Microglia’s morphological responses to insufficient sleep and recovery sleep in ventral hippocampus

Johanna Emilia Immonen

Master´s Thesis

Psychology

Faculty of Medicine

September 2019

Supervisors: Iiris Hovatta & Sarah Steffens

Tiedekunta – Fakultet – Faculty Lääketieteellinen tiedekunta		Koulutusohjelma – Utbildingsprogram – Degree Programme Psykologian maisteriohjelma
Tekijä – Författare – Author Johanna Emilia Immonen		
Työn nimi – Arbetets titel – Title Microglia's morphological responses to insufficient sleep and to recovery sleep in ventral hippocampus		
Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track Psykologia		
Työn laji – Arbetets art – Level Pro Gradu -tutkielma	Aika – Datum – Month and year Elokuu 2019	Sivumäärä – Sidoantal – Number of pages 39

Tiivistelmä – Referat – Abstract

Aims

Sleep is needed to maintain brain homeostasis. Chronic insufficient sleep has been associated with elevated levels of inflammatory markers. Microglia are the resident immune cells of the brain. As microglial morphology correlates with their functional state, the current study aimed to characterize microglial morphology after insufficient sleep and recovery sleep. We hypothesised that microglia adopt an activated state after insufficient sleep, indicated by a deramification of the branches and an enlargement of cell bodies compared with the controls.

Methods

We caused insufficient sleep with acute sleep deprivation by 9 h of gentle handling, and conducted sleep fragmentation for 14 days in mice. The tissue was collected after perfusing the animals with PFA. The brain tissue from ventral hippocampus was immunostained for microglia and imaged with a confocal microscope. Ramification and soma size were quantified by tracing the branches and segmenting the somas.

Results

Neither the acute sleep deprivation nor the chronic fragmented sleep did result in any differences in morphology compared with their control groups. Surprisingly, the soma size was significantly smaller following the recovery sleep after fragmented sleep compared with the controls.

Conclusions

Microglial morphology and thus function may not be affected by acute sleep deprivation and chronic fragmented sleep in ventral hippocampus. Microglial soma size was significantly smaller after recovery sleep following chronic fragmented sleep compared with the control. This could have been due to larger soma sizes in this control group compared with other controls. Further studies are needed.

Avainsanat – Nyckelord – Keywords
Microglia, Acute sleep deprivation, Chronic fragmented sleep, Recovery sleep, Morphology

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
Iiris Hovatta, Sarah Steffens

Säilytyspaikka – Förvaringställe – Where deposited
E-thesis

Muita tietoja – Övriga uppgifter – Additional information

Tiedekunta – Fakultet – Faculty Lääketieteellinen tiedekunta		Koulutusohjelma – Utbildingsprogram – Degree Programme Psykologian maisteriohjelma
Tekijä – Författare – Author Johanna Emilia Immonen		
Työn nimi – Arbetets titel – Title Microglia's morphological responses to insufficient sleep and to recovery sleep in ventral hippocampus		
Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track Psykologia		
Työn laji – Arbetets art – Level Pro Gradu -tutkielma	Aika – Datum – Month and year Elokuu 2019	Sivumäärä – Sidoantal – Number of pages 39

Tiivistelmä – Referat – Abstract

Tavoitteet

Unta tarvitaan aivojen tasapainotilan ylläpitämiseksi. Krooninen riittämätön uni voi aiheuttaa akuutin tulehdustilan. Mikrogliat ovat aivojen omia immuunipuolustussoluja. Tutkimukseni pyrki kartoittamaan, miten mikroglian morfologia muuttuu riittämättömän unen sekä siitä toipumisen jälkeen. Mikroglian morfologiaa tutkitaan, sillä mikroglian morfologia on yhteydessä microglian toiminnalliseen tilaan. Hypoteesimme oli, että mikrogliat aktivoituvat riittämättömän unen jälkeen. Aktivoitunut tila näkyy mikroglian haarakkeiden vähäisempänä haaroittumisena ja haarakkeiden vähentyneenä lukumääränä, sekä mikroglian sooman koon kasvamisena.

Menetelmät

Hiiriä käsiteltiin hellävaroen yhdensän tunnin ajan, jotta ne valvoisivat päivän ja siten mallintaisivat akuuttia unettomuutta. Kroonista katkonaista unta taas mallinnettiin hyödyntäen erityistä häkkiä, jossa tanko liikkui häkin läpi kahden minuutin välein havahduttaen hiiret unestaan 14 peräkkäisenä päivänä. Osa hiiristä sai toipua katkonaisesta unesta nukkumalla rajoittamattomasti kolmen vuorokauden ajan. Hiiriä käytettiin, jotta aivonäytteitä voitaisiin kerätä. Kummankin unettomuuden mallin jälkeen eläimet lopetettiin ja perfusoitiin. Aivonäytteet otettiin ventraalisesta hippokampuksesta. Mikroglia värjättiin immunohistokemiallisesti ja kuvattiin konfokaali-mikroskoopilla. Mikroglian haarakkeiden ramifikaatio ja sooman koko kvantifioitiin piirtämällä haarakkeet ja segmentoimalla soomat kuvista.

Tulokset

Akuutti unettomuus eikä krooninen unen katkonaisuus aiheuttaneet mikrogliassa morfologisia muutoksia kontrolliryhmiin verrattuna. Yllättäen sooman koko oli merkittävästi pienempi kroonisen katkonaisen unen ja sitä seuraavan toipumisunen jälkeen kontrolliryhmään verrattuna.

Johtopäätökset

Mikroglian morfologia ja siten toiminta ei ole välttämättä muuttunut ventraalisessa hippokampuksessa akuutissa unettomuussa ja kroonisessa katkonaisen unessa. Mikroglian sooman koko oli merkittävästi pienempi kroonisen katkonaisen unen ja siitä toipumisen jälkeen kontrolliryhmään verrattuna. Tämä voi johtua siitä, että tässä kontrolliryhmässä mikroglian sooman koko oli suurempi kuin muissa kontrolliryhmissä, joten jatkotutkimuksia tarvitaan vaikutuksen varmentamiseksi.

Avainsanat – Nyckelord – Keywords
Microglia, Acute sleep deprivation, Chronic fragmented sleep, Recovery sleep, Morphology

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
Iiris Hovatta, Sarah Steffens

Säilytyspaikka – Förvaringställe – Where deposited
E-thesis

Muita tietoja – Övriga uppgifter – Additional information

TABLE OF CONTENTS

Abbreviations

EEG	Electroencephalolography
IL-1β	Interleukin 1 Beta
LPS	Lipopolysaccharide
NREM	Non rapid eye movement
REM	Rapid eye movement
S1 - S4	Sleep stages 1 - 4
SWA	Short wave activity
TLR	Toll-like receptors
TNFα	Tumor necrosis factor alpha

INTRODUCTION

Sleep has a crucial role in regulating and maintaining homeostatic state with regards to energy metabolism, inflammation, and neuronal plasticity (Porkka-Heiskanen et al., 2013). Furthermore, sleep and mental health are interconnected in many important ways. Psychiatric patients experience more sleep disturbances, but the effect has been suggested to be bidirectional – worse sleep quality can also be a risk factor for mental health disorders (Krystal, 2012). Anxiety disorders and major depression are the most common diagnoses associated with insomnia (Monti & Monti, 2000).

What is sleep?

Sleep is a behavioral state which is defined by reduced mobility and an increased sensory threshold (Wigren & Porkka-Heiskanen, 2018). Beyond the behavioral signs, the vigilance state can be measured with electroencephalography (EEG). EEG allows to identify and to divide the sleep into non-rapid-eye-movement (NREM) and rapid-eye-movement (REM) sleep, and to further divide the NREM sleep into three stages (S1, S2 and S3) of increasing order of slow wave activity (SWA). SWA refers to low-frequency, high-amplitude waves. In REM sleep, EEG is characterized by low-amplitude and high-frequency waves similar to waking, but the muscle tone is lost. During sleep, these stages alternate in 90 minutes cycle starting from the S1, proceeding through stages S2 and S3 into REM sleep (Wigren & Porkka-Heiskanen, 2018). Both REM and NREM sleep are important for memory consolidation (Peigneux et al., 2001). REM sleep is also important for integrity of blood-brain barrier, as REM sleep restriction increases the blood-brain barrier permeability (Gomez-Gonzalez et al., 2013).

Sleep is regulated by homeostatic and circadian processes (Borbély et al., 2016). The homeostatic aspect of sleep regulation depends on the sleep and waking states. When the wakefulness prolongs, the sleep pressure or sleep need builds up and induces sleep (Porkka-Heiskanen et al., 2013). According to the sleep factor theory, there are substances such as adenosine that accumulate in the brain during wakefulness and inhibit brain activity, and again decrease during sleep (Porkka-Heiskanen, 1999). The amount of SWA in the EEG of NREM sleep is a reliable marker for sleep need (Tononi & Cirelli, 2014). The other aspect of sleep regulation - the circadian process – is independent of sleep and waking states. Instead, various circadian oscillations that are dependent on the time of the day are drivers of this

process, producing more sleepiness during the night and less during the day (Borbély et al., 2016).

Sleep deprivation experiments can help trying to disentangle what happens during sleep. Much of what is known about why we sleep comes from these studies (Porkka-Heiskanen, 2013). Various experimental models of insufficient sleep are used to gain insights on what happens in the brain and the body during acute or chronic sleep deprivation. Acute, or total, sleep deprivation refers to a lack of sleep for 24 hours or more from the time of awakening. In partial sleep deprivation, the sleep time is only reduced. Applied for several subsequent nights, it becomes chronic insufficient sleep (Mullington et al, 2010). Beside sleep loss, fragmented sleep is a sleep phenotype that has been associated with day-time sleepiness and cognitive dysfunction in human (Martin et al., 1996) and in rodents (McCoy et al., 2007; McKenna et al., 2007; Ramesh et al., 2012). In fragmented sleep, short arousals disrupt the sleep. This occurs for example in obstructive sleep apnea (Nadjar et al., 2017). Fragmented sleep increases the delta power in NREM during subsequent recovery sleep, indicating that fragmented sleep induces a homeostatic sleep need in rats (McKenna et al., 2007). Delta waves are high amplitude waves seen in EEG recordings, and power is the square of the magnitude of these waves. Delta power is associated with the depth of the sleep, and an increase in delta power shows recovery sleep after sleep deprivation.

Different research methods can be utilized when studying sleep in human and in animals. To study the effects of insufficient sleep in human, EEG can be used to mark changes in sleep architecture or blood samples for instance to see changes in inflammatory markers. With the use of positron emission tomography, the glucose uptake and thus brain metabolism levels (Wu et al., 1996)) has been studied in human after sleep deprivation. However, in human studies brain samples cannot be collected. With the use of animals, such studies can be conducted, and the sleep and wake can be experimentally studied on cellular and molecular level (Rihel & Schier, 2013). Mice are a common model organism in the biomedical field, and in sleep studies (e.g. Bellesi et al., 2017; Meetu et al. 2017; Ramesh et al., 2012). Even non-mammalian organisms such as drosophila can be used in sleep research for example in the study of circadian rhythm, which is regulated by only a few genes that are conserved across species (Zimmerman et al., 2008). The neuroanatomy and neural circuitries of non-mammalian model organisms however considerably differs from the human brain, which presents a major pitfall to their usage in sleep research (Zimmerman et al., 2008). Therefore, the current study utilises mice. Nevertheless, it is to be noted that in contrast to human, mice

are nocturnal animals and thus sleep during the day and are wakeful during the night. Furthermore, mice do not display the substages of S1 to S4 during NREM which are seen in human, although recent research have identified substages in mice that could be human-like (Lacroix et al., 2018).

The neural circuitry regulating sleep and wakefulness

One of the most striking features of sleep is **the reversible disconnection** from the environment. The disconnection is induced by thalamus, which acts as a gate of sensory information to the cortex (Porkka-Heiskanen et al., 2013). In this state, the sleeper does not respond to external stimuli. Periodic high frequency bursts of action potentials in thalamic relay neurons are associated with NREM sleep, while tonic depolarization of these neurons results in a suppression of these bursts and in a wakeful or REM sleep state (McCormick & Bal, 1994).

The ascending arousal system, or the waking system, consists of nuclei in the brain stem, hypothalamus and basal forebrain, and can be divided into two major branches (Saper et al., 2005). The first branch ascends mainly from pedunculopontine and laterodorsal tegmental nuclei in the brainstem to the thalamus, and is responsible for activating the relay neurons, maintaining the transmission between the thalamus and the celebral cortex; and the second branch is a pathway originating from upper brain stem and caudal hypothalamus, which activates neurons in the hypothalamic area, basal forebrain and cerebral cortex. In rats, lesion in this pathway increased the amount of REM and NREM sleep, and rendered the sleep more fragmented (Gerashchenko et al., 2003).

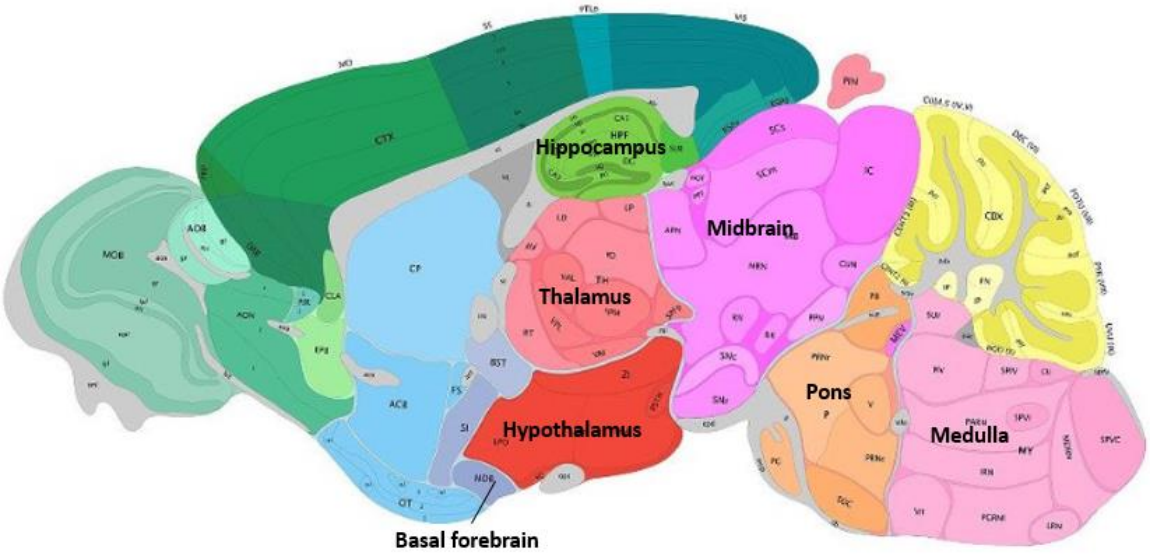
The ventrolateral preoptic area and **the median preoptic nucleus** are small nuclei in hypothalamus that are more active during NREM sleep than waking, and these nuclei are called sleep nuclei (Porkka-Heiskanen et al., 2013). During sleep, they inhibit the arousal system, whereas the arousal system inhibits the sleep nuclei during waking, creating a self-reinforcing loop (Saper et al., 2005). This loop, termed **flip-flop circuit**, is thought to explain why transitions between waking and sleep are rapid.

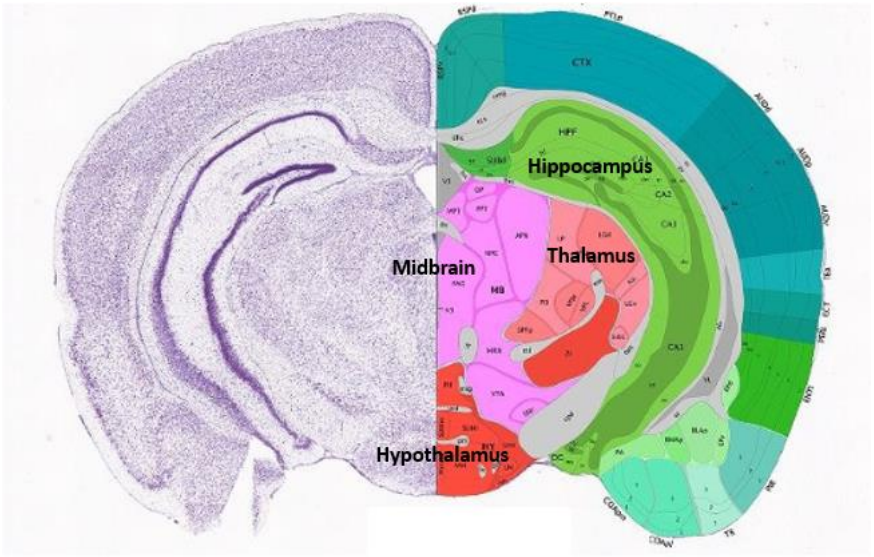
Hippocampus is not a part of the regulatory system of the wakefulness and sleep, however, it is of importance for the sleep-dependent memory consolidation. According to the active system consolidation hypothesis of the two-stage memory system, the events are initially encoded parallelly in the hippocampus and neocortical networks. Subsequently by repetitive re-activation during slow wave sleep the memory traces are redistributed, and the neocortical

connections are strengthened (Diekelmann & Born 2010). Besides system consolidation, the memory representations are thought to be strengthened on a synaptic level, for which long-term potentiation induced by hippocampus during REM sleep is crucial (Diekelmann & Born 2010). Furthermore, the synaptic homeostasis hypothesis proposes that the reason we sleep is to maintain the synaptic plasticity in the brain (Tononi & Cirelli, 2014). According to this hypothesis, wakefulness strengthens connections throughout the brain, but the net synaptic strength is normalized during sleep, which is required for learning.

Ventral hippocampus is a distinct region in its function (Fanselow & Dong, 2010) and its connections to other regions (Swanson and Cowan, 1977). Whereas dorsal hippocampus is associated with cognitive functions such as spatial memory, ventral hippocampus is linked with emotions. Ventral hippocampus projects into the amygdala, and this pathway has been implicated in fear learning (Fanselow & Dong, 2010). The amygdala–hippocampal network is also involved in the emotional processing and in the memory consolidation of emotional material that occurs during sleep. The "Sleep to Forget and Sleep to Remember" hypothesis proposes that during REM sleep, the activation of the amygdala–hippocampal network supports long-term retention of informational aspects of the emotional memory (Walker & van Der Helm, 2009). According to the model, the emotional charge associated with the emotional experience is instead decoupled from the memory, and then forgotten. Nevertheless, the topic is still a matter of discussion, and an opposite view states that the emotional charge along with the emotional memory would be consolidated during REM sleep (Tempesta et al., 2018). Maladaptive consolidation of the memory and its emotional charge during REM sleep is thought to be implicated in dysfunctional emotional memories such as in post-traumatic stress disorder (Murkar & De Koninck, 2018).

A)





B)

Figure 1. Sagittal (A) and coronal (B) sections on the mouse brain showing the brain regions important in sleep and wakefulness. Midbrain, pons and medulla form together brainstem. Pictures are created based on The Allen Mouse Brain Atlas (© 2010 Allen Institute for Brain Science. Allen Mouse Brain Atlas. Available from: https://mouse.brain-map.org).

How does insufficient sleep affect the inflammatory system?

Many sleep inducing factors have proinflammatory properties when occurring in bigger amounts. Numerous proteins that are associated with immune system have been seen to affect sleep (Imeri & Opp, 2009). These proteins are called cytokines, and they are involved in the

activation and regulation of the immune system (Hiscott & Ware, 2011). Many cytokines have multiple functions, and they can act on several receptors. Interleukin 1 Beta (IL-1β) and tumor necrosis factor alpha (TNFα) in particular have been studied for their sleep factor - like properties (Imeri & Opp, 2009). Interleukins are cytokine proteins that modulate immune responses and can regulate the differentiation and proliferation of certain immune cells (Shebert, 2011). TNFα is secreted by activated macrophages, and it can induce regulated cell death, apoptosis (Shebert, 2011). In the brain, inflammatory cells called microglia both release and receive cytokines as part of their communication with other cells (Hanisch et al., 2002). Cytokines from the peripheral immune system can also pass the blood brain barrier and enter the central nervous system, which can occur excessively in pathological states (Hanisch et al., 2002).

After various durations of sleep restriction, sleep loss (Mullington et al, 2010) and fragmented sleep (Ramesh et al., 2012) has been seen to increase the levels of acute inflammatory system markers, including IL-1 beta and TNF-alpha. Acute phase response is the early activation of an immune cascade, which is mediated by the toll-like receptors (TLRs). TLRs are expressed and functional also in the central nervous system (Hanke & Kielian, 1979). TLRs recognize molecular patters of micro-organisms, and by activating the nuclear-factor kappa-beta (NKfB) stimulating gene transcription, inflammatory cytokines are produced (Takeuchi & Akira, 2010). Injection or stimulation of endogenous production of IL-1β or TNFα enhances NREM sleep, whereas inhibiting either one reduces the spontaneous sleep (Krueger, 2008). Furthermore, intracerebroventricular injection of IL-1 β inhibitor reduces the amount of NREM sleep after sleep deprivation in rats (Opp & Krueger, 1994). It is unclear whether the changes in the inflammatory cytokine levels are sustained after recovery sleep following fragmented sleep; however, after chronic sleep restriction and two nights of recovery sleep several pro-inflammatory markers have been reported to remain elevated (van Leeuwen et al., 2009).

Microglia

Microglia are the resident phagocytic cells of the central nervous system (Kettenman et al., 2011). Microglia are derived from mesodermal tissue, and their progenitors take up residence in the nervous system during embryonic and fetal development (Chan et al., 2007). Microglia enter all brain regions and are found ubiquitously in the mature brain, each cell occupying a defined, non-overlapping territory (Kettenman et al., 2011). Microglia in the healthy nervous

system were named to be resting as microglia's primary function was thought to be gained in response to an inflammatory challenge. However, the resting microglia has turned out to be highly motile (Nimmerjahn et al., 2005). Microglia constantly monitor the surrounding brain parenchyma with their dynamic branches (Nimmerjahn et al., 2005). In response to a disruption in tissue homeostasis, microglia adopt an activated state (Hanisch & Kettenmann, 2007). This state can be modelled with an inflammatory stimulant, such as lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria (Fan et al., 2015). This pro-inflammatory activated state is characterized by morphological changes such as an enlarged soma size and deramification, which means decreased number of microglial branches. These morphological changes are tightly coupled with a change in the microglial functional state. Therefore these changes are often used in research to characterize the functional changes in microglia (Kettenmann et al., 2007). Upon activation, microglia release inflammatory signaling molecules and cytotoxic factors to destroy the invading pathogens (Lull & Block, 2010). Once the activated state is evoked, microglia can remain activated for a considerable period of time, even up to months (Qin et al., 2007).

Besides the immune response, microglia have a plethora of other functions such as clearance of debris, and regulation of neuronal death and survival (Tay et al., 2017). They also partake in neuronal spine formation (Parkhurst et al., 2013) and synaptic pruning during the development (Paolicelli et al., 2011) and in mature brain (Ikegami et al., 2019). Microglial branches contact synapses and sense neural activity; these contacts are dependent on the neural activity and are prolonged after a disruption to the homeostatic state in the brain (Wake et al., 2009). Furthermore, the branches establish contacts with neuronal somas, preferably neurons with high spontaneous activity (Li et al., 2012). A spontaneous activity decrease was observed after microglial interaction, suggesting that microglia can have an inhibitory influence on neuronal excitability (Li et al., 2012).

Microglial activation in response to insufficient sleep

Chronic activation of the microglia is a common pathological feature in neurodegenerative disorders (Hickman et al., 2018). In diseases such as Alzheimer's, the initially useful microglia progress into deleterious drivers of the neuronal damage (Hickman et al., 2018). Recently, it has been recognised that microglial dysfunction may contribute to the pathophysiology of the sleep disorders, potentially mediating the detrimental consequences of

sleep loss via neuronal circuit remodeling or a loss of physiological functions (Nadjar et al., 2017).

Recent studies in mice have shown that chronic sleep reduction, although not acute sleep deprivation, can induce morphological signs of microglial activation, indicated by significant reduction in the microglial process length and in the counts of well ramified cells compared with control mice (Bellesi et al., 2017; Wadhwa et al. 2017). Furthermore, microglia were expressing up-regulated levels of complement type 3 receptors in rats' hippocampus following 5 days long sleep deprivation (Hsu et al., 2003). Complement type 3 is a crucial part of the complement system, which is a first line defense in the peripheral immune system as well as in the brain, where the complement system acts as a rapid and local immune surveillant (Stephan et al., 2012).

Aim of the thesis

The current study aimed to characterize the microglia's morphological changes in the ventral hippocampus after acute sleep deprivation, chronic fragmented sleep and a recovery sleep after fragmented sleep. Specifically, I measured branch length, branch number and soma area after fragmented sleep in mice to address this aim.

The hypotheses were:

1. Fragmented sleep is expected to be associated with shorter microglial branch length, lower branch count, greater soma volume and thicker branches compared with the control.

2. Acute sleep deprivation is not expected to be associated with the above-mentioned morphological changes.

3. After three days of recovery sleep following fragmented sleep, the microglia is expected to show an incomplete transformation back to their ramified, resting state.

METHODS

I participated in taking care of the animals and in conducting acute sleep deprivation. I did the immunostaining for a part of the samples. I set up the protocol for automatically segmenting the microglial somas and validated it against manual segmentations that were also done by me. I automatically segmented the somas and semiautomatically traced the microglial branches, and then conducted all the statistical analyses. Rest of the work involving the data preparation was done by my supervisor Sarah Steffens.

Animals

Thirty-eight male C57BL/6J mice, age ranging from 8 to 12 weeks and with a bodyweight of 30±4 g, were purchased from Envigo, Netherlands. The mice were housed in groups in a 12 h light/dark cycle (lights on 8 am - 8 pm) and at a temperature (21 - 23°C). Up to four animals shared cages in which food and water were provided *ad libitum*. All efforts were made to minimize animal suffering and to reduce the number of animals used (Animal license number by the Provincial Government of Southern Finland: ESAVI/5752/04.10.07/2017).

Treatment conditions

There were four experimental groups accompanied with appropriate control groups (Figure 1).

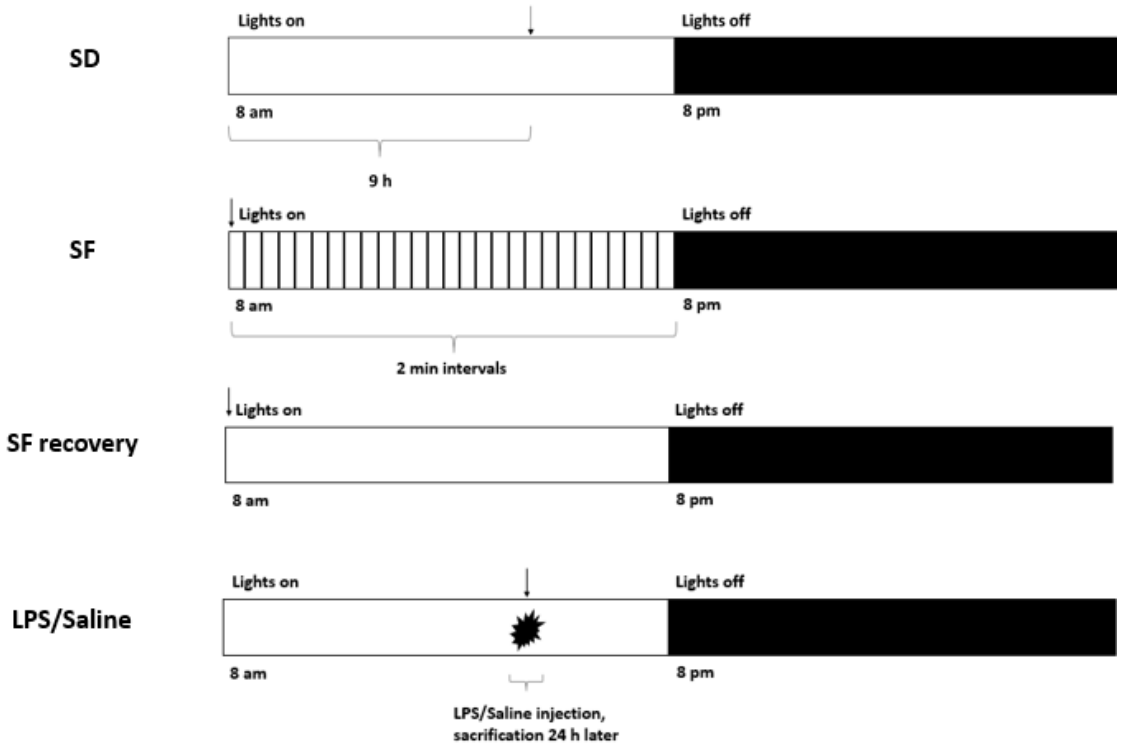


Figure 1. The timelines of treatment groups. Arrows indicate the timepoint of sacrification. SD = Acute sleep deprivation; SF = Sleep fragmentation; SF recovery = Recovery after sleep fragmentation; LPS = Lipopolysaccharide.

To set a biological control of microglia activation, a group of mice was intraperitoneally injected with 5mg/kg LPS (Qin et al., 2007; Verdonk et al., 2016) from *Escherichia coli* (055:B5, Sigma, USA; diluted in 0.9% saline) and another group was injected intraperitoneally with 0.9 % saline solution. LPS causes a peripheral inflammation, which also activates microglia as there is communication between the peripheral immune system and the neuroimmune system (Fan et al., 2015; Qin et al., 2007). The brain tissue was collected 24 h after the injections. The LPS group was compared with the saline group that underwent the same process of injection with the vehicle (saline).

Another group of mice underwent acute sleep deprivation for 9 h during the lights-on phase, which is resting time for these nocturnal animals, and the control mice were kept in similar conditions other than that they were not disturbed. For the experimental group, the acute sleep deprivation was initiated at the beginning of the lights-on period at 08:00 am by introducing novel objects into their cage, which keeps the animals awake without forcing activity (Franken et al., 1991). Mice that were falling asleep regardless of the experimental settings were gently poked with a painter's brush by the investigator. The brain tissue of the

experimental and the control group were collected immediately after the acute sleep deprivation phase.

Chronic sleep fragmentation was conducted for two weeks with a sleep fragmentation chamber (Model 80391, Lafayette Instruments, USA) as in previous studies (Hakim et al., 2015; Kaushal et al., 2012). A sweeper arm crossed the cage close to the ground every two minutes during the lights-on period, which aroused the mice briefly and forced them to climb over the arm. The use of a sleep fragmentation chamber minimizes the animal's activity and stress compared with inducing fragmented sleep by human interaction (Balcombe et al., 2004). The control animals were housed in similar cages, but the sweeper arm was turned off and the animals were thus undisturbed. To adapt the animals to the novel cage before the sleep fragmentation experiment, they were housed for two days in the sleep fragmentation chamber with the sweeper arm turned off and for one further day with the sweeper arm crossing the chamber once in every twenty minutes. The brain tissue was collected at the end of the last lights-off period.

Chronic sleep fragmentation with a recovery period was performed the same as the chronic sleep fragmentation, but it was followed with three days when the sweeper arm was switched off and the animals were undisturbed to allow recovery sleep. The brain tissue was collected at the end of the last lights-off period.

Tissue Preparation

Before the tissue collection, the animals were anesthetized with Pentobarbital (120 mg/kg, Mebunat, Orion, Finland) and perfused with phosphate-buffered saline (PBS, pH 7.4). Then the tissue was preserved by the perfusion with 100 ml paraformaldehyde (PFA) per animal.

The tissue was fixed for 24 h in 4% PFA in PBS and cryoprotected in 20% sucrose solution and frozen down to -80 degree Celsius. The tissue samples were sliced on coronal plane with a cryostat (Leica CM3050 S) into 35 μm thick slices (bregma -50 to –70 mm) and placed in 0.01 M tris-buffered saline solution (with 0.05 % Tween-20, TBS-T).

Immunohistochemistry

The tissue samples were first washed three times in 0.01 M TBS-T for 10 min. The samples were then incubated for 90 min at room temperature in 10 % normal goat serum (NGS) in 0.001 M TBS-T to block unspecific binding. Thereafter, the samples were incubated overnight at +4 degrees Celsius with primary antibody (anti-Iba-1; rabbit; Synaptic Systems,

Germany, resolved 1:2000 in TBS-T), which stains the ionized calcium binding adaptor 1 protein (IBA-1) that is localised specifically in microglia (Ito et al., 1998). The second day started by washing the samples three times in 0.01 M TBS-T for 10 min. This was followed by a 2 h light-protected incubation at room temperature with the secondary antibody (anti-rabbit; 1:500; Alexafluor568; Invitrogen; Lot: 1670154), which binds to the primary antibody and gives the samples a fluorescent red colour. The three washing steps were then repeated, and then the slices were mounted onto uncoated specimen slides (Superfrost Ultra Plus, Thermo Scientific, Germany), dried at approximately 40 degrees Celsius, and then cover slipped.

Image Acquisition

A confocal microscope (SPX 8, Motorized DMi8 inverted microscope, Leica, Germany) with a 63x magnification oil objective and controlled by LAS X software was used to image samples on the ventral hippocampus. Confocal microscope allows to gather a z-stack series of images of the sample (image resolution 1024 x 1024 pixels; pixel size: 0.24 x 0.24 microns; step size 0.20 microns; 33-79 z-layers per slice depending on the tissue quality) and thus the three-dimensional form of the microglia could be captured.

Morphometric Analysis

The morphometric features were analysed with Fiji ImageJ 1.51. Using the Simple Neurite Tracer plugin, microglial processes were semi automatically traced and the skeletonised paths analysed. The traces were drawn starting from a common point in the centroid of the soma, reaching to the furthest possible route along microglial process. These traces were then complemented with their branches, until all the visible branches were traced (see Figure 1A). The number of branches was calculated in a way that is sensitive for ramification. If a new branch emerged from a branch, the original branch was divided into two branches, one reaching from the beginning of the original branch to the starting point of the new branch, and the other starting from the new branch and ending where the original branch ends. The average length of these branches describes the mean of the branch length per cell, and together with branch count define the degree of ramification of the microglia. Microglia that were centred to fit the image in their full length were considered for tracing.

The soma sizes were measured with an automatic script adapted from Salinas-Navarro et al., (2017) from maximal projections that were created with a script by Hoehne (2015) (see Figure 1B and 1C). These measures were validated against manually segmented soma

volumes, which showed a high positive correlation tested with Pearson's correlation coefficient (Figure 2).

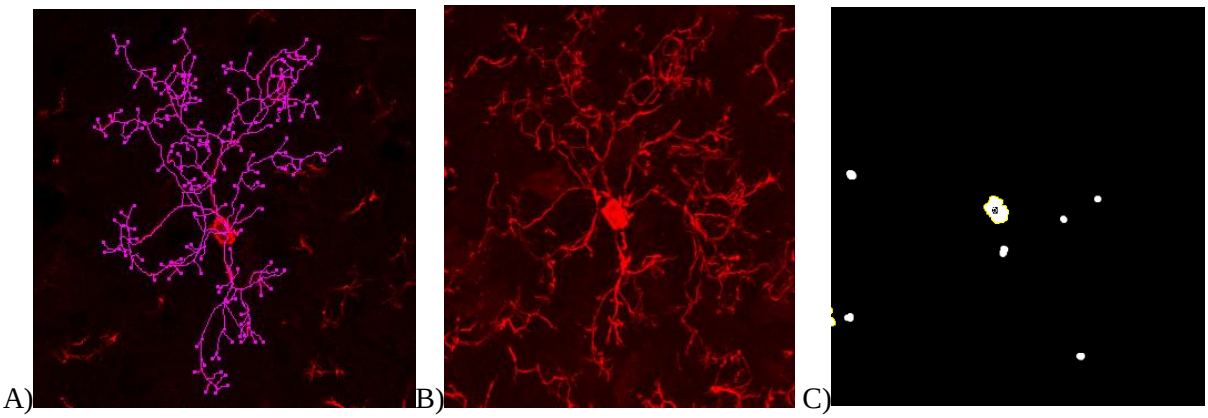


Figure 2. Traces (A), a maximal projection (B) and the soma area (C) of a microglial cell from a control group.

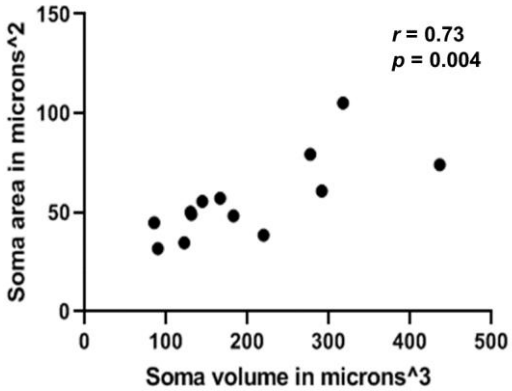


Figure 3. A correlation between manually segmented soma volumes and automatically segmented soma areas in somatosensory cortex. Somatosensory cortex was used because the manual soma volume segmentations were available from this region.

All the available confocal microscopy pictures were used to extract the soma areas. The automatically segmented areas were manually confirmed to be somas. Only somas that were fully seen in the picture were selected. The semiautomatic branch measurements were balanced to include a range of 2 to 5 animals per group. The number of pictures and animals used for each measurement per group are shown in table **1.**

Table 1. The data structure of the treatment groups.

	Soma area			Branches		
	Animals	Pictures	Cells per animal	Animals	Pictures	Cells per animal
	N	N	Mean [Min;Max]	N	N	Mean [Min;Max]
SD	4	10	9.00 [12;42]	4	8	2.50 [1;4]
SD ctr	3	8	21.33 [12;33]	2	6	5.00 [4;6]
SF	5	16	30.40 [7;32]	4	10	2.50 [2;3]
SF ctr	7	21	22.00 [13;33]	5	9	2.00 [1;3]
SF rcv	5	15	20.20 [15;29]	NA	NA	NA
SF rcv ctr	5	11	19.80 [4;33]	NA	NA	NA
LPS	7	21	23.14 [10;38]	5	10	2.00 [1;3]
Saline	2	5	25.50 [14;37]	2	6	5.00 [5;5]

Note. Min = Minimum; Max = Maximum. SD = Acute Sleep Deprivation, SD ctr = Acute Sleep Deprivation Control, SF = Sleep Fragmentation, SF ctr = Sleep Fragmentation control, SF rcv = Sleep Fragmentation with Recovery Sleep, SF rcv = Sleep Fragmentation with Recovery Sleep Control, LPS = Lipopolysaccharide.

Statistical Analyses

Statistical analyses were performed with SPSS (IBM SPSS statistics 25). Generalised estimating equations (GEE) were used to compare the treatment groups while considering that the microglia measurements coming from the same mouse may have had shared variance. Visual inspection and the Shapiro-Wilk test were used to test the normality of the data. All data were normalised with logarithmic transformations. When three comparisons per treatment group were made, the threshold for statistical significance was set with Bonferroni correction to $p < 0.017$.

RESULTS

Descriptive statistics

The effects of insufficient sleep on microglia morphology were investigated. The morphology of the microglia was measured by the soma size, number of the microglial branches and mean branch length. These features were studied after acute sleep deprivation, chronic fragmented sleep, and LPS injection, along with the appropriate control groups. The soma size was measured also after recovery sleep following the chronic fragmented sleep. The mean and standard errors of the mean are shown for the soma area, number of branches and mean branch length per treatment group in table **2**.

Table 2. Descriptive statistics on the morphological features of the treatment groups.

	Soma area		Number of branches		Mean branch length	
	Mean (± SEM)	Number of Cells	Mean (± SEM)	Number of Cells	Mean (± SEM)	Number of Cells
SD	37.02 (± 1.75)	90	202.40 (± 25.67)	10	4.45 (± 0.29)	10
SD ctr	33.11 (± 2.06)	64	164.20 (± 16.48)	10	4.62 (± 0.21)	10
SF	39.47 (± 1.76)	152	141.00 (± 33.60)	10	4.25 (± 0.24)	10
SF ctr	33.83 (± 1.33)	154	183.30 (± 27.66)	10	4.86 (± 0.54)	10
SF rcv	32.51 (± 1.46)	101	NA	NA	NA	NA
SF rcv ctr	35.55 (± 1.41)	99	NA	NA	NA	NA
LPS	42.44 (± 1.64)	162	181.10 (± 23.06)	10	4.24 (± 0.27)	10
Saline	32.79 (± 2.26)	51	208.50 (± 23.82)	10	4.01 (± 0.19)	10

Note. SEM = Standard error of the mean. NA = Not available. SD = Acute Sleep Deprivation, SD ctr = Acute Sleep Deprivation Control, SF = Sleep Fragmentation, SF rcv = Sleep Fragmentation with Recovery Sleep, SF rcv = Sleep Fragmentation with Recovery Sleep Control SF ctr = Sleep Fragmentation control, LPS = Lipopolysaccharide.

Treatment effects

The soma size, number of the microglial branches and mean branch length were compared in acute sleep deprivation, chronic fragmented sleep, recovery following the chronic fragmented sleep and LPS injection to the appropriate control groups. Pairwise comparisons of mean differences between the experimental group and the appropriate control group were tested with GEEs.

There were no significant changes in the morphological features after acute sleep deprivation or fragmented sleep (tables 4 and 5).

Table 3. Generalised estimating equations for morphological differences between acute sleep deprivation and control groups.

	Treatment effect				
Outcome variable	*Mean Difference*	*95% Wald CI*	*Std. Error*	*df*	*p-value*
Number of branches	0.19	[-0.08; 0.46]	0.14	1	0.17
Mean branch length	-0.047	[-0.14; 0.49]	0.049	1	0.34
Soma area	0.15	[0.037; 0.34]	0.1	1	0.12

Note. Wald CI = Wald confidence interval, Df = degrees of freedom; Std. error = Standard error, p < .05.*

Table 4. Generalised estimating equations for morphological differences between fragmented sleep and control groups.

	Treatment effect				
Outcome variable	*Mean Difference*	*95% Wald CI*	*Std. Error*	*df*	*p-value*
Number of branches	0.21	[-0.13; 0.55]	0.18	1	0.23
Mean branch length	-0.11	[-0.30; 0.089]	0.099	1	0.29
Soma area	0.16	[-0.036; 0.35]	0.1	1	0.11

Note. Wald CI = Wald confidence interval, Df = degrees of freedom; Std. error = Standard error, p < .05.*

The soma area was significantly greater in the LPS treated microglia compared with saline treated microglia. However, the number of branches or the mean branch length were not significantly different between the LPS and saline treated microglia (table 3).

Table 5. Generalised estimating equations for morphological differences between LPS and saline treatment.

	Treatment effect				
Outcome variable	*Mean Difference*	*95% Wald CI*	*Std. Error*	*df*	*p-value*
Number of branches	-0.177643	[-0.56; 0.23]	0.21	1	0.39
Mean branch length	0.049	[-0.08; 0.19]	0.07	1	0.48
Soma area	0.25	[0.11; 0.39]	0.072	1	0.001**

*Note. Wald CI = Wald confidence interval, Df = degrees of freedom; Std. error = Standard error, LPS = Lipopolysaccharide. ** p < .01.*

The tracing of the branches was not completed for the fragmented sleep with recovery sleep groups. As the branch features were unchanged after acute sleep deprivation and chronic fragmented sleep, there was no effect to recover from. The automatic soma area

measurements were however conducted, because there was an insignificant 13.27% increase in the soma area after fragmented sleep.

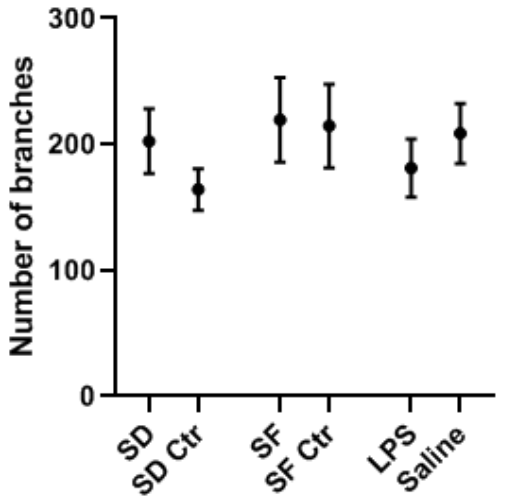
The soma area showed a significant 8.55 % decrease after fragmented sleep with recovery period compared with its control group (table 6).

Table 6. Generalised estimating equations for morphological differences between fragmented sleep with recovery and control group.

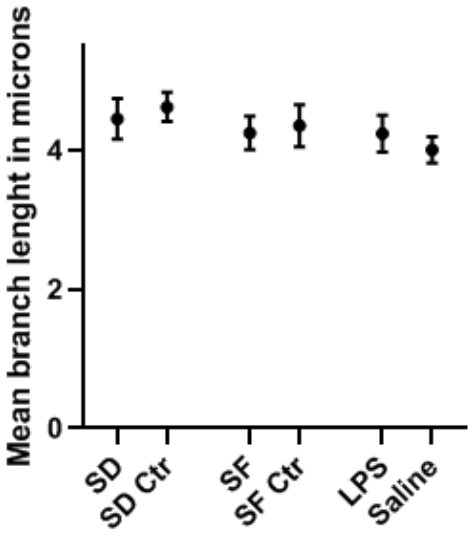
	Treatment effect				
Outcome variable	*Mean Difference*	*95% Wald CI*	*Std. Error*	*df*	*p-value*
Soma area	-0.11	[-0.21; 0.017]	0.0.050	1	0.022*

Note. Wald CI = Wald confidence interval, Df = degrees of freedom; Std. error = Standard error, p < .05.*

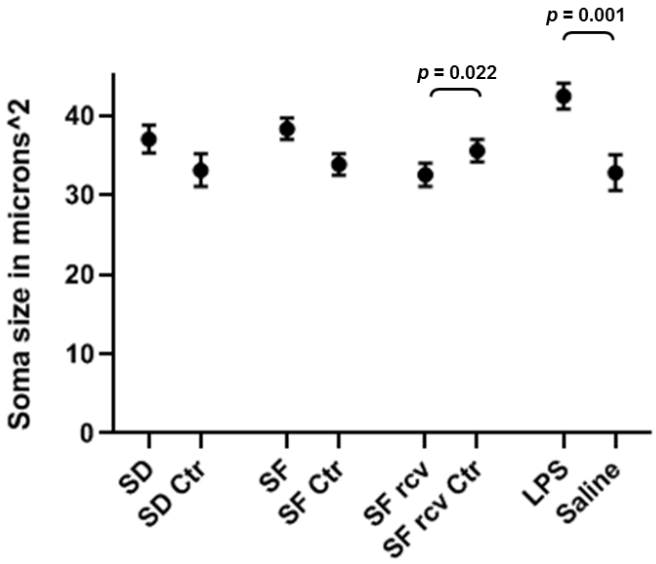
The means and the standard errors of the mean are plotted (Figures 2A, 2B and 2C) to allow visual estimation of the morphological differences between the experimental group compared with the appropriate control group.



A)



B)

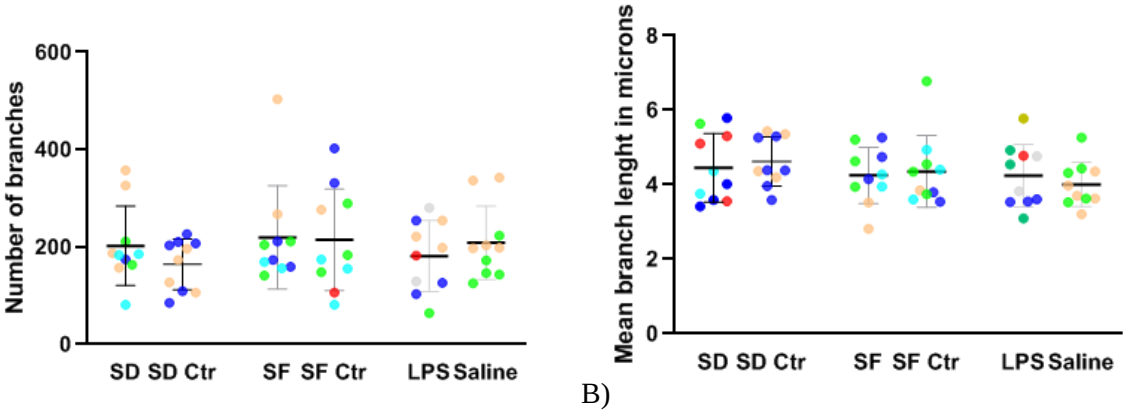


C)

Figure 2. The means of the number of branches (A) and mean branch length (B) and soma area (C) plotted with standard errors of the mean for each of the groups.

Animal effect

Several microglia from one animal were used to extract the morphological features. As such, there could be variation related to the animals within the treatment groups. The individual microglia cells were colour coded according to the mice (Figure 3). The cells from the same animals did not cluster, which suggests that there are no effects of the animal.



A) B)

Figure 3. The cells plotted and colour coded by the animal.

DISCUSSION

The current study aimed to characterise microglia's morphological response to acute sleep deprivation, chronic fragmented sleep and to recovery sleep following fragmented sleep. The microglia's morphology was not altered after acute sleep deprivation nor the chronic fragmented sleep in the ventral hippocampus compared with the control groups. Surprisingly, the soma area following the chronic fragmented sleep with a three days long recovery period was significantly smaller, showing an opposing effect compared with the classical activation induced by LPS. The microglial soma area was larger in LPS treated mice in comparison to saline treated mice, which indicates a classical activation state of the microglia. However, the mean branch length and branch count did not differ between the LPS and saline injected microglia. The hypothesised activated state was thus not seen after insufficient sleep nor the LPS injection.

Acute sleep deprivation

We did not find evidence that acute sleep deprivation would cause neuroinflammation indicated by microglial activation in the ventral hippocampus. However, acute sleep deprivation can induce peripheral inflammation as seen by increases in inflammatory markers such as circulating numbers of monocytes and neutrophils (Hurtado-Alvadaro et al., 2013). It has been hypothesized that the peripheral inflammation might be triggered by alterations in stress responses and vascular changes during loss of sleep (Mullington et al., 2010), but the central nervous system could be more resilient to these or other adverse events caused by acute sleep deprivation. Acute sleep deprivation may cause differential effects in the peripheral and neuroinflammatory systems.

The lack of morphological changes after acute sleep deprivation in the ventral hippocampus is in line with a previous study that reported no alteration in the microglia branch length or the number of branch end points after 8 h of sleep deprivation in murine cerebral cortex (Bellesi et al., 2017), a similar sleep deprivation time as in the current study. Together these studies compile that microglia do not alter their branch or soma morphology after acute sleep deprivation in ventral hippocampus or cerebral cortex. Nevertheless, acute sleep deprivation could induce morphological changes in microglia in other brain regions.

After chronic sleep reduction for 5 days previous studies have found a reduction in the microglia branch length and a decrease in the count of ramified cells cerebral cortex in mice (Bellesi et al., 2017) and hypertrophy of microglia in hippocampus in rats (Hsu et al., 2003).

In the study by Wadhwa et al. (2017), microglial ramification and soma area were not altered in the rat hippocampus after 24 hours of sleep deprivation, but in response to 48 hours sleep deprivation, the microglial morphology adopted the activated state characteristics. These changes were coupled with the increase of pro-inflammatory markers. A severe sleep deprivation sustained for several days may thus be required for causing microglial activation in these regions.

Chronic fragmented sleep

The current study suggests that chronic fragmented sleep alone without restriction to the sleep time does not induce changes to the microglia morphology in the ventral hippocampus. In previous studies that have used the same frequency of sleep interruptions as the current study, already 24 hours exposure to fragmented sleep has been associated with sleepiness and cognitive dysfunction in mice (Ramesh et al., 2012) and in rats (McKenna et al., 2007; McCoy et al., 2007). The fragmented-sleep induced cognitive dysfunction and sleepiness seem to require the activation of the inflammatory TNF-α-dependent pathways, as TNF-α double receptor knockout mice or mice treated with TNF-α neutralizing antibody do not exhibit sleepiness or cognitive dysfunction after fragmented sleep (Ramesh et al., 2012). Activated microglia are the primary source for TNF-α release in the brain (Gregersen et al., 2000), and the microglial release of TNF-α can also recruit more microglia (Hanisch, 2002). Despite the role of TNF-α-dependent pathways in the maladaptive effects of fragmented sleep, we found no evidence of the microglial activation. Chronic fragmented sleep may not lead to a fully developed neuroinflammation.

Chronic fragmented sleep with a recovery period

The microglia soma size was significantly smaller in mice that had had recovery sleep following chronic fragmented sleep than in control mice that had not had chronic fragmented sleep. Microglia may reduce their soma size after having been exposed to chronic fragmented sleep and then to an opportunity of recovery sleep. However, the microglia's soma size in the control group is larger compared with the other control groups. This suggests that potentially the effect is driven by an increase in the soma size in the control group instead of a decrease in the recovery sleep group. For instance, various stressors have been reliably seen to activate microglia in the hippocampus (Calcia et al., 2016).

After recovery from sleep restriction, persistent pro-inflammatory changes in the peripheral immune system have been reported in humans (van Leeuwen et al., 2009; Pejovic et al.,

2013), but no studies have yet addressed the recovery from chronic fragmented sleep in the peripheral or neuroimmune system in human or animals. Future studies are needed to determine how microglia respond to recovery sleep following chronic fragmented sleep.

The effects of LPS

The size of microglial cell bodies were significantly larger after LPS injection compared with saline. Enlargement of the microglial cell bodies is associated with classical microglial activation (Ransohoff & Cardona, 2010). However, there were no significant effects seen in the branch measurements associated with deramification after the LPS injection compared with saline injection. LPS, consisting of cell wall components of gram-negative bacteria (Fan et al., 2015), is a powerful stimulus for microglia and a classical model for stimulating inflammation. Its effects on the microglia function and morphology are well documented (Kettenmann et al., 2011; Abd-El-Basset & Fedoroff, 1995). The dosage used in the current study has been seen to result in microglial activation, measured with cathepsin H expression, at the time point used also in the current study, 24 hours after intraperitoneal injection (Fan et al., 2015). Nevertheless, while the current study did not find morphological differences in the microglial branches induced by the LPS treatment in the ventral hippocampus, a significant decrease in average branch length has been seen in the somatosensory cortex and in basal forebrain (Unpublished data). This could be due to regional differences in the microglial activation induced by peripheral inflammation. For instance, cathepsin H expression that has been associated with neuroinflammation, has been reported to be lower in the hippocampus than in the cortex 24 hours after LPS injection (Fan et al., 2015).

The Morphological features

The average soma size was 32.79 ± 2.26 (mean ± SEM) μm^2 in saline injected mice. The automatized soma area measurements were based on the script by Salinas-Navarro et al. (2017), who reported previously an average soma size of 53.10 ± 1.3 (mean ± SEM) μm^2 in naïve murine retina. The microglial cell bodies reactive to the optic nerve crush were previously reported to have an increased size of 74.3 ± 2.7 μm^2 which is also likely to be significantly greater than the average size of the somas in the LPS injected mice (42.44 ± 1.64 μm^2) in the current study. Instead of a difference in the script performance, these results likely reflect regional differences between retinal and hippocampal microglia. In murine hippocampus, average soma sizes of 22.72 ± 2.24 μm^2 and 26.47 ± 1.33 μm^2 in naïve and LPS -treated mice, respectively, have been reported (Verdonk et al., 2016). These

results indicate similar although slightly smaller numbers than in the current study. As the current study used semiautomatic tracing instead of usual automated tracing to reveal morphological changes in branch features on a more sophisticated level, comparison of the branch features with other studies is not straightforward. Also, in the current study the branches were defined differently than in the studies such as Bellesi et al., (2017), making the numbers incomparable. In the current study, the branch length also reflects the ramification, as the further branching cut the primary branch in two. Instead, Bellesi et al. (2017) calculated the whole length of microglia's branches per cell. Nevertheless, the careful visual determination of the branches can be considered reliable even in the absence of other converging measures.

The Strengths and limitations

The current study utilised three different experimental models for insufficient sleep and a recovery from it, along with a biological control for the microglial activation. The study was carefully planned, and all the experimental groups had their own control groups to avoid any bias from factors related to, for instance, the mice's circadian rhythm, housing or age. Moreover, this was the first study to address the morphological response of the microglia to a recovery sleep after chronic fragmented sleep.

Furthermore, a strength of the current study was the high level of detail that could be captured on the semiautomatic tracing of the microglia. Sometimes the activated state is analysed by thresholding the picture and analysing the overall level of thresholded material (Beynon & Walker, 2012) or by contrasting the soma size to the total area occupied by the cell (Bellesi et al., 2017). These approaches offer only a very crude measure of the morphological changes occurring in microglia. A detailed morphological analysis, as done in the current study, may enable to distinguish the morphological and thus the functional state of the microglia from various possible morphological states. Besides the classical deramified state, microglia may become hyper-ramified in response to chronic stress (Hinwood et al., 2012), and also the phagocytic amoeboid state microglia have been proposed to have three different stages – transitional, motile and locomotor type stages (Stence et al., 2001). Furthermore, the characterisation of the morphological changes is of biological importance, as it can thus capture alterations in the microglia that might not be seen, for example, in the cytokine expression level. In response to non-pathological events, microglia have shown morphological signs of activation and hyper-ramification in absence of changes in

inflammatory marker levels after chronic sleep deprivation (Bellesi et al., 2017) and chronic stress (Hinwood et al., 2012), respectively.

However, there is a trade-off between the time and the level of detail in the morphological analysis, and the current study was underpowered to study the variability in microglia's responses. For example, both hyper- and deramified microglia have been observed after ischemia (Morrison & Filosa, 2013), suggesting that the microglial responses to a non-homeostatic state can be diverse. When the responses may vary between the cells, it is of particular relevance not to select only some of the cells for morphological analysis. As such, the future studies could consider fully automated methods for extracting information on the microglia morphology and thus maximising the information gained from the mice used. The measurements could also be more objective, as in manual tracing the researcher may be inclined to choose the most representative cells for the measurement even when the files are blinded.

As another limitation to the current study is that it is not known if and how much the mice slept during the lights-off period during the fragmented sleep. However, previous studies have indicated that the mice subjected to fragmented sleep do not sleep during the night even when permitted (Ringgold et al., 2013; Trammel et al., 2014).

Future directions

The current and previous studies (Hsu et al., 2003; Meetu et al., 2017, Bellesi et al., 2017) point out that microglia's responses to insufficient sleep might peak only after the sleep deprivation has sustained for more than a day, suggesting that the future studies should consider prolonging the sleep deprivation and to further study chronic sleep deprivation. At least 6 days of partial sleep deprivation can also increase the permeability of the blood brain barrier in mice (He et al., 2014). This could potentially alter the integrity of the immune privileged state of the central nervous system and cause microglial activation. Elevated stress might not be a confounding factor even when the sleep deprivation is prolonged, as mice's corticosterone levels have not been reported to be elevated after acute (Hagewoud et al., 2010a) or 4 days partial sleep deprivation (Hagewoud et al., 2010b).

Genome-wide expression profiling is an approach that has been adopted in studying the murine microglia in many neurodegenerative disease models (Wes et al., 2016). While the morphological features are associated with microglia's function, the morphology alone does not reveal how or if the microglia respond to insufficient sleep at transcriptional level. An

unbiased, single cell gene expression study could potentially capture transcriptional changes associated with the morphology and suggest which pathways are involved. This could help in elucidating the functional meaning of the microglia after insufficient sleep, and partly help answering whether the changes are adaptive or maladaptive.

Furthermore, using in vivo two-photon microscopy and thinned-skull preparation, time lapse images on microglia have been achieved transcranially from transgenic mice that were expressing enhanced green fluorescent protein specifically in microglia (Nimmerjahn et al., 2005). Considering the microglia's importance in monitoring synapses (Wake et al., 2009) and increased microglial synaptic phagocytosis after chronic sleep deprivation (Bellesi et al., 2017), a dynamic inspection of both morphological changes and contacts with synapses could be investigated. This could be done if both neurons and microglia were labelled and imaged with two-photon microscopy during sleep deprivation or chronic fragmented sleep. It has been suspected that the sustained neuronal activity and synaptic membrane damage during prolonged wakefulness could be activating signals for microglia after chronic sleep deprivation (Bellesi et al., 2017), which might be seen in such an experiment.

Future studies could also count the numbers of microglia and to assess any differences between regions and treatment groups compared with the controls. A notably higher amount of OX-42 positive cells in hippocampus and dentate gurys has been reported after 5 days chronic sleep restriction compared with the control (Hsu et al., 2003). OX-42 is a marker for complement type 3 receptors, which are expressed in microglia. The higher cell count suggests that either a greater number of microglia were expressing complement type 3 receptors, or that the total amount of microglia were increased. If the numbers of microglia are increased, either migration or microgliosis could be occurring in response to insufficient sleep. Microglia are known to migrate to a site of injury (Kettenman et al., 2011), and microglia can migrate also in cases where the tissue homeostasis is disrupted although direct damage due to injury is not present, such as in Alzheimer's disease where microglia co-localizes with amyloid deposits (Mirzaei et al., 2016). In leech nervous system, microglia can migrate to a site of nerve injury and to be present in almost 6 folded numbers compared with controls at the site of injury within 24 hours after the injury (Morgese et al., 1983) and to start migrating within 15 minutes from the occurrence of injury (McGlade-McCulloh et al., 1989). Similarly in mice, majority of the microglia has been observed to be motile after 1 day from injury, with peak speed bursts beyond 10 μm/min (~600 μm/h) (Carbonell et al., 2005). As such, the dynamics of the microglial migration may be rapid, and selective to only some of

the cells. By including a wide range of brain regions and with the use of e.g. flow cytometry, which can efficiently and reliably sort and calculate cells, potential differences in microglia counts due to migration or microgliosis after insufficient sleep could be screened for.

Conclusions

The acute sleep deprivation nor the chronic fragmented sleep did not induce morphological changes in murine microglia in the ventral hippocampus, suggesting that hippocampal microglia do~~es~~ not respond morphologically to acute sleep deprivation or chronic fragmented sleep. Potentially, only a prolonged sleep deprivation causes a microglial response in the ventral hippocampus (Meetu et al., 2017; Hsu et al., 2003; Bellesi et al., 2017). The microglial responses to acute sleep deprivation could be focused on other brain regions. Microglia in the ventral hippocampus may also not be involved in the maladaptive effects that are associated with the fragmented sleep (McCoy et al., 2007; McKenna et al., 2007; Tartar et al., 2010; Ramesh et al., 2012). The microglia soma size was significantly smaller after recovery sleep following chronic fragmented sleep compared with the control, indicating an opposite effect to the classical microglia activation. However, this control group seemed to have a larger soma size when compared with other control groups, which alternatively could explain the finding. LPS injection caused an enlargement of the microglia soma size, as expected as a sign of classical microglial activation. However, the mean branch length and branch count were not changed upon the LPS treatment. Ventral hippocampus is associated with processing of emotional memories during sleep. Prolonged wakefulness and insufficient sleep may disrupt this processing and change the neuronal activity in the ventral hippocampus. While microglia are active sensors of their surroundings, they might not change their morphology and functional state in ventral hippocampus in response to insufficient sleep.

REFERENCES

Abd-El-Basset, E., & Fedoroff, S. (1995). Effect of bacterial wall lipopolysaccharide (LPS) on morphology, motility, and cytoskeletal organization of microglia in cultures. Journal of neuroscience research, 41(2), 222-237.

Aho, V., Ollila, H. M., Rantanen, V., Kronholm, E., Surakka, I., van Leeuwen,W. M., et al. (2013). Partial sleep restriction activates immune response related gene expression pathways: experimental and epidemiological studies in humans. PLoS ONE 8:e77184. doi: 10.1371/journal.pone.0077184

Aloisi, F. (2001). Immune function of microglia. Glia 36, 165–179.

B. Gomez-Gonzalez, G. Hurtado-Alvarado, E. Esqueda-Leon, R. Santana-Miranda, J. A. Rojas-Zamorano, and J. Velazquez-Moctezuma. (2013). REM sleep loss and recovery regulates blood-brain barrier function," Current Neurovascular Research, vol. 10, no. 3, pp. 197–207.

Balcombe, J. P., Barnard, N. D. & Sandusky, C. (2004). Laboratory routines cause animal stress. Contemp. Top. Lab. Anim. Sci. 43, 42–51.

Bellesi, M., de Vivo, L., Chini, M., Gilli, F., Tononi, G., & Cirelli, C. (2017). Sleep loss promotes astrocytic phagocytosis and microglial activation in mouse cerebral cortex. Journal of Neuroscience, 37(21), 5263-5273.

Bellesi, M., de Vivo, L., Tononi, G., & Cirelli, C. (2015). Effects of sleep and wake on astrocytes: clues from molecular and ultrastructural studies. BMC biology, 13(1), 66.

Borbély, A. A., Daan, S., Wirz-Justice, A., & Deboer, T. (2016). The two-process model of sleep regulation: a reappraisal. Journal of sleep research, 25(2), 131-143.

C. Smith. (1995). Sleep states and memory processes. Behavioural Brain Research, vol. 69, no. 1-2, pp. 137–145.

Calcia, M. A., Bonsall, D. R., Bloomfield, P. S., Selvaraj, S., Barichello, T., & Howes, O. D. (2016). Stress and neuroinflammation: a systematic review of the effects of stress on microglia and the implications for mental illness. Psychopharmacology, 233(9), 1637-1650.

Carbonell, W. S., Murase, S. I., Horwitz, A. F., & Mandell, J. W. (2005). Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. Journal of neuroscience, 25(30), 7040-7047.

Chan, W. Y., Kohsaka, S., & Rezaie, P. (2007). The origin and cell lineage of microglia—new concepts. Brain research reviews, 53(2), 344-354.p

Davis, B. M., Salinas-Navarro, M., Cordeiro, M. F., Moons, L., & De Groef, L. (2017). Characterizing microglia activation: a spatial statistics approach to maximize information extraction. Scientific reports, 7(1), 1576.

Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01.

Späth-Schwalbe, E., Hansen, K., Schmidt, F., Schrezenmeier, H., Marshall, L., Burger, K., ... & Born, J. (1998). Acute effects of recombinant human interleukin-6 on endocrine and central nervous sleep functions in healthy men. The Journal of Clinical Endocrinology & Metabolism, 83(5), 1573-1579.

Everson, C. A. (2005). Clinical assessment of blood leukocytes, serum cytokines, and serum immunoglobulins as responses to sleep deprivation in laboratory rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R1054–R1063. doi: 10.1152/ajpregu.00021.2005

Fan, K., Li, D., Zhang, Y., Han, C., Liang, J., Hou, C., ... & Ma, J. (2015). The induction of neuronal death by up-regulated microglial cathepsin H in LPS-induced neuroinflammation. Journal of neuroinflammation, 12(1), 54.

Fanselow, M. S., & Dong, H. W. (2010). Are the dorsal and ventral hippocampus functionally distinct structures?. Neuron, 65(1), 7-19.

Fonken, L. K., Frank, M. G., Kitt, M. M., Barrientos, R. M., Watkins, L. R., and Maier, S. F. (2015). Microglia inflammatory responses are controlled by an intrinsic circadian clock. Brain Behav. Immun. 45, 171–179. doi: 10.1016/j.bbi.2014.11.009

Franken, P., Dijk, D. J., Tobler, I. & Borbely, A. A. (1991). Sleep deprivation in rats: effects on EEG power spectra, vigilance states, and cortical temperature. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 261, R198–R208.

Gerashchenko, D., Blanco-Centurion, C., Greco, M. A., & Shiromani, P. J. (2003). Effects of lateral hypothalamic lesion with the neurotoxin hypocretin-2–saporin on sleep in Long–Evans rats. Neuroscience, 116(1), 223-235.

Gregersen, R., Lambertsen, K., & Finsen, B. (2000). Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. Journal of Cerebral Blood Flow & Metabolism, 20(1), 53-65.

Hakim, F., Wang, Y., Carreras, A., Hirotsu, C., Zhang, J., Peris, E., & Gozal, D. (2015). Chronic sleep fragmentation during the sleep period induces hypothalamic endoplasmic reticulum stress and PTP1b-mediated leptin resistance in male mice. Sleep, 38(1), 31-40.

Hanisch, U. K. (2002). Microglia as a source and target of cytokines. Glia, 40(2), 140-155.

Hanisch, U.-K. & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 10, 1387–1394.

Hanke, M. L., & Kielian, T. (2011). Toll-like receptors in health and disease in the brain: mechanisms and therapeutic potential. Clinical science, 121(9), 367-387.

Hayashi, Y., Koyanagi, S., Kusunose, N., Okada, R., Wu, Z., Tozaki-Saitoh, H., et al. (2013). The intrinsic microglial molecular clock controls synaptic strength via the circadian expression of cathepsin S. Sci. Rep. 3:2744.

He, J., Hsuchou, H., He, Y., Kastin, A. J., Wang, Y., & Pan, W. (2014). Sleep restriction impairs blood–brain barrier function. Journal of Neuroscience, 34(44), 14697-14706.

Hickman, S., Izzy, S., Sen, P., Morsett, L., & El Khoury, J. (2018). Microglia in neurodegeneration. Nature neuroscience, 21(10), 1359.

Hinwood, M., Tynan, R. J., Charnley, J. L., Beynon, S. B., Day, T. A., & Walker, F. R. (2012). Chronic stress induced remodeling of the prefrontal cortex: structural re-organization of microglia and the inhibitory effect of minocycline. Cerebral cortex, 23(8), 1784-1797.

Hiscott, J., Ware, C., (2011). Cytokines. Curr. Opin. Immunol. 23, 561–563.

Hoehne, M. (2015). Produce max projections from LIF files in imageJ. Retrieved 21.04.2019 from: https://forum.image.sc/t/produce-max-projections-from-lif-files-in-imagej/93/5.

Hsu JC, Lee YS, Chang CN, Chuang HL, Ling EA, Lan CT. (2003). Sleep deprivation inhibits expression of NADPH-d and NOS while activating microglia and astroglia in the rat hippocampus. Cells Tissues Organs. 173(4):242-54.

Hurtado-Alvarado, G., Pavón, L., Castillo-García, S. A., Hernández, M. E., Domínguez-Salazar, E., Velázquez-Moctezuma, J., & Gómez-González, B. (2013). Sleep loss as a factor to induce cellular and molecular inflammatory variations. Clinical and Developmental Immunology, 2013.

Ikegami, A., Haruwaka, K., & Wake, H. (2019). Microglia: Lifelong modulator of neural circuits. Neuropathology.

Imeri, L., & Opp, M. R. (2009). How (and why) the immune system makes us sleep. Nature Reviews Neuroscience, 10(3), 199.

Ito, D., Imai, Y., Ohsawa, K., Nakajima, K., Fukuuchi, Y., & Kohsaka, S. (1998). Microglia-specific localisation of a novel calcium binding protein, Iba1. Molecular brain research, 57(1), 1-9.

Krueger, J. M., Walter, J. A. M. E. S., Dinarello, C. A., Wolff, S. M., & Chedid, L. (1984). Sleep-promoting effects of endogenous pyrogen (interleukin-1). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 246(6), R994-R999.

Kaushal, N., Ramesh, V. & Gozal, D. (2012). TNF-α and temporal changes in sleep architecture in mice exposed to sleep fragmentation. PloS One 7, e45610.

Kettenmann, H., Hanisch, U. K., Noda, M., & Verkhratsky, A. (2011). Physiology of microglia. Physiological reviews, 91(2), 461-553.

Krueger, J. M. (2008). The role of cytokines in sleep regulation. Current pharmaceutical design, 14(32), 3408-3416.

Krystal, A. D. (2012). Psychiatric disorders and sleep. Neurologic clinics, 30(4), 1389-1413.

L. Imeri, M.R. Opp. (2009). How (and why) the immune system makes us sleep. Nature Reviews Neuroscience, (10), 199-210

Lacroix, M. M., de Lavilleon, G., Lefort, J., El Kanbi, K., Bagur, S., Laventure, S., ... & Benchenane, K. (2018). Improved sleep scoring in mice reveals human-like stages. bioRxiv, 489005.

Li, Y., Du, X. F., Liu, C. S., Wen, Z. L., & Du, J. L. (2012). Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. Developmental cell, 23(6), 1189-1202.

Lull, M. E. & Block, M. L. (2010). Microglial activation and chronic neurodegeneration. Neurotherapeutics 7, 354–365.

Martin, S. E., Engleman, H. M., Deary, I. J., & Douglas, N. J. (1996). The effect of sleep fragmentation on daytime function. American journal of respiratory and critical care medicine, 153(4), 1328-1332.

McCormick, D.A. & Bal, T. (1994). Sensory gating mechanisms of the thalamus. Curr Opin Neurobiol 4, 550–556.

McCoy, J. G., Tartar, J. L., Bebis, A. C., Ward, C. P., McKenna, J. T., Baxter, M. G., ... & Strecker, R. E. (2007). Experimental sleep fragmentation impairs attentional set-shifting in rats. Sleep, 30(1), 52-60.

McGlade-McCulloh, E., Morrissey, A. M., Norona, F., & Muller, K. J. (1989). Individual microglia move rapidly and directly to nerve lesions in the leech central nervous system. Proceedings of the National Academy of Sciences, 86(3), 1093-1097.

Meerlo, P., Mistlberger, R. E., Jacobs, B. L., Heller, H. C., & McGinty, D. (2009). New neurons in the adult brain: the role of sleep and consequences of sleep loss. Sleep medicine reviews, 13(3), 187-194.

Mirzaei, N., Tang, S. P., Ashworth, S., Coello, C., Plisson, C., Passchier, J., ... & Sastre, M. (2016). In vivo imaging of microglial activation by positron emission tomography with [11C] PBR28 in the 5XFAD model of A lzheimer's disease. Glia, 64(6), 993-1006.

Monti, J. M., & Monti, D. (2000). Sleep disturbance in generalized anxiety disorder and its treatment. Sleep medicine reviews, 4(3), 263-276.

Morgese, V. J., Elliott, E. J., & Muller, K. J. (1983). Microglial movement to sites of nerve lesion in the leech CNS. Brain research, 272(1), 166-170.

Mullington, J.M., Simpson, N. S.,Meier-Ewert, H. K., and Haack,M. (2010). Sleep loss and inflammation. Best Pract. Res. Clin. Endocrinol. Metab. 24, 775–784. doi: 10.1016/j.beem.2010.08.014

Murkar, A. L., & De Koninck, J. (2018). Consolidative mechanisms of emotional processing in REM sleep and PTSD. Sleep medicine reviews, 41, 173-184.

Nadjar, A., Wigren, H. K. M., & Tremblay, M. E. (2017). Roles of microglial phagocytosis and inflammatory mediators in the pathophysiology of sleep disorders. Frontiers in cellular neuroscience, 11, 250.

Nimmerjahn, A., Kirchhoff, F., & Helmchen, F. (2005). Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science, 308(5726), 1314-1318.

Opp, M. R., & Krueger, J. M. (1994). Anti-interleukin-1 beta reduces sleep and sleep rebound after sleep deprivation in rats. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 266(3), R688-R695.

Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT. (2011) Synaptic pruning by microglia is necessary for normal brain development. Science 333:1456–1458. 10.1126/science.1202529

Parkhurst, C. N., Yang, G., Ninan, I., Savas, J. N., Yates III, J. R., Lafaille, J. J., ... & Gan, W. B. (2013). Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. Cell, 155(7), 1596-1609.

Pease, J. E., & Williams, T. J. (2002). Chemokines. In *Asthma and COPD* (pp. 255-260). Academic Press.

Peigneux, P., Laureys, S., Delbeuck, X., & Maquet, P. (2001). Sleeping brain, learning brain. The role of sleep for memory systems. Neuroreport, 12(18), A111-A124.

Pejovic, S., Basta, M., Vgontzas, A. N., Kritikou, I., Shaffer, M. L., Tsaoussoglou, M., ... & Chrousos, G. P. (2013). Effects of recovery sleep after one work week of mild sleep restriction on interleukin-6 and cortisol secretion and daytime sleepiness and performance. American Journal of Physiology-Endocrinology and Metabolism, 305(7), E890-E896.

Porkka-Heiskanen, T., Strecker, R. E., & McCarley, R. W. (2000). Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an in vivo microdialysis study. Neuroscience, 99(3), 507-517.

Porkka-Heiskanen, T. (1999). Adenosine in sleep and wakefulness. Annals of medicine, 31(2), 125-129.

Porkka-Heiskanen, T., Zitting, K. M., & Wigren, H. K. (2013). Sleep, its regulation and possible mechanisms of sleep disturbances. Acta physiologica, 208(4), 311-328.

Qin, L., Wu, X., Block, M. L., Liu, Y., Breese, G. R., Hong, J. S., ... & Crews, F. T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia, 55(5), 453-462.

Ramesh V, Nair D, Zhang SX, Hakim F, Kaushal N, Kayali F, Wang Y, Li RC, Carreras A, Gozal D. Disrupted sleep without sleep curtailment induces sleepiness and cognitive dysfunction via the tumor necrosis factor-α pathway. J Neuroinflammation. 2012 May 11; 9():91.

Ransohoff R. M., Cardona A. E. (2010). The myeloid cells of the central nervous system parenchyma. Nature 468, 253–262. 10.1038/nature09615

Ransohoff, R. M. (2016). A polarizing question: do M1 and M2 microglia exist?. Nature neuroscience, 19(8), 987.

Rihel, J., & Schier, A. F. (2013). Sites of action of sleep and wake drugs: insights from model organisms. Current opinion in neurobiology, 23(5), 831-840.

Saper, C. B., Scammell, T. E., & Lu, J. (2005). Hypothalamic regulation of sleep and circadian rhythms. Nature, 437(7063), 1257.

Sherbet, G. V. (2011). Growth factors and their receptors in cell differentiation, cancer and cancer therapy. Elsevier.

Stence, N., Waite, M., & Dailey, M. E. (2001). Dynamics of microglial activation: A confocal time-lapse analysis in hippocampal slices. Glia, 33(3), 256-266

Stephan, A. H., Barres, B. A., & Stevens, B. (2012). The complement system: an unexpected role in synaptic pruning during development and disease. Annual review of neuroscience, 35, 369-389.pr

Swanson, L. W., & Cowan, W. M. (1977). An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. journal of Comparative Neurology, 172(1), 49-84.

Takeuchi, O., & Akira, S. (2010). Pattern recognition receptors and inflammation. Cell, 140(6), 805-820.

Tartar JL, McKenna JT, Ward CP, McCarley RW, Strecker RE, Brown RE. (2010) Sleep fragmentation reduces hippocampal CA1 pyramidal cell excitability and response to adenosine. Neurosci Lett. 2010 Jan 18; 469(1):1-5.

Tartar, J. L., McKenna, J. T., Ward, C. P., McCarley, R. W., Strecker, R. E., & Brown, R. E. (2010). Sleep fragmentation reduces hippocampal CA1 pyramidal cell excitability and response to adenosine. Neuroscience letters, 469(1), 1-5.

Tay, T. L., Savage, J. C., Hui, C. W., Bisht, K., & Tremblay, M. È. (2017). Microglia across the lifespan: from origin to function in brain development, plasticity and cognition. The Journal of physiology, 595(6), 1929-1945.

Tempesta, D., Socci, V., De Gennaro, L., & Ferrara, M. (2018). Sleep and emotional processing. Sleep medicine reviews, 40, 183-195.

Tononi, G., & Cirelli, C. (2014). Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. Neuron, 81(1), 12-34.

van Leeuwen, W. M., Lehto, M., Karisola, P., Lindholm, H., Luukkonen, R., Sallinen, M., ... & Alenius, H. (2009). Sleep restriction increases the risk of developing cardiovascular diseases by augmenting proinflammatory responses through IL-17 and CRP. PloS one, 4(2), e4589.

Verdonk, F., Roux, P., Flamant, P., Fiette, L., Bozza, F. A., Simard, S. & Chrétien, F. (2016). Phenotypic clustering: a novel method for microglial morphology analysis. Journal of neuroinflammation, 13(1), 153.

Wadhwa, M., Kumari, P., Chauhan, G., Roy, K., Alam, S., Kishore, K., ... & Panjwani, U. (2017). Sleep deprivation induces spatial memory impairment by altered hippocampus neuroinflammatory responses and glial cells activation in rats. Journal of neuroimmunology, 312, 38-48.

Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. (2009) Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci 29:3974–3980. 10.1523/JNEUROSCI.4363-08.2009

Walker, M. P., & van Der Helm, E. (2009). Overnight therapy? The role of sleep in emotional brain processing. Psychological bulletin, 135(5), 731.

Wes, P.D., Holtman, I.R., Boddeke, E.W., Möller, T. & Eggen, B.J. (2016) Next generation transcriptomics and genomics elucidate biological complexity of microglia in health and disease. Glia 64, 197–213 (2016).

Wigren, H. K., & Porkka-Heiskanen, T. (2018). Novel concepts in sleep regulation. Acta Physiologica, 222(4), e13017.

Wollmer M.A., Lucius R, Wilms H., Held-Feindt J., Sievers J., Mentlein R. (2001) ATP and adenosine induce ramification of microglia in vitro. J Neuroimmunol 115: 19–27, 2001.

Wu, J. C., Klein, E. A., Gillin, J. C., & Keator, D. (1996). Brain metabolism and sleep deprivation: A PET study. Biological Psychiatry, 39(7), 605.

Zeitzer, J. M., Morales-Villagran, A., Maidment, N. T., Behnke, E. J., Ackerson, L. C., Lopez-Rodriguez, F., ... & Wilson, C. L. (2006). Extracellular adenosine in the human brain during sleep and sleep deprivation: an in vivo microdialysis study. Sleep, 29(4), 455-461.

Zimmerman, J. E., Naidoo, N., Raizen, D. M., & Pack, A. I. (2008). Conservation of sleep: insights from non-mammalian model systems. Trends in neurosciences, 31(7), 371-376.