes in microglial expression (Tynan et al., 2010, Kreisel et al., 2014, Roumier et al., 2004) , mostly by a decrease of microglial activity, and sometimes not (Ohgidani et al., 2016, Bollinger et al., 2016, Kopp et al., 2013). One reason could be the time delay between the acute stressor and the sacrifice of the animals, since activation of microglia takes around 45 min to occur (Dailey, Waite, 1999) and maybe the effects of the stress itself only start to manifest after termination of the stressful event. Therefore, studies in which the animals were sacrificed prior to this 45 min mark might see less or no change at all in microglial activity attributable to the acute stressor. This is not to say that microglia are not reacting prior to these 45 min, but rather that Iba-1 as a marker only then starts to change. TNF-α for example seems to be a cytokine reliably released by microglia immediately after the stressful event (Ohgidani et al., 2016, Walker, Nilsson & Jones, 2013, Riazi et al., 2008, Denieffe et al., 2013).

b) Glucocorticoids

An alternative way of influencing microglia in the present study could be the negative feedback mechanism that is introduced by glucocorticoids that might have led to a reduced expression of Iba-1 within the ILC as this area is very densely packed with glucocorticoid receptors (GCRs) (Calcia et al., 2016). Moreover, it has been shown that glucocorticoids are a significant factor in stimulation of microglia mediated remodeling of the neural structures within the mPFC through neuronal colony stimulating factor-1 signalling (Horchar, Wohleb, 2019). However, in an earlier experiment Reichmann et al. (2013) could demonstrate that under similar conditions as in this work neither DSS induced colitis nor WAS led to a significant rise in cortisol levels, which opposes the findings by Do et al. (2018) who demonstrated an increase in cortisol levels after 7 days of DSS treatment in C57BL/6J mice. In the present study glucocorticoids were not measured and given the

contradictory findings I cannot assess whether or not glucocorticoids might have influenced the findings.

c) Water-avoidance-stress

WAS stimulates c-Fos expression in colonic myenteric neurons and PVN, LC and BNST neurons in the brain and increases fecal pellet output in rodents (Bonaz, Tache, 1994, Miampamba et al., 2007). Yet we have to acknowledge that WAS, when compared to chronic unpredictable stress, foot shock, social defeat or RS, is a very mild stressor as exemplified by the significant increase in glucocorticoids release by chronic unpredictable stress, foot shock, social defeat or RS (Ohgidani et al., 2016, Calcia et al., 2016, Horchar, Wohleb, 2019) compared to no significant increase of glucocorticoid by WAS (Reichmann et al., 2013). Most experiments focussing on microglia use some variation of the above mentioned stressors (Ohgidani et al., 2016), yet WAS has currently not been used experimentally to study its effects on microglia and therefore the effect of WAS on microglia is not documented in scientific literature. Since all mice in the present experiment did experience WAS I cannot make definitive statements as to whether or not WAS had an influence in the experiment. Yet since it is only a mild stressor and since the mice were sacrificed only shortly after experiencing WASs it can be assumed that WAS is not accountable for any changes on microglia seen in the results.

4.2 Where and how does norBNI act

The main effect I saw in the present study was that norBNI downregulated microglial activation independently of DSS colitis within the ILC and tended to downregulate microglial activity within the PVH. Since we know that an intraperitoneal injection of norBNI can have effects on behaviour as mentioned above (Maraschin et al., 2017), I need to ask the question how intraperitoneal administration of norBNI does achieve that. This could be due to the simple fact, that norBNI changes the pain perception on a spinal level. However, since norBNI acts as an antagonist on KORs (Portoghese et al., 1987) it will all but inhibit the effects of endogenous dynorphins acting as analgesic substrates on KORs (Chavkin, Cohen & Land, 2019). Therefore, it should rather be assumed that, even though intraperitoneally injected, norBNI can enter the blood stream and access the brain through the BBB (Maraschin et al., 2017).

Currently, there are only a handful of studies investigating the effects of norBNI on microglia. One of these could show that norBNI, in addition to being a KOR antagonist, is also a potent inhibitor of TLR-4 receptors (X. Zhang et al., 2019). The paper suggests an

implication in neuropathic pain and drug addiction, but the fact that norBNI inhibits both KORs and TLR-4 in microglia (Chao et al., 1996), which frequently express both receptors (Colonna, Butovsky, 2017), might add an additional hypothetical pathway by which norBNI might have influenced microglia in the present experiment.
However, this information only provides us with a possible answer to the reason why we see a reduced Iba-1 signal, but not an answer as to why only the ILC is most affected. Most experiments seem to agree that the mPFC is one of the primary brain regions in context to psychological stress (Ulrich-Lai, Herman, 2009, Bollinger et al., 2016, Y. Zhang et al., 2019). It seems that regions within the mPFC like the ILC regularly exhibit significant changes in regard to various stressors and are of vital importance regulating the cardiovascular response to acute stressors (Fassini et al., 2015). One might therefore infer a higher susceptibility of this brain region to stress. Since the ILC and the PVH are vital parts of the PP (Figure 1), the current findings might be explained by assuming that these regions reacted first. What we see might be the above mentioned reduction in microglial activity by acute stress, which has not yet affected other regions of the PP, with norBNI being only a facilitator to increase the speed at which theses effects take place.
As was shown by (DePaoli et al., 1994), of the ROIs examined here only the SN is amongst those regions with the highest density of KOR expression. Other areas like the PVH, the CA3 and the ILC only show moderate expression of KOR. Therefore, it seems that there is no correlation between the density of KOR expression within a region and the effect of norBNI in the present experiment.

4.3 How does neuronal activation influence microglia: c-Fos vs Iba-1

Looking at the results obtained by a similar experiment conducted by (Reichmann et al., 2013), I can draw some conclusions regarding the expression of Iba-1 compared to the expression of c-Fos, a marker for neuronal activation, under a chronic stressor like DSS and an acute stressor like WAS. Both experiments used a very similar protocol, with the DSS treatment and the WAS paradigm being the same and the selected brain areas were very similar as well. The ROIs that were the same in both experiments are the DG, CA1, CA3, MeA, PVH, CC and the ILC. Within those regions (Reichmann et al., 2013) showed a significant reduction in the number of c-Fos expressing cells for mice treated with DSS (except for the CA3 region), yet the present study did not show any effect of the DSS on the expression of Iba-1. This could mean that microglia is indifferent to the effects of the

DSS-induced colitis, as opposed to neurons. It could mean that the DSS-induced colitis did not yet have had a chance of sufficiently changing the microglia or that WAS masks the differences in Iba-1 expression. Based on the above mentioned schemes of stress-related circuitries in the brain (Figure 1 and 2), I could also expect changes in the PBN, the PAG, the CeA and the PVH. Since the PBN, PAG and CeA are part of the SP (Figure 2) and WAS is a psychological stressor using the PP, those regions should not be affected. However, since these regions were not investigated further research is needed.

4.4 Sex differences

One aspect that can be easily overlooked when considering stress and its effects on the brain, is the fact that almost all experiments are conducted on cohorts of male rodents only. Using male rodents is common in scientific literature due to the fact that the menstrual cycle of female rodents might have an unintentional or unwanted and ultimately unpredictable impact on the outcome of the experiment. Therefore, this study as well focuses on male mice only.

It should be noted though that there are significant differences between male and female populations concerning stress, post-traumatic stress disorder, depression and anxiety in rodents as well as humans (Bollinger et al., 2016, Maeng, Shors, 2013, Villa et al., 2018, Doyle et al., 2017). Women are twice as likely as men to suffer from post-traumatic stress disorder or anxiety following a stressful live experience and this fact seems to be coherent with different brain regions being activated during stress in males and females, the mPFC, especially the PLC and the ILC, being one of the primary regions to act in a sex dependent manner when it comes to inhibition of learning due to stress (Maeng, Shors, 2013). Differences in microglia morphology and distribution have been observed and the fact that microglia express estrogen-receptors and that estrogen acts anti-inflammatory may be additional reasons for different susceptibilities to stress in males and females (Villa et al., 2018, Bollinger et al., 2016). It thus would be interesting to study the effects of WAS, colitis and KOR antagonism on microglial phenotypes in female mice as well.

4.5 Colitis as an influencing factor

In the current experiment, colitis did not significantly influence the expression of microglia, although within the CA3 region, DSS induced colitis tended to decrease Iba-1 immunoreactivity. There is one study documenting that inflammation in the colon led to an increase of Iba-1 expression within microglia of the retina in C57BL/6J mice (Maneu et al., 2016). Furthermore, (Feng et al., 2019) could demonstrate that DSS-induced colitis led to

an increase in the infarction area, 3 and 7 days after stroke, compared to a control group, probably attributable to an imbalance of different microglial subtypes at the stroke site. But just as colonic inflammation can affect the brain, the brain itself can affect the gastrointestinal tract, the hippocampus being especially active during gastrointestinal inflammation (Riazi et al., 2008). RS in particular led to a disturbance in the gut microbiome and colonic inflammation which was accompanied by an increase in hippocampal microglial activation especially in the CA3 region (Jang et al., 2018). Whether RS, colonic inflammation or the changed gut microbiome were causally related to each other is not clear, but this association reinforces once again the fact that the brain and the gut stand in direct communication with one another and changes in one of the two lead inevitably to changes to the other as well. Consistent with the above findings is the fact that inflammation changed the permeability of the BBB accompanied by an increase in inflammatory molecules like TNF-α (Denieffe et al., 2013) as was also observed by other studies (Riazi et al., 2008, Feng et al., 2019, Jang et al., 2018). Interestingly enough the CA3 region was the only region other than the PVH and the ILC that did see changes in my experiment. norBNI does not seem to influence learning by itself when injected directly into the CA3 region but does prevent the negative effects on learning mediated through KOR overstimulation by alcohol intoxication (Sandin et al., 1998, Kuzmin et al., 2013). Overstimulation of KOR in the CA3 region seems to be a primary factor for inhibition of memory formation during stress (Sandin et al., 1998, Kuzmin et al., 2013, Vanz et al., 2018). This overstimulation can also be achieved by cortisol, and stress induced cortisol release seems to be an additional pathway by which stress can impair memory formation (Stokes, 1995). The exact way by which KORs and GCRs interact with each other are a subject of further research, yet the results of my study leave room to speculate. Assuming an increase in cortisol levels due to DSS treatment (Do, Woo, 2018), antagonism at the KOR could override the cortisol mediated overstimulation of the GCR within the CA3 region of the hippocampus resulting in a decreased expression of Iba-1 compared to no norBNI treatment and compared to no DSS treatment as was seen in my study. This effect of norBNI would then only be visible in the presence of elevated cortisol levels but not when cortisol levels are normal like with no DSS treatment.

4.6 Conclusions and further directions

The significance of my study is highlighted by numerous reports in very recent years all identifying microglia in its importance to the brain and its functions and their relationship

to the gut microbiome in colonic inflammation (Riazi et al., 2008, Berg et al., 2019, Y. Zhang et al., 2019, Zella et al., 2019, Feng et al., 2019, Mravec et al., 2018, Ong et al., 2017, Lin et al., 2017, Colonna, Butovsky, 2017). Although my study did not show a clear relationship between DSS-induced colitis and microglial activation it demonstrated a visible effect of the KOR antagonist norBNI on two extremely important sites involved in the stress response, the ILC and the PVH. The ILC in particular serves as an important integrator and mediator in the PP, with WAS being the psychological stress paradigm in this experimental setup. These changes are likely attributable to the fact that these two regions are amongst the first to be involved in the stress response and therefore are the first to show an effect to stress as could be seen in other studies facilitated by norBNI (Ulrich-Lai, Herman, 2009). Yet there are still many open questions that need to be answered and that leave much room for speculation. One of the most important ones is the time dependency of the microglial stress response, which has not yet been unambiguously been answered, or the fact that there are many different kinds of stressors being used to activate microglia. Firstly, one would need a clear picture of when and how long microglia reacts to stress and how it changes over time with the stressor either being acute or chronic, especially exploring the early phases of the microglial response with immunohistochemical and/or molecular methods. Secondly, in order to get a clearer picture of how different kinds of stressors activate microglia, one could explore different brain regions with above mentioned methods to see an emerging picture of when and how each region in the brain reacts in a time dependent manner to those different kinds of stressors. Ideally, all of above mentioned studies should be carried out on male and female mice, in order to see potential sex-specific differences in stress reactions as have already been described (Maeng, Shors, 2013, Bollinger et al., 2016, Villa et al., 2018, Doyle et al., 2017). With this knowledge the effects of different substances like norBNI and others will be more easy to evaluate, so that one day valuable treatments for different stress-dependent ailments can be found.

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