EFFECTS OF CHRONIC MILD SLEEP RESTRICTION FOLLOWING REPEATED ENDOTOXIN EXPOSURE ON ALZHEIMER'S DISEASE PATHOLOGY IN HEALTHY WILD TYPE MICE

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Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

December 10, 2018

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ABSTRACT

An estimated 46.8 million people are living with dementia, and those numbers are expected to rise to 74.7 million by 2030. Alzheimer's disease (AD) is the most common underlying cause of dementia and accounts for up to 70% of dementia cases. AD is a chronic neurodegenerative disorder characterized by aggregation and accumulation of β-amyloid (Aβ) peptides, resulting in neuron loss and eventually cognitive decline. The microglial cells of AD patients become dysfunctional and less able to clear Aβ from the blood brain barrier (BBB), but maintain their ability to detect Aβ peptides and produce proinflammatory cytokines in response. As a result, AD patients experience a self-perpetuating condition of neuroinflammation, resulting in accumulation of Aβ and eventually cognitive deficits. These pathological alterations seen in AD patients are similar to those induced by injections of lipopolysaccharide (LPS). Additionally, both AD patients and mice injected with LPS experience reduced durations of rapid eye movement (REM) sleep and enhanced duration of non-REM sleep. REM sleep plays an essential role in memory consolidation and non-REM sleep plays an essential role in the recovery from Aβ accumulation that occurs during wakefulness. The rising number of individuals who are receiving insufficient sleep may fail to receive these restorative functions provided by sleep. As a result, failing to obtain sufficient sleep could increase an individual's risk for developing dementia and eventually AD. The goal of this study is to examine this possibility further by investigating the effects of chronic mild SR alone and in the presence of an inflammatory insult (LPS), on cognition and hippocampal Aβ. It is hypothesized that chronic mild sleep restriction (SR) will lead to cognitive deficits and increased levels of hippocampal Aβ. It is also hypothesized that SR, following repeated LPS injections, will lead to greater cognitive deficits and higher levels of hippocampal Aβ than exposure to LPS or SR may induce alone. To test these hypotheses, fifty-five 4-6-month-old C57BL/6J mice were divided into SR, large cage

control (LCC), and home cage control (HCC) groups. SR was achieved using the modified multiple platform method (MMPM), which deprived mice of REM sleep 10 hours every day for 6 weeks. LPS injections were administered during the last 7 days of the experiment. Cognition was examined using contextual fear conditioning (CFC) and Aβ was quantified using Aβx−42 ELISA. The results indicate that SR failed to significantly increase hippocampal Aβ and cognitive deficits, as described in previous studies. LPS also failed to alter hippocampal Aβ and cognitive deficits, as previously demonstrated in our lab. As a result, the data presented was unable to provide a firm conclusion on the effects of chronic mild SR following repeated endotoxin exposure on AD pathology. This data draws attention to the lack of consistency among the effects of SR and LPS on Aβ. Since this study was unable arrive at an answer to the question, we set out to investigate, it is necessary to explore the relationship between SR following LPS exposure in future studies. Understanding how sleep and LPS impact the onset and progression of AD can aid in the development of strategies that may one day decrease its prevalence.

ACKNOWLEDGEMENTS

There are several people who I would like to thank, without whom completion of this honors thesis would not have been possible. Firs of all I would like to thank Dr. Gary Beohm. His constant encouragement and guidance throughout the project allowed me to keep persisting and growing as a member of the scientific community.

Secondly, I would like to thank Kelly Bryce. Kelly worked patiently and attentively with me to help me develop the skills and knowledge that allowed me to complete this project. Her constant dedication to the field and to others in the lab is a true inspiration for me. She helped me develop a new appreciation for the field of science, and the researchers behind it that work tirelessly to cure and prevent diseases like Alzheimer's disease.

I would also like to thank Dr. Michael Chumley, who was instrumental in providing the feedback necessary for me to create this project. His guidance and feedback on my thesis is what allowed me to continue improving. This project would ultimately not be complete without his help.

Additionally, I would like to thank Dr. Marlo Jeffries for her support as both a committee member and a mentor. Dr. Jeffries not only provided wonderful feedback on my thesis, but she also helped guide it in a direction that aligned with the field of science. Additionally, many of the skills I learned during her class allowed me to complete this project with greater confidence then I could have ever imagined.

Lastly, I would like to thank all the graduate and undergraduate students in the Neurobiology of Aging lab. I would especially like to thank Christopher Hagen, Briar Hill, Jack Figg, and Lauren McCue. Without them, I would not have completed this project. Even in times they may have been busy, they took time out of their day to help me move animals for this experiment, on a countless number of occasions.

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INTRODUCTION

AD is a chronic neurodegenerative disorder characterized by aggregation and accumulation of β-amyloid (Aβ) peptides and hyperphosphorylated tau proteins in the brain [1]. These Aβ peptides aggregate to form oligomers that block cell to cell signaling between neuronal synapses and senile plaques that attract immune cells to produce cytotoxic proinflammatory cytokines and reactive oxygen species that result in neuron loss [2]. When tau, a protein normally responsible for microtubule assembly in neurons, becomes inactive and toxic upon hyperphosphorylation and aggregation, neurodegeneration ensues [3]. As these pathologies increase in severity, the resulting synaptic dysfunction and neurodegeneration leads to a gradual decline in cognition known as dementia [4]. An estimated 46.8 million people are currently living with dementia, and those numbers are expected to rise to 74.7 million by 2030 [5]. Alzheimer's disease is the most common underlying cause of dementia, accounting for up to 70% of dementia cases [6].

In early stages of AD, microglia, resident immune cells of the brain, actively promote Aβ clearance and hinder AD progression [7]. As the disease progresses, microglia become dysfunctional and less able to clear Aβ [8]. Reduced clearance of Aβ across the blood brain barrier (BBB) may lead to greater accumulation in the brain, resulting in a more toxic environment for neurons [9]. Despite their reduced ability to clear Aβ, microglial cells retain their ability to detect Aβ peptides and produce proinflammatory cytokines in response [7]. Pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) induce neuroinflammation and eventually lead to neuron loss [10]. Additionally, neuroinflammation has been found to result in an increase in production and deposition of Aβ [11], leading to a self-perpetuating condition of neuroinflammation [7].

A similar state of inflammation to those seen in AD patients also arises after injections with gram-negative bacteria called lipopolysaccharide (LPS). The endotoxin, LPS, is a molecular

fragment from the cell wall of gram-negative bacteria that acts as a nonspecific activator of the immune system by influencing cytokine secretion from microglia [12]. Our lab has previously demonstrated that LPS injections lead to increases in hippocampal Aβ and greater cognitive deficits in C57BL/6J mice [13]. Previous research has also demonstrated that middle-aged rats injected with LPS experienced reduced duration of rapid eye movement (REM) sleep and enhanced duration of non-REM sleep [14].

AD has also been found to bring about changes in sleep patterns [15] similar to those induced by LPS. Sleep disruption currently affects nearly 45% of the individuals with AD [16]. During the night, REM sleep and non-REM sleep alternate in a cyclic pattern [15]. Non-REM sleep produces an electroencephalography (EEG) pattern characterized by high voltage, low frequency waves and reduced activity in postural muscles, whereas, REM (also known as paradoxical) sleep produces a EEG pattern characterized by low voltage, high frequency waves and loss of postural muscle tone, similar to that of wakefulness [17]. REM sleep plays an essential role in memory consolidation [18] and non-REM sleep plays an essential role in the recovery from Aβ accumulation that occurs during wakefulness [19]. Sleep disruption in AD patients is characterized by reduced duration of REM sleep and enhanced duration of non-REM sleep [6]. These changes in sleep precede the onset of cognitive decline and sleep quality further declines in parallel with progression of the disease [20], suggesting that a bidirectional relationship may exist.

Sleep disruption increases with age [21] and is not exclusive to AD patients. Non-AD patients also experience alterations and disruptions in their sleep. Older adults experience declines in REM sleep and more frequent awakenings during the night [21]. Individuals who work long hours, overnight shifts, or experience stress at their workplace, are at risk for sleep loss [22]. Similar to AD, these individuals, who work long hours or do shift work have also been

found to exhibit reduced REM sleep [23]. As a result, insufficient sleep is now considered a public health epidemic affecting 65 million adults in the United States [22].

Aβ has been found to increase during wakefulness and decrease during sleep in a diurnal pattern [6]. Aβ production is intimately linked to neuron activity and, therefore accumulates during periods of wakefulness when neurons are active [24]. When overall neuronal activity is lowered, as in non-REM sleep, less Aβ is released [20] and clearance of Aβ increases by two-fold [19]. This leads to an overall reduction in Aβ, creating a less toxic environment for neurons, which improving cognitive function [20]. A period of extended wakefulness, as in sleep restriction (SR) or sleep deprivation (SD) can therefore lead to a decreased opportunity for Aβ clearance resulting in cognitive deficits. Additionally, it has been found that aggregation of Aβ for longer periods will disrupt the sleep cycle, leading to a destructive positive-feedback loop [15].

SD has been shown to induce cognitive deficits and increase Aβ. A paradoxical SD study that deprived healthy rats of paradoxical/REM sleep led to memory impairment and increased Aβ peptides [25]. Mild SR studies in transgenic 3xTgAD mice that have 3 of the genetic mutations associated with AD demonstrated an increase in memory deficits and cortical Aβ [26]. Even a short period of SR of 6 hours per day for 6 weeks in mice can lead to cognitive impairments in object recognition [27].

SD has also been found to alter the body's ability to fight off a pathogen. Circulating levels of inflammatory cytokines such as IL-1, IL-6 and TNF have been found to peak during early onset of sleep [28]. Since cytokines coordinate functional immune responses [28], increases in them during sleep suggests that sleep plays a vital role in fighting off pathogens. Even one night of partial SD has been found to alter IL-6 [29]. A brief period of SD (4-8 hours) has also been found to significantly decrease activity of nonspecific immune cells called natural-killer-cell

[28]. Extended periods of wakefulness have resulted in a reduction in the body's ability to fight off pathogens [28]. Patients restricted to 4 hours of sleep for 6 days before inoculation with influenza virus experienced a reduced antibody response compared to individuals with a normal sleep schedule of 7.5 to 8.5 hours of sleep [30].

Considering the restorative function of non-REM sleep, it is suggested that cytokines released in response to an inflammatory insult increase non-REM to allow enhanced clearance of neurotoxic waste (including Aβ) across the BBB [31]. However, in conditions such as SR, when REM sleep is restricted [32], the body may be limited in its ability to adjust its sleep in response to an inflammatory insult. As a result, these organisms might experience difficulty clearing Aβ across the BBB, resulting in greater cognitive deficits. To better understand this relationship, it is necessary to investigate the effects of SR following repeated endotoxin exposure on AD pathology.

The goal of the present study is to investigate the effects of chronic mild SR alone and following endotoxin exposure on cognition and Aβ. To accomplish this, C57BL/6J mice were subjected to 10 hours of SR every day for 6 weeks. During the last seven days of the experiment, mice were injected with either saline or LPS. Cognition was examined using contextual fear conditioning (CFC) and Aβ was be quantified using ELISA. It is hypothesized that chronic mild SR conditions will lead to cognitive deficits and increased levels of Aβ. Additionally, it is hypothesized that SR following exposure to an infectious agent will lead to greater cognitive deficits and higher levels of hippocampal Aβ than LPS [13] or SR induces alone.

MATERIALS AND METHODS

Animals

Adult male and female 4-6-month-old C57BL/6J mice were bred and raised in the Texas Christian University vivarium. The animals were housed in groups of 3-4 in standard

polycarbonate mouse cages (12.5 x 15 cm x 25 cm) maintained at 22°C. All mice were on the same 12-h light/dark schedule (where lights were turned on at 0700 and off at 1900) and given food and water ad libitum. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) and in accordance with protocols approved by the Institutional Animal Care and Use committee (IACUC) of Texas Christian University.

Experimental Design

Mice were separated into 3 groups: SR, large cage control (LCC) and home cage control (HCC). Experimental SR groups (n=19) were subjected to paradoxical SR that deprived mice of REM sleep using the modified multiple platform method (MMPM). In MMPM, 3-4 mice from the same cohort were placed inside cages (26.67 x 48.26 x 15.56 cm) containing 15 circular platforms (3 cm in diameter) filled with 1 cm of water (Figure 1). Mice were allowed to move freely around the inside of the cage by jumping from one platform to another. In this method, small amounts of non-REM sleep can still be obtained, but when the mice enter REM sleep and lose muscle tone, they fall into the water, causing them to wake. Experimental groups were placed in their respective cages with fresh water every day for 6 weeks at 0800 h and then removed at 1800 h and placed back into their home cages until 0800 h the next day. A black heating pad from Vivosun was placed underneath the SR cages of the experimental groups to regulate the temperature of the water. The heating pad was set to 36.7°C to maintain the temperature of the water at 25°C. To test the potential influence of environmental stress on behavior, mice in the LCC group (n=18) were placed in cages identical to that of experimental groups with 3-4 mice per cage but were not filled with water. The LCC groups were allowed to sleep on the floor of the cage, which allowed them to obtain both REM and non-REM sleep. SR and LCC cages were cleaned daily. Mice in the HCC group (n=18) were kept in their home cages

but moved to clean cages when the animals were moved to their experimental cages. This accounted for any variability induced by handling and moving the animals into new cages. All mice had access to food pellets and water ad libitum.

*Figure 1. Modified multiple platform method***.** For SR groups, cages were filled with 1 cm of water. LCC groups utilized the same cages as SR groups, but were not filled with water.

Injections

Animals from each group (LCC, HCC, and SR) were randomly assigned to either LPS (n=28) or saline conditions (n=27). Intraperitoneal (i.p.) injections of 250 µg/kg of LPS derived from Escherichia coli stereotype 055:B5 (Sigma, St. Louis, MO) were administered to LPS groups. The dose of 250 µg/kg of LPS was chosen because previous studies in our lab have shown that this dose reliably induces sickness behavior, which can include weight loss, lethargy, piloerection and decreased grooming [13]. LPS has also been shown to increase hippocampal Aβ and lead to greater learning deficits in C57BL/6J mice [13]. When LPS mice were given their injections, 200 µg/kg of saline was administered to saline groups. Injections (LPS or saline) were administered during the last week (week 6) each day for 7 days. Animal weights were measured daily during the 7 days of injections.

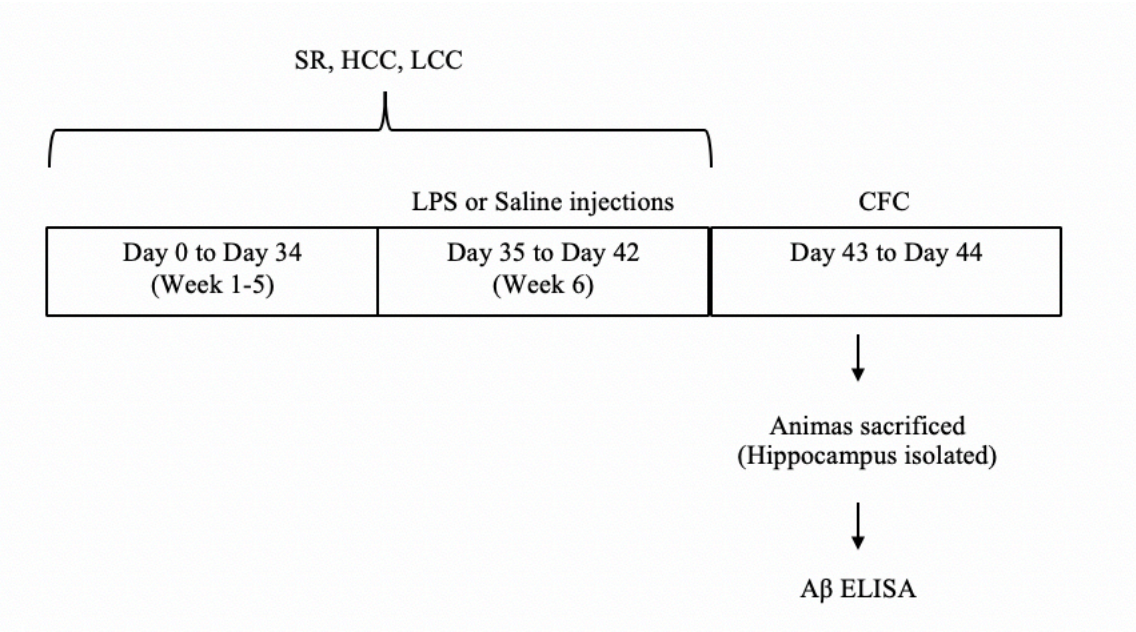

Figure 2. Experimental timeline. All animal groups were injected with LPS during week 6, followed by CFC 24 hours later. 24 hours after CFC animals were sacrificed and hippocampus isolated for further analysis via Aβ ELISA.

Contextual Fear Conditioning (CFC)

CFC was used to assess hippocampus-dependent contextual learning. The CFC protocol that was used was previously described by Khan et al. [13]. CFC began 24 hours after the 7th injection of LPS or saline. Each testing chamber (Coulbourn Instruments, Whitehall, PA, 7W x 7D x 12H) had an electric grid floor, a small light bulb attached to the ceiling, and infrared photo beams along the walls to monitor movement of the animal continuously. A salient wall design consisting of black polka dots was used on each side of the testing chamber, which has been found to increase the context-shock pairing [13]. Peppermint oil (Now Foods, Bloomingdale, IL) mixed with water at a ratio of 1:10 was placed in a container underneath the grid floor to serve as the olfactory cue. Together, the paradigm paired a mild aversive stimulus (2-s 0.5mA shock) with polka-dotted walls and peppermint olfactory cues. Training sessions began with a 90-s acclimation period followed by a 2-s 0.5mA shock (delivered by the electric grid floor). Animals remained in the chamber until 182-s and were then transported back to their home cages in clear

polycarbonate transport boxes. Testing began 24 hours after the training session. During testing, mice were placed back in the chambers for 90-s, consisting of the same environment with polka-dot walls and peppermint olfactory cues, however, no shock was administered. Freezing behavior (an innate fear response in rodents) was monitored using the Freeze Monitor System and software (San Diego Instruments, San Diego, CA). Mice were considered to be freezing when they did not break photo beams during a 2-s interval of time. Freezing time during training was utilized as the dependent variable. Total freezing time was used to indicate if an association between the context and aversive stimulus was learned.

Brain Dissection

24 hours after behavioral testing, animals were sacrificed via rapid decapitation and the hippocampus isolated from both brain hemispheres. The hippocampus was stored in Pro-Prep at -80°C until Aβ quantification.

Aβ ELISA Procedure

The brain tissue was homogenized with protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL) containing protease inhibitors at 1600 x g for 30 min and were allowed to lyse further for 30 min on ice. The lysate was then centrifuged at 16,000 x g for 40 min and the clear lysate was removed for the Aβx−42 ELISA (Covance Research Products, Dedham, MA). The BetaMark Aβx−42 ELISA was then performed in accordance with the manufacturer's instructions. The samples were diluted 2:1 with working incubation buffer, which includes the horseradish peroxidase (HRP)-labeled detection antibody, loaded into triplicate wells. The plate was incubated overnight at 2-8 °C. The wells were then washed and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well. The plate was then incubated for 45 min at room temperature in the dark, and the optical density was read at 620 nm (BMG LabTech FLUOstar Omega, Cary, NC).

Statistical Analyses

Behavioral and biological data was analyzed (IBM SPSS Statistics 23) using a two-way analysis of variance (ANOVA) with group and treatment as main factors. The alpha level used for all statistical analyses was 0.05. Any significant main effects or interactions were further analyzed by Turkey's Honest Significance Difference (HSD) test post hoc comparisons.

RESULTS

The effects of chronic mild sleep restriction on cognitive deficits

To determine the effects of chronic mild SR following repeated LPS exposure on cognitive function, freezing behavior in CFC was evaluated. If experimental groups freeze more than controls, it indicates a learned association between the context and aversive stimulus. If experimental groups freeze less than the controls, it indicates a cognitive deficit. A two-way ANOVA was performed and indicated that the main effect of SR was not significant (SR, LCC, HCC; $F(2,40) = 2.875$, $p = 0.068$). The SR groups froze significantly less than the HCC groups in both LPS and saline conditions ($p = 0.026$). SR groups did not freeze significantly less when compared to the LCC groups that account for environmental factors ($p = 0.083$). The main effect of LPS exposure was not significant (LPS, Sal; $F(1,40) = 0.551$, $p = 0.551$). The main effect of interaction between SR and repeated LPS exposure was also not significant ($F(2,40) = 0.717$, $p = 0.494$; Figure 3). Overall, this data indicates that SR, LPS and the interaction between SR and LPS exposure does not significantly alter cognitive deficits.

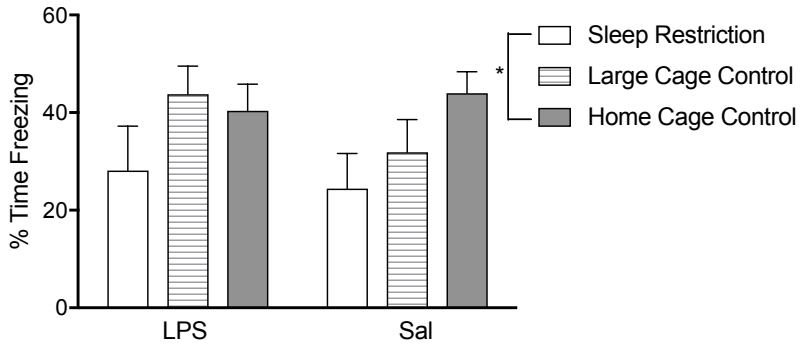

Figure 3. The effects of chronic mild sleep restriction on cognitive deficits. The overall main effects of SR, LPS, and the interaction between the two are not significant. Bars represent means ± SE, n = 7-9 mice per condition. Significant differences ($p<0.05$) are designated by *.

The effects of chronic mild sleep restriction on hippocampal Aβ

To determine the effects of chronic mild SR following repeated LPS exposure on the levels of Aβ in the hippocampus, Aβ was quantified with an Aβx-42 ELISA. A two-way ANOVA was performed and indicated that the effects of SR on hippocampal Aβ were not significant (SR, LCC, HCC; $F(2,49) = 3.156$, $p = 0.051$). The main effect of LPS exposure on hippocampal Aβ was not significant (LPS, Sal; $F(1,49) = 0.830$, $p = 0.367$). The main effect of interaction between SR and LPS exposure was not significant ($F(2,49) = 2.382$, $p = 0.103$; Figure 4). Overall, this data indicates that the effects of SR, LPS and the interaction between SR and LPS exposure on hippocampal Aβ are not significant.

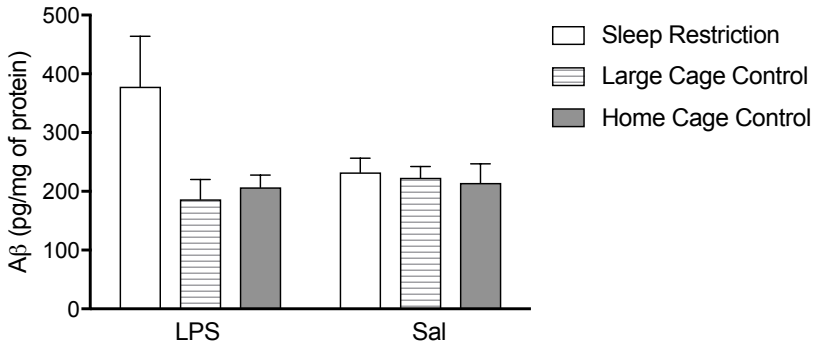

Figure 4. The effects of chronic mild sleep restriction on hippocampal Aβ. Concentration of hippocampal Aβ was not significantly altered by chronic mild sleep restriction, LPS exposure or a combination of the two. Bars represent means ± SE; n= 9-10 mice per condition. Significant differences ($p<0.05$) are designated by *.

DISCUSSION

The purpose of this study was to determine how chronic mild SR effects levels of hippocampal Aβ and cognition alone and if this effect is altered when administered in combination with exposure to an infectious agent. It was hypothesized that chronic mild SR will lead to cognitive deficits and increased levels of Aβ. Additionally, it was hypothesized that SR following exposure to LPS leads to greater cognitive deficits and higher levels of hippocampal Aβ than LPS or SR alone. These hypotheses were not supported by the data.

SR was not found to increase cognitive deficits, as predicted by the hypothesis. SR groups demonstrated significantly lower freezing time compared to HCC groups, but because SR groups were not significantly different from LCC groups, the overall main effect of SR was not significant. SR also did not significantly alter hippocampal Aβ. A large majority of the literature on SR has demonstrated greater cognitive deficits [26] and increases in Aβ [33], as a result of SR. Cognitive deficits following SR seem to be consistent among previous studies, but a few studies have had difficulty replicating a statically significant alteration in Aβ [26], [34]. In a study conducted by Meco et al., 3xTg mice that were deprived of sleep for 4 hours a day for 8

weeks developed significant cognitive impairments and alterations in tau, but not in Aβ [34]. The lack of complete consistency demonstrates the obvious need for future SR and sleep deprivation studies to assess Aβ as a parameter. Doing so may aid in determining if SR is a risk factor or simply a biomarker of AD that does not directly contribute to it [34].

LPS did not significantly affect hippocampal Aβ or cognitive deficits, even though it has previously been shown to alter these parameters in our lab [13]. Previously, LPS has been shown to reliably induce sickness behavior, increase hippocampal Aβ, and cognitive deficits [13]. LPS-induced sickness related symptoms include weight loss, lethargy, piloerection, and decreased grooming [13]. Of which, weight was used to indicate LPS injections were administered properly, which demonstrated that mice lost 2-3 grams within the first three days of LPS injections (data not shown). This indicates that LPS was administered properly and induced sickness-related symptoms. Despite this, alterations in hippocampal Aβ and cognitive deficits as a result of LPS injections failed to emerge, which could demonstrate the need to increase the volume of LPS administered in future studies. Even alterations in endotoxin units in the lot of LPS provided by the manufacturer could affect the experimental outcome. If endotoxin units are too low, LPS may fail to reliably induce inflammation significant enough to elevate hippocampal Aβ and cognitive deficits.

Since LPS failed to reliably induce increases in hippocampal Aβ and cognitive deficits, the data presented cannot support a firm conclusion in regard to the effects of SR following LPS exposure. Despite this, groups who received SR following repeated LPS injections had a 62.65% increase in Aβ compared to groups who received SR alone. While this interaction was not significant, an increase as such suggests the need to investigate this relationship further to determine conclusive evidence on the potential synergistic effects between LPS and SR. Determining conclusive evidence regarding SR in combination with exposure to an

inflammatory insult, such as LPS can shed light on the importance of sleep as a preventative measure against AD. Due to inadequacy of literature on the subject and lack of fully conclusive evidence in the present study, it is important that future research continues to explore this topic in more detail.

Future studies should consider using other behavioral methods in combination with CFC, such as novel object recognition, to obtain a more wholistic and conclusive understanding of the impact of SR and LPS exposure on cognition. Although the MMPM has already been shown to successfully decrease REM sleep [32], verifying this during the experiment using electroencephalography (EEG) may still provide beneficial data. Additionally, future studies should look into levels of brain-derived neurotrophic factor (BDNF). BDNF is a proinflammatory cytokine that plays a role in synaptic plasticity, cognition, and sleep regulation [35]. More specifically, BDNF has been found to increase after acute sleep loss [35]. Investigating BDNF in greater detail may provide a better understanding of the relationship between inflammation and sleep.

CONCLUSION

In conclusion, chronic mild SR did not significantly increase hippocampal Aβ and cognitive deficits, as many previous studies have demonstrated. The lack of complete consistency in regard to the effects of SR, particularly on that of Aβ, demonstrates the need for future SR studies to further explore this relationship. Additionally, LPS also failed to alter hippocampal Aβ and cognitive deficits, as previously demonstrated in our lab. Since LPS injections did not alter our parameters as expected, the data presented cannot provide a firm conclusion on the effects of chronic mild SR following repeated endotoxin exposure on AD pathology. It is therefore necessary to explore the relationship between SR following LPS

exposure in future studies. Understanding how sleep and LPS impact the onset and progression of AD can aid in the development of strategies that may one day decrease its prevalence.

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