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The Effect of Age on Neurological Inflammation to Acute Sleep Fragmentation in Mice

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THE EFFECT OF AGE ON NEUROLOGICAL INFLAMMATORY RESPONSES TO ACUTE SLEEP FRAGMENTATION IN MICE

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By

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ABSTRACT

Obstructive sleep apnea is identified by recurring events of airway collapse during sleep, intermittent hypoxia, and perturbations in sleep continuity, known as sleep fragmentation. There is evidence to suggest that elderly patients are more at risk of developing obstructive sleep apnea. The purpose of this study was to assess whether age affects neurological inflammatory responses to acute sleep fragmentation. This assessment was made by subjecting young (4-5 months old) and old (10-11 months old) male C57BL/6j mice to automated sleep fragmentation, as well as having mice in both age categories as a control with no sleep fragmentation, for twenty-four hours. Immediately after, brains were collected and hypothalami, hippocampi, and the prefrontal cortices were removed. The tissues were analyzed for the gene expression of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) using real time PCR. It was hypothesized that neuroinflammation would be exacerbated in older mice compared with young mice in response to sleep fragmentation. Results indicate that the inflammatory response to sleep fragmentation is variable among the examined brain tissues. In the prefrontal cortex and hypothalamus, sleep fragmentation had a significant effect on TNF-α gene expression. In the hypothalamus, age also had a significant effect on TNF-α gene expression. However, there was no significant interaction between sleep fragmentation and age and TNF-α gene expression in the regions assessed, which does not support the original hypothesis. These results can lead to better understanding of the relationship between age and obstructive sleep apnea, and lead to further investigations.

I dedicate this thesis to my parents, Bobbi and Michael, my sisters Katie and Carey, and my grandparents Phyllis and Jerry, who inspire and support me in everything I do. I also dedicate this thesis to Zackary, who has believed in me and encouraged me from the moment we met.

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VITA

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Taylor, M. (2021, March) *The Effect of Age on Neurological Inflammatory Responses to Sleep Fragmentation in Mice.* Oral Presentation at the WKU Student Research Conference. Bowling Green, KY.

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INTRODUCTION

Obstructive Sleep Apnea and Aging

Obstructive sleep apnea (OSA) is often identified by recurring events of airway collapse during sleep, intermittent hypoxia, and perturbations in sleep continuity, known as sleep fragmentation. Previous studies have demonstrated that the risk for developing obstructive sleep apnea increases with age. In the same study, evidence suggests that the collapsibility of the pharyngeal airway increases in older adults, potentially creating a different phenotype in older individuals (Edwards, et al., 2014). It is known that individuals with obstructive sleep apnea show similar cognitive deficits to those that are associated with aging. OSA is an independent risk factor for depression and cognitive impairment. Studies have shown in older patients with OSA, their cognitive function is significantly impaired due to the brain becoming overwhelmed and unable to compensate for neurocognitive decline associated with both aging and the effects of obstructive sleep apnea (Edwards, et al., 2014).

Sleep Fragmentation and the Inflammatory Response

Sleep plays an important role in many of life's processes. It is known that sleep influences memory, cognitive ability, hormone secretion, metabolism, and immune function. Although the precise function of sleep has been heavily debated over the years, it is heavily argued that adequate sleep is essential to living a healthy life. Previous studies have shown that significant sleep loss increases sympathetic nervous system activity, therefore activating the immune system and generating inflammatory cytokines

that play an important role in metabolic and cardiovascular disorders (Kheirandish-Gonzal & Gonzal, 2019).

There is significant evidence that suggests that obstructive sleep apnea is a low-grade chronic inflammatory disease. Previous studies have shown that after only one night of sleep deprivation, there is a buildup of inflammatory cytokines in the tissues, creating an acute inflammatory response (Center for Disease Control, 2020). TNF-α, a pro-inflammatory cytokine associated with sleep regulation, is associated with circadian rhythms and is often elevated after periods of sleep deprivation (Kheirandish-Gonzal & Gonzal, 2019). Typically, TNF-α and other inflammatory cytokines will continue to increase in sleep-deprived individuals until they regain the ability to establish a proper sleep-wake cycle. In patients with undiagnosed and/or untreated OSA, the proper cycle is never established, and pro-inflammatory cytokine levels continuously increase.

Sleep fragmentation is a common disorder that causes many problems such as metabolic disorders, cardiovascular disease, and neurocognitive decline. Because sleep fragmentation is associated with an increased proinflammatory immune response, it is important to study this relationship so that diagnosis and treatment of these conditions can be improved and better understood.

Age and Inflammation

Aging is often thought of as a degenerative process that is often coupled with increased levels of inflammation. The exact relationship between aging and increase in pro-inflammatory cytokines is not quite known. Previous studies indicate that many age-related diseases such as diabetes, cardiovascular disease, and metabolic syndrome are associated with increased levels of inflammation (Jenny, 2012). These age-related

inflammatory diseases are also often thought to be associated with obstructive sleep apnea.

The relationship between the effect of age on the inflammatory response to sleep fragmentation has not been extensively studied but is important to analyze because if the relationship can be better understood, then there can be optimization of diagnosis and treatment of obstructive sleep apnea in regard to age. To study this, a mouse model was used to compare gene expression of the cytokine TNF-α between age groups of sleep fragmented mice. Sections of the brain (hypothalamus, prefrontal cortex, and hippocampus) were extracted, and TNF-α levels in each tissue were analyzed and compared against the endogenous control gene, 18s. It is hypothesized that neuroinflammation would be exacerbated in older mice compared with young mice in response to acute sleep fragmentation.

MATERIALS AND METHODS

Sleep Fragmentation

This research was conducted using a highly regulated timeline. Thirty-six male C57BL/6j mice were used from a breeding colony at Western Kentucky University. The mice had constant access to food and tap water. The mice were grouped into four categories for the study as follows: nine young mice subjected to automated sleep fragmentation, nine young mice with no sleep fragmentation (control), nine old mice subjected to sleep fragmentation, and nine old mice with no sleep fragmentation (control). The young mice were between four and five months of age, and the old mice were between ten and eleven months of age. The mice were placed into a Model 80390 Sleep Fragmentation Chamber for twenty-four hours. Food and water were provided. For the mice that were subjected to automated sleep fragmentation, the computerized swipe bar within the sleep fragmentation chamber was turned on and programmed to sweep across the bottom of the cage every sixty seconds, requiring the mice to move and awaken. For the controls, the bar did not move.

Tissue Collection

Mice were removed from the Sleep Fragmentation Chamber, anesthetized with isoflurane gas (<1 min), and decapitated at the end of the 24 h sleep fragmentation period. The brain was removed from each mouse and stored in RNAlater solution at 4°C. Within thirty days of harvesting, the hypothalamus, hippocampus, and prefrontal cortex were dissected from each brain and stored in RNAlater solution at -18°C.

RNA Isolation and Reverse Transcription

The tissues were homogenized, and RNA was extracted using a Qiagen RNeasy Mini Kit, which included all of the necessary materials for successful RNA extraction and purification of each tissue. After isolation, total RNA concentration was measured using a NanoDrop 2000. RNA was then reverse transcribed using a Thermo Scientific High-Capacity cDNA Reverse Transcription Kit. The samples were run according to the manufacturer's protocol using a thermocycler.

RT-PCR

RT-PCR was performed by loading a 96-well PCR plate with cDNA, 18s (endogenous control) and TNF-α (gene of interest) probes, and Taqman Gene Expression Master Mix (Thermofisher Scientific). The plate was loaded into the Applied Biosystems 7300 machine for amplification. The amplification protocol for each plate was as follows: 50°C for two minutes, 95°C for ten minutes, and forty cycles at 95°C for fifteen seconds and 60°C for one minute. The manufacturer's protocol was followed except that reaction volumes were reduced from the manufacturer protocol of 50 μL to 20 μL, and each sample was run in duplicate. A comparative C_t analysis was performed. C_t values were averaged for each sample, resulting in a value termed the ΔC_t. C_t values for each TNF-α sample against the lowest C_t value of the respective control sample were then normalized, resulting in a value termed the $\Delta\Delta C_t$. The negative value of this powered to 2 ($2^{-\Delta\Delta Ct}$) was plotted.

Statistical Analysis

The results of the RT-PCR amplification were analyzed using a two-way ANOVA test. The two independent variables of the study were sleep treatment (SF or

NOSF) and age (young or old). Relative expression was the continuous variable. Statistical significance was designated at $p < 0.05$.

RESULTS

Prefrontal Cortex

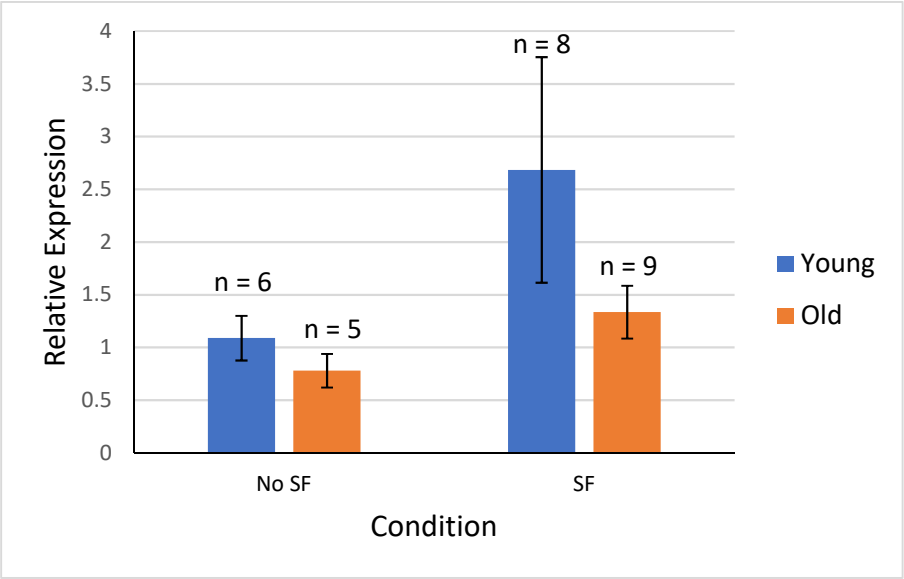

Figure 1: Prefrontal Cortex TNF-α Expression

	DF	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	0.371	4.789	0.0396	4.789	0.545
Age	1	0.188	2.436	0.1329	2.436	0.306
Treatment * Age	1	0.003	0.043	0.8378	0.043	0.054
Residual	22	0.077				

Table 1: ANOVA Table for log TNF-α in the Prefrontal Cortex

Within the prefrontal cortex, there was a significant effect of the sleep treatment on TNF-α gene expression (Two-way ANOVA, F=4.79, P=0.04). However, age and the interaction between sleep treatment and age did not have significant effects on TNF-α gene expression in the prefrontal cortex.

Hippocampus

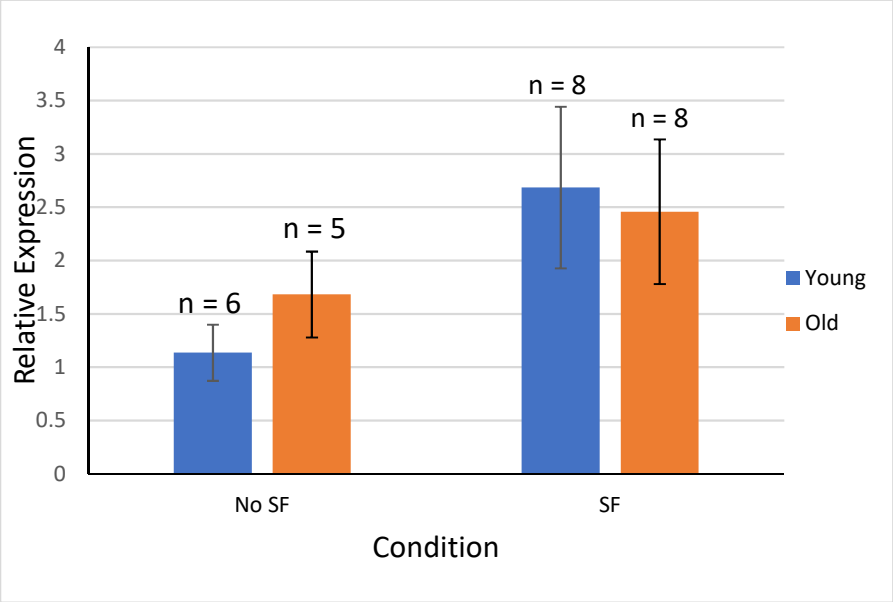

Figure 2: Hippocampus TNF-α Expression

	DF	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	8.512	3.392	0.0790	3.392	0.407
Age	1	0.161	0.064	0.8022	0.064	0.057
Treatment * Age	1	0.941	0.375	0.5466	0.375	0.088
Residual	22	2.509				

Table 2: ANOVA Table for TNF-α in the Hippocampus

Within the hippocampus, there was no significant effect of sleep treatment, age, or the interaction between sleep treatment and age on TNF-α gene expression.

Hypothalamus

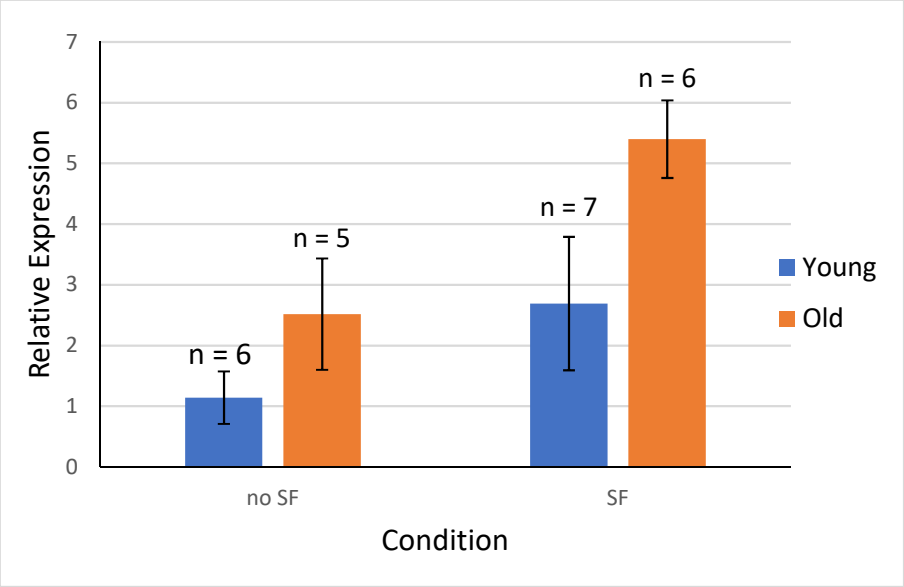

Figure 3: Hypothalamus TNF-α Expression

	DF	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	1	29.051	12.552	0.0020	12.552	0.936
Age	1	24.654	10.652	0.0039	10.652	0.889
Treatment * Age	1	2.626	1.135	0.2995	1.135	0.165
Residual	20	2.314				

Table 3: ANOVA Table for TNF-α in the Hypothalamus

Within the hypothalamus, there was a significant effect of sleep treatment on TNF-α gene expression (Two-way ANOVA, F=12.6, P=0.02). There was also a significant effect of age on TNF-α gene expression (Two-way ANOVA, F=10.7, P=0.004). There was not a significant effect of the interaction between sleep treatment and age on TNF-α gene expression within the hypothalamus.

DISCUSSION

It was hypothesized that neuroinflammation would be exacerbated in older mice compared with young mice in response to sleep fragmentation. However, my results did not provide support for this hypothesis.

In the prefrontal cortex, sleep treatment had a significant effect on TNF-α gene expression. This is consistent with what was expected, as it is known from previous studies that sleep fragmentation induces an exacerbated inflammatory response (Dumaine and Ashley, 2015). However, in the prefrontal cortex, neither age nor the interaction between age and sleep fragmentation had significant effects on TNF-α gene expression. Relative expression levels of TNF-α were actually higher in the younger mice of both the sleep fragmented and control groups, which is the opposite of what was expected. This might be explained by the lack of knowledge on longitudinal TNF-α levels in the brain as it ages.

In the hippocampus, there was no significant effect of the sleep treatment, age, or sleep treatment with age on TNF-α gene expression. The relative expression levels do suggest higher levels of the cytokine in the sleep fragmented mice, but not high enough to be deemed significant. There were also higher levels of TNF-α in the young sleep fragmented group for the hippocampus than the old mice in the sleep-fragmented group, which is the opposite of what was expected. These results might be explained by the fact that this study involved acute sleep fragmentation. Neurocognitive decline is gradual

while aging, and if a chronic study was completed, the results may show a significant relationship between age with sleep fragmentation and TNF-α gene expression.

In the hypothalamus, there was a significant effect of the sleep treatment on TNF-α gene expression. There was also a significant effect of age on TNF-α gene expression. However, the interaction between age and sleep fragmentation did not have a significant effect on TNF-α gene expression. The results were partially in support of the hypothesis, because TNF-α gene expression did increase when mice were in the experimental condition, and with age, but the statistical analysis suggests that sleep treatment with age was not significant. This is where the hypothesis is not supported.

Many other studies suggest that there is significant evidence for the increase of inflammation as one ages. However, the findings in this study are somewhat inconsistent with these findings. This may be due to the length of the study. Since this was an acute sleep fragmentation study, the long-term effects of sleep fragmentation were not examined. Perhaps if a chronic study were conducted, then the findings would have been consistent with what was originally hypothesized in this study.

The results in this study may also be attributed to observed errors within the study. The dissected tissues were mistakenly kept above freezing temperature for an extended period of time, when they should have been kept below freezing temperatures for preservation purposes, although RNAlater is an effective preservative for RNA. This might have denatured the RNA in the tissues. Because of this, not all the RNA samples were useable for further analysis, so the sample size of the study was smaller than originally anticipated.

Overall, this study suggests that age with sleep fragmentation does not have an effect on TNF-α gene expression. In all three tissues, the statistical analyses for sleep treatments with age had P-values that suggest that there is no significant effect on TNF-α gene expression. However, the results were consistent with previous findings that TNF-α gene expression levels were elevated in mice that were in the sleep fragmented groups, regardless of age. This study gives a better understanding of the interaction between age and sleep apnea and opens many gateways for further research. This can ultimately lead to better treatment of patients with obstructive sleep apnea, drug optimization, and a better understanding of obstructive sleep apnea disease progression in humans.

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