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Effects of Sleep Fragmentation on the Progression of Alzheimer's Disease

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Abstract:

Alzheimer's Disease (AD) is a neurodegenerative disease that is characterized by amyloid-beta (Aβ) plaques, neurofibrillary tangles, neuronal death, and profound cognitive impairment. Previous studies have indicated that increased Aβ and alterations in the daily sleep-wake cycle are early risk factors and possible predictors of AD. Acute sleep deprivation decreases Aβ clearance, and increased Aβ levels stimulate neuroinflammation and accelerate loss of neurons and synapses. Likewise, it has been shown that there are higher rates of sleep disorders in AD patients. However, limited studies have investigated whether sleep fragmentation accelerates the progression of AD pathology. This partial review will discuss experiments investigating the link between sleep and AD. Additionally, we completed three pilot studies exploring whether chronic disruption of daily sleep-wake cycles with sleep fragmentation (SF) increases Aβ and neuroinflammation in the brains of transgenic mice that serve as an experimental model for AD. Mice were sorted into an undisturbed sleep (US) group and an SF group, involving stimulation for one-hour periods during the light phase, 4 times/day, 5 days/week for 4 weeks. Sleep monitoring using the noninvasive piezoelectric system showed that the US mice slept as expected during the light phase; however, SF mice had greatly reduced sleep during the SF intervals, and sleep loss was only partially restored during the dark period. Protein analysis showed that hippocampal levels of Aβ40 and Aβ42 were significantly increased in SF compared to US mice. Additionally, gene expression markers of neuroinflammation in the hippocampus were significantly elevated in SF mice. These results suggest that fragmentation of the daily sleep-wake cycle stimulates hippocampal levels of Aβ and neuroinflammation. If future rodent studies support these findings that chronic SF advances AD pathology, then improving sleep consolidation would be a potential therapeutic strategy for reducing the progression of AD in humans.

For years, it has been known that there is an important link between sleep and neurodegeneration. We may know from experience that even one night of poor sleep can cause our cognitive function to decline. The past two and a half years, I have worked in a research lab testing the effects of sleep fragmentation on the progression of Alzheimer's disease (AD) in a mouse model. Before I go over the methods and results of these experiments, I will go into depth about the background of sleep, AD, and other studies that have found similar results showing the interconnectedness of sleep and AD.

Part I: Literature Review of Sleep and Alzheimer's Disease

We spend nearly one-third of our lives sleeping, so clearly sleep must be an important physiological process. Additionally, while the amount of sleep may differ between species, almost every animal sleeps, including mammals, birds, reptiles, amphibians, and some insects and fish (Hobson et al., 2005). Despite the universality of sleep, it is still somewhat of a mystery why we sleep. For a while, scientists believed that sleep was a time of greatly reduced brain activity; however, with the discovery of rapid eye movement (REM) sleep, they realized that the brain is very much active during sleep, and may control many important processes (Hobson et al., 2005). Sleep is divided into REM sleep and non-rapid eye movement (NREM) sleep, which is further divided into 3 stages: N1, N2, and N3. Scientists use electroencephalography (EEG), which records electrical activity of the brain, electromyography (EMG), which records muscle activity, and electrooculography (EOG), which measures eye movements, to differentiate among the different sleep stages. One first enters N1 sleep from wake, where EEG waves begin to slow in frequency, and EMG and EOG activity is low, but still present. One progresses through N2 into N3 sleep, which is labelled as slow wave sleep due to the presence of very low frequency (0.5-4 Hz) and high amplitude brain waves. EOG and EMG activity are very low in N3. Slow wave sleep is longest towards the beginning of the night while REM sleep lengthens towards the end of the night. REM sleep is characterized by a very active brain, as illustrated by high frequency waves on the EEG. It can be distinguished from wake because muscles are paralyzed during REM sleep, so there is no EMG activity, and eye movements are very rapid (hence the name rapid eye movement sleep), as shown by increased activity in the EOG. Each sleep cycle through NREM and REM sleep lasts about 90 minutes. We complete sleep cycle after cycle throughout the night, preferably passing through four to six cycles a night, until we awaken, normally out of REM or light sleep. Rodents also show sleep cycles, but they are much shorter than in humans, only lasting about 1-2 minutes. These short sleep cycles are called sleep bouts, and rodents have many sleep bouts throughout the 24-hour day, with increased numbers of sleep bouts during the light phase, or rest period in nocturnal rodents.

There have been many studies addressing the importance of sleep in general and the purpose or function of REM and NREM sleep. One theory of why we sleep is that sleep drives metabolic clearance from the brain. According to this hypothesis, without sleep, harmful substances would build up in the brain and cause problems, such as neurodegeneration. One study that backs up this theory with evidence is from Xie et al. (2013). The brain doesn't have a conventional lymphatic system; instead, it has a glymphatic system, which describes the convective exchange between the cerebrospinal fluid (CSF) and interstitial fluid (ISF). CSF

circulates throughout the brain, which allows for the removal of ISF proteins, like amyloid-beta (Aβ), a major protein involved in AD (as discussed later). This study by Xie et al. (2013) hypothesized that the sleep-wake cycle regulates glymphatic clearance and Aβ clearance is increased during sleep. Using a fluorescent tracer, they discovered that CSF influx is suppressed in conscious/awake mice, meaning less CSF enters the brain and exchanges with ISF. Additionally, Aβ was cleared two times faster in sleeping mice compared to awake mice. This supports the theory that sleep is essential for glymphatic clearance of harmful proteins from the brain.

Another proposed function of sleep is that it helps consolidate memories and improves learning. One study looked at how early nocturnal sleep, dominated by slow wave sleep, and late nocturnal sleep, dominated by REM sleep, affected the consolidation of declarative and procedural memory (Plihal and Born, 1997). Declarative memory is long-term memory associated with facts and experiences while procedural memory is long-term memory associated with performance of tasks without conscious awareness. This study asked healthy men to recall a paired-associate word list (task of declarative memory) and complete a mirror-tracing task (task of procedural memory). Results compared how participants did on each task after wakefulness, early nocturnal sleep, and late nocturnal sleep. Early sleep increased recall of the declarative memory task compared to late sleep, and both were improved compared to wakefulness. Additionally, late sleep improved performance on the procedural memory task compared to early sleep, and both were improved compared to wakefulness. Therefore, this study showed not only that sleep is important for memory consolidation and learning, but that NREM sleep specifically helps improve declarative memory to a higher degree while REM sleep helps improve procedural memory to a greater degree (Plihal and Born, 1997).

Finally, other proposed purposes of sleep include theories related to energy conservation because metabolism is greatly reduced during sleep. One of these theories is the restorative theory, which was proposed because muscle growth, tissue repair, protein synthesis, and growth hormone release occur mainly during sleep. The other is the brain plasticity theory, which states sleep helps brain development and maintenance through synaptic growth and pruning (Tononi and Cirelli, 2003; Tononi and Cirelli, 2014).

Now that the basics of sleep have been covered, we will dive into the fundamentals of Alzheimer's disease (AD). AD affects millions of people, and there is no known cure for it yet. What used to be a mysterious disease has been researched extensively, and we now know some information about its mechanism of action and risk factors. As many people are aware, signs of AD can include memory loss, aphasia (language disturbance), apraxia (impaired motor function), agnosia (failure to recognize objects despite intact sensory functioning), forgetfulness, disorientation/confusion, and disturbance in organization/planning for future events (Schachter and Davis, 2000). At the pathological level, AD is characterized by amyloid-beta (Aβ) plaques and neurofibrillary tangles in the brain, as well as loss of synapses and eventually, neuronal death. Aβ plaques are deposits of insoluble Aβ, which is a protein generated from the amyloid precursor protein (APP). APP can get cleaved along two pathways: the non-amyloidogenic pathway and the amyloidogenic pathway. The non-amyloidogenic pathway involves the enzyme

α-secretase cleaving APP into sAPPα and C83, which is then cleaved by γ-secretase to create p3 and AICD. These molecules are not pathogenic. The amyloidogenic pathway uses the enzyme β-secretase instead to cleave APP to sAPPβ and C99. C99 is cleaved by γ-secretase to form Aβ and AICD (Soria Lopez et al., 2019). It is this Aβ peptide that can aggregate and cause problems in AD patients. γ-secretase can cleave at variable spots along C99, producing Aβ with varying lengths, from 37 to 42 amino acids. The longer the variants, especially Aβ40 and Aβ42, the more toxic they are because they are more likely to aggregate into plaques (Soria Lopez et al., 2019). The accumulation of Aβ causes many downstream effects to occur, such as the hyper-phosphorylation of tau protein. This hyperphosphorylated tau is what makes up neurofibrillary tangles, another hallmark of AD, and results in neuronal death due to neurofibrillary tangles blocking neurons from effectively communicating with each other. Aβ is also toxic because it causes inflammation, oxidative stress, and excitotoxity. Ultimately, the formation of senile Aβ plaques and neurofibrillary tangles leads to neuronal death, synapse loss, and the progression of AD. Typically, there is accelerated neuronal death in the hippocampus and entorhinal cortex first in AD patients (Schachter and Davis, 2000). These brain areas correspond to those involved in memory and learning, giving rise to the common AD symptoms of impaired learning and memory. Ventricles, fluid-filled spaces within the brain, grow larger as the hippocampus and other brain areas shrink. Next, neuronal death will occur in the cerebral cortex, which is responsible for language, reasoning, long-term memory storage, and social behavior, causing a variety of impairments. As time goes on, more neurodegeneration spreads to other brain areas and results in death.

There are several genes and risk factors involved with developing AD. Three mutations were identified in patients with familial early-onset autosomal dominant AD, but these mutations account for fewer than 1% of all AD cases (Schachter and Davis, 2000). These mutations are in the APP, presenilin-1, and presenilin-2 genes, which all cause an increase in the amount of Aβ42 produced (Homolak et al., 2018). The fourth gene that increases the risk of developing AD is apolipoprotein E (APOE) (Schachter and Davis, 2000). One allele of this gene, APOE4, has been shown to increase one's risk of AD, and many patients with both early and late-onset AD have this allele. It is thought that APOE4 enhances Aβ aggregation or decreases its clearance from the brain, leading to AD pathology (Soria Lopez et al., 2019). Finally, another risk factor is age—as one gets older, their chances of developing AD increase substantially.

Next, let's explore how sleep changes in individuals with AD. One of the main reasons AD patients are institutionalized is due to sleep-wake disturbances, including sundowning and nocturnal wandering (Ju et al., 2013). Sundowning describes the restlessness, confusion, and irritability of AD patients as daylight begins to fade, sometimes continuing into the night. This makes it difficult for AD patients to fall asleep and stay in bed. In mild to moderate AD, sleep-wake disturbances such as increased inadvertent daytime napping and insomnia at night affect 25-40% of patients (Ju et al., 2013). Wakefulness after sleep onset (WASO) and sleep latency, or amount of time it takes to fall asleep, increase as AD progresses, and total sleep time often decreases (Peter-Derex et al., 2015). Even more importantly, the pattern of sleep changes to become very fragmented, with less consolidated sleep in the evening and excessive tiredness during the day, which can result in multiple naps. A study by Ju et al. (2013) was conducted to

determine how Aβ deposition in preclinical AD affects sleep quality and quantity. Preclinical AD refers to the period of pathological changes characteristic of AD (such as increased Aβ) but before cognitive symptoms have arisen; it has been found that increased levels of Aβ and tau as well as impaired sleep precludes the arrival of cognitive decline symptoms in AD patients by as many as 10-20 years (Wang and Holtzman, 2019). This study (Ju et al., 2003) used 142 cognitively normal adults aged 45+ and measured their sleep with actigraphy, which is a sensor on one's wrist that monitors rest and activity. They also measured the amount of Aβ42 in the cerebrospinal fluid (CSF), where the less Aβ that is detected in the CSF means more Aβ is accumulating into plaques in the brain instead of being cleared. The 32 participants with amyloid deposition had worse sleep quality as measured by decreased sleep efficiency (percentage of time in bed spent asleep) compared to those without amyloid deposition. Their WASO was also increased, again showing their poorer sleep quality. Additionally, frequent daytime napping (3+ days per week) was significantly associated with amyloid deposition.

Another study by Musiek et al. (2018) found similar results when looking at how the circadian rest-activity pattern changes in preclinical AD. Cognitively normal adults wore actigraphs to measure their rest and activity patterns, and the experimenters also quantified the amount of Aβ (both Aβ42 and plaques) and phosphorylated tau. Individuals with amyloid deposition had significantly more circadian fragmentation as measured by intradaily variability. In other words, these individuals showed an abnormal circadian rhythm with increased nighttime activity and decreased daytime activity. Furthermore, increased CSF levels of phosphorylated tau compared to Aβ42 is a marker of neurodegeneration. This study found that increasing this ratio of phosphorylated tau to Aβ42 was associated with an increase in circadian fragmentation. Once again, this supports the idea that sleep and AD are interconnected—those with AD have altered sleep and circadian rhythms, and this alteration can be seen years before the cognitive symptoms of AD begin. Furthermore, clinical follow up studies have shown that cognitively normal older individuals with high sleep fragmentation had a 1.5-fold increased risk of developing AD while self-reported reduced sleep was associated with a 2-fold increased risk of AD development (Holth et al., 2017). Additionally, worse sleep efficiency and diminished slow wave sleep (SWS) duration are associated with rate of future Aβ accumulation (Winer et al., 2020). This begs the question if sleep plays a causal role in AD pathology and progression, which is what my lab investigates—this will be discussed further below in Part II.

Many studies have shown a bidirectional relationship between sleep alterations and AD progression. Besides poor sleep leading to AD, AD can also contribute to poor sleep. As neurons die and synapses are lost in AD brains, important brain areas for sleep may be degenerated. For instance, loss of cholinergic neurons in the basal forebrain or noradrenergic neurons in the locus coeruleus could affect sleep-wake regulation (Holth et al., 2017). AD can make sleep problems worse for patients due to the destruction of brain areas essential for proper sleep. However, as mentioned above, sleep changes are often seen 15-20 years before AD symptoms begin (Wang and Holtzman, 2019); therefore, it is likely that sleep problems may lead to the progression of AD, and as AD progresses further, it worsens patients' sleep, creating a vicious cycle.

Several studies have shown that sleep deprivation and fragmentation cause increased levels of Aβ and tau to accumulate in the brain, which can lead to the progression of AD. Here, we will explore the specifics of some of these studies that aimed to determine a causal link between sleep and AD. The first study was done by Kang et al. (2009), and they monitored hippocampal levels of Aβ in mice expressing a mutated form of human APP that was known to be associated with AD. First, they looked at how Aβ levels fluctuate through the light/dark cycle. During the night when mice are more active, levels of Aβ are higher; on the contrary, during the light phase when mice tend to sleep more, levels of Aβ are lower. This is the normal, diurnal pattern of Aβ caused by the sleep-wake cycle, and it is likely due to the increased clearance of Aβ observed during sleep. Next, experimenters exposed these mice to six hours of sleep deprivation at the beginning of the light phase. This caused mice to have significantly higher levels of ISF Aβ compared to those not sleep deprived. Additionally, when the sleep deprived mice were allowed to have rebound sleep, their Aβ levels were reduced. Finally, since one instance of sleep deprivation caused significantly increased Aβ levels, experimenters were interested in what chronic sleep deprivation would do to Aβ levels in the hippocampus. Mice were sleep deprived for twenty hours a day for 21 days; after those 21 days, sleep deprived mice showed a significant increase in Aβ plaque formation, supporting the hypothesis that sleep deprivation can directly impact Aβ levels and AD progression.

Another study by Minakawa et al. (2017) investigated the effects of chronic sleep fragmentation on Aβ deposition in the APP/PS1 mouse model of AD. Sleep fragmentation was carried out by allowing mice access to a running wheel in cages with a low volume of water in the bottom. In order to avoid the water, the mice had to sit or run on the wheel. This housing condition altered the sleep/wake pattern of the mice as evidenced by their increased activity during the light phase (when mice are normally less active) and decreased activity during the dark phase (when mice are normally more active). This sleep fragmentation induced in the mice resembles the sleep fragmentation seen in human AD patients who sleep more during the day and have frequent nighttime awakenings. The sleep fragmentation protocol resulted in mice having significantly more Aβ plaque load, including larger plaques and an increased number of plaques in the brain, compared to control. Moreover, this effect was dose-dependent—the greater the sleep fragmentation, the greater the Aβ plaque load.

Ju et al. (2017) aimed to determine how disrupted slow wave sleep (SWS) specifically affects Aβ levels in human CSF. 17 participants slept in the lab and were awoken once the EEG indicated they were in SWS/N3. Those participants that had SWS disruption showed greater increases in levels of Aβ40 and Aβ42 compared to those who slept through the night without any disruption. This study also discovered that participants with worse sleep quality at home, as measured by actigraphy for 6 days, had increased levels of hyperphosphorylated tau, another marker of AD.

Qiu et al. (2016) investigated the effects of chronic sleep deprivation on AD pathology as well as learning and memory in the APP/PS1 mouse model of AD. Mice underwent two months of chronic sleep deprivation (SD) for four hours each day, and experimenters found that chronic SD increased senile Aβ plaque deposition as well as insoluble levels of Aβ in the hippocampus

and cortex. This effect was even long-lasting when tested again at 3 months post-experiment. Additionally, chronic SD increased levels of hyperphosphorylated tau, which also persisted out to 3 months. Interestingly, the study found that chronic SD caused mitochondrial dysfunction and neuronal apoptosis in the hippocampus, leading the experimenters to believe this may be the mechanism by which SD acts to induce pathological changes in the brain. Finally, the study also looked at how SD affected learning and memory using an operant learning chamber with four corners. A water bottle was placed in one corner and mice were trained to go to that corner and "nosepoke" for a drink. Later, the water was placed in a different corner of the chamber and the frequency of correct and incorrect visits along with nosepokes were counted. Chronic SD resulted in more incorrect visits and nosepokes. This study painted a wonderful picture of how sleep deprivation increases levels of Aβ and tau, causes neuronal death, and even detrimentally impacts spatial learning and memory in a mouse model of AD.

Finally, Lim et al. (2013) ran a large study investigating if sleep fragmentation increases one's risk of developing AD. 737 people without dementia wore actigraphs for ten days to measure their sleep and activity and were followed for up to six years to determine the prevalence of AD. Experimenters measured sleep fragmentation using k_{RA}, which is the probability per 15 second epoch of having an arousal after at least five minutes of sleep; thus, a higher k_{RA} would mean greater sleep fragmentation. After the follow-up, 97 people (13%) developed AD, and the sleep fragmentation metric k_{RA} was positively associated with the risk of developing AD. An individual with high (90th percentile) sleep fragmentation had a 1.5-fold increased risk for developing AD as compared to an individual with low (10th percentile) sleep fragmentation. Likewise, increased k_{RA} was associated with lower baseline cognitive performance and a more rapid rate of cognitive decline. This study was instrumental in establishing sleep fragmentation as a risk factor for AD. It joins the studies mentioned above in illustrating the relationship between disrupted/fragmented sleep and AD neuropathology, such as Aβ and tau.

Part II: Methodology and Results of Our Three Pilot Studies

There has been a great deal of growth in the sleep and AD research fields, as evidenced by the studies described above; however, limited studies have shown a causal relationship and investigated whether sleep fragmentation accelerates the progression of AD pathology. We completed three pilot studies exploring whether chronic disruption of daily sleep-wake cycles with sleep fragmentation (SF) increases Aβ and neuroinflammation in the brains of transgenic mice that serve as an experimental model for AD.

Methods:

Experimental Animals and Housing Conditions:

All three pilot studies used 16 female 3xTg-AD mice since females have been shown to be at increased risk for AD as well as show more symptoms than males (Ju et al., 2013). The 3xTg-AD mouse model expresses 3 dementia-related mutations in amyloid precursor protein

(APP), presenilin 1, and tau, resulting in this mouse model developing Aβ plaques and neurofibrillary tangles (Oddo et al., 2003). The Aβ plaques develop first, around 4 months, and continue to increase as the mice age; tau pathology occurs later, at around 12 months (Oddo et al., 2003; Sterniczuk et al., 2010). The mice were kept on a 12:12-hr light/dark cycle with lights on at 7:00 am and lights off at 7:00 pm (19:00). Food and water were provided ad libitum. Mice were group housed with 4 mice/cage during weeks 2 and 3 of the experiment when sleep recording via the piezoelectric system was not happening; however, during the one-hour sleep fragmentation intervals, SF mice were singly housed and then returned to group housing when the SF interval was complete. During weeks 1 and 4, all mice were singly housed in the piezoelectric system setup to measure sleep, which will be discussed more in depth below. The three pilot studies (called SF studies) used female 3xTg-AD mice of different ages: SF 2 used 8-month-old mice, SF 3 used 11-month-old mice, and SF 4 used 14-month-old mice.

Experimental Design:

The three SF studies each ran for a total of 4 weeks. The 16 mice were randomly split into 2 groups, an undisturbed (US) group and an SF group, with 8 mice in each group. The SF group underwent SF intervals that were one hour long and occurred 4 times a day, 5 days a week (Monday-Friday). These SF intervals were equally spaced throughout the light phase, occurring from 9:00-10:00 am, 11:30 am-12:30 pm, 2:00-3:00 pm, and 4:30-5:30 pm. During these SF intervals, the SF mice were kept awake by placing novel toys in their cages or gently tapping the mice with a paintbrush; meanwhile, the US mice were left undisturbed. We did sleep fragmentation this way to best match the sleep profile of human Alzheimer's patients. Many Alzheimer's patients show fragmented/disrupted sleep across the night (normal rest phase), which is what we are mimicking with SF during the light phase, when mice prefer to be asleep. After the 4 weeks of SF were over, all mice were euthanized between 5:30-7:30 pm using CO_2 and decapitated. The brains were dissected and right and left regions of the hippocampus and cerebral cortex were kept for further analysis. One side was used for protein analysis while the other side was used to detect expression of neuroinflammatory markers.

Sleep Monitoring:

Sleep was monitored noninvasively during the first and fourth weeks of each study via the piezoelectric system (Signal Solutions; Lexington, KY). This involves a sensor placed beneath the cages of individual animals that can transform mechanical pressure into electrical signals (Mang et al., 2014). The sensors are very sensitive to vibration and can even detect the rhythmic breathing of mice at ~3 Hz. Awake and active mice that are grooming, eating, running, etc. show high amplitude and frequency waves that are more erratic/irregular in nature. However, mice that are asleep show very regular waves that are low in amplitude and have a frequency of about 3 Hz. The piezo system computes a "decision statistic" for every 2-second interval where a high value indicates a regular signal and the mouse is most likely asleep and a low value indicates an irregular signal and the mouse is most likely awake. An overall decision statistic threshold is computed based on the saddle point of the distribution of decision statistics collected over time—everything above this threshold is considered sleep and everything below this threshold is considered wake. Mang et al. (2014) compared results from the piezoelectric system

to the typical way of monitoring sleep through EEG and found that the piezo system had 90% accuracy. Since EEG is an invasive procedure that requires the surgical implantation of electrodes into the mice's skulls and recovery time, the piezoelectric system is an accurate, noninvasive alternative to EEG.

Using data from the piezoelectric system, average percent daily sleep was calculated for each mouse in addition to percent sleep in both the light and dark phases. Average sleep bout length was also calculated for 24 hours, light phase, and dark phase. A sleep bout begins when a 30-second interval contains greater than 50% sleep and terminates when a 30-second interval contains less than 50% sleep.

Aβ and Neuroinflammatory Marker Analysis:

In order to assess neuropathology, Aβ was extracted from brain samples and quantified. Two forms of Aβ were measured—Aβ40 and Aβ42, with Aβ42 considered more toxic due to its longer length and increased capability of aggregating into plaques. Additionally, two buffers—DEA and RIPA—were used to extract Aβ from the brain samples. DEA-soluble Aβ includes diffusible Aβ monomers and oligomers while RIPA-soluble Aβ includes aggregated Aβ. Once the Aβ was extracted from the hippocampus and cortex brain samples, the amounts were analyzed by sandwich ELISA, or enzyme-linked immunosorbent assay, for all three experiments. This procedure measures the amount of antigen, or Aβ, present between two layers of antibodies, a capture and detection antibody. The detection antibody used is fluorescent, so the concentration of Aβ was determined based off how much fluorescence was given off by the sample. More fluorescence indicated more Aβ present in the sample, which points to that particular mouse having greater AD pathology.

Markers of reactive microglia, reactive astrocytes, proinflammatory cytokines, and inflammatory chemokines were measured in the hippocampus and cortex by TaqMan low density gene expression array in SF 2 and SF 3 mice. The TaqMan technique uses real-time PCR to measure the mRNA expression of genes. Markers of reactive microglia were measured because microglia are macrophages and act as the main immune cells in the brain. It is thought that as Aβ aggregates into plaques, microglia become activated so that they can phagocytize and get rid of this toxic protein (Song and Colonna, 2018). In early stages of AD, microglial activation likely has positive effects in helping to clear Aβ from the brain; however, in later stages of AD, too much microglial activation can actually be detrimental. Chronic, excessive microglial activation can cause the excessive loss of neurons and synapses, accelerating neurodegeneration and cognitive decline. The markers of reactive microglia that were measured in this study included Ctsd, Cst7, and Clec7a. Reactive astrocytes were another cell measured in this study. Astrocytes are the most abundant glial cells in the brain, and have many functions including secretion of nutrients, maintenance of the neuronal microenvironment/homeostasis, and regulation of the blood-brain barrier (Li et al., 2019). However, it has been found that Aβ can disrupt the normal functioning of astrocytes, and these reactive astrocytes can be neurotoxic and produce inflammatory cytokines and reactive oxygen species; thus, this leads to even more neuroinflammation and neurodegeneration (Li et al., 2019). The specific markers of reactive astrocytes measured in this study included GFAP, LCN2, and PTX3.

In addition to microglia and astrocytes, signaling molecules involved in inflammation were measured, including inflammatory cytokines and chemokines. Proinflammatory cytokines are recruited by the immune system when it is activated. This activation can be in response to a foreign invader or, in the case of AD, an accumulation of the toxic protein Aβ. With increased release of cytokines, more immune cells and inflammatory molecules are recruited to the brain, causing neuroinflammation to worsen, leading to neurodegeneration (Song and Colonna, 2018). Several markers of important proinflammatory cytokines were measured in this study, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β. Finally, markers of inflammatory chemokines, including CCL2, CCL3, and CXCL1, were measured. Chemokines are a family of cytokines that play a role in the regulation of the immune system. When functioning properly, they are involved in cell communication/signaling and recruiting immune cells to the site of infection or damage; however, they can become over-active in AD and cause neuroinflammation (Martin and Delarasse, 2018). Chemokines can increase the production of Aβ as well as recruit T cells and over-activate microglia in the brain, leading to neuroinflammation and neuronal death (Martin and Delarasse, 2018).

Results:

Effects of SF on Sleep:

Four time periods were analyzed for sleep differences: baseline, week 1 SF, recovery, and week 4 SF. Baseline was the mice's normal sleep as measured by the piezoelectric system on the weekend prior to the first SF shift. Week 1 and 4 SF was the sleep measured during the first and fourth weeks of the SF protocol respectively (Monday-Friday) when mice were in their piezo cages. Recovery is the weekend of rest between weeks 3 and 4 of the SF study when mice were moved from group housing into piezo cages for sleep recording. The data showed a large reduction in sleep for the SF mice during their sleep fragmentation intervals compared to the US mice (Figure 1). This proves that our SF protocol was successful in keeping SF mice awake during the four SF intervals a day while not overly disturbing the US mice. Figure 1 shows that the US mice slept as expected during the 24-

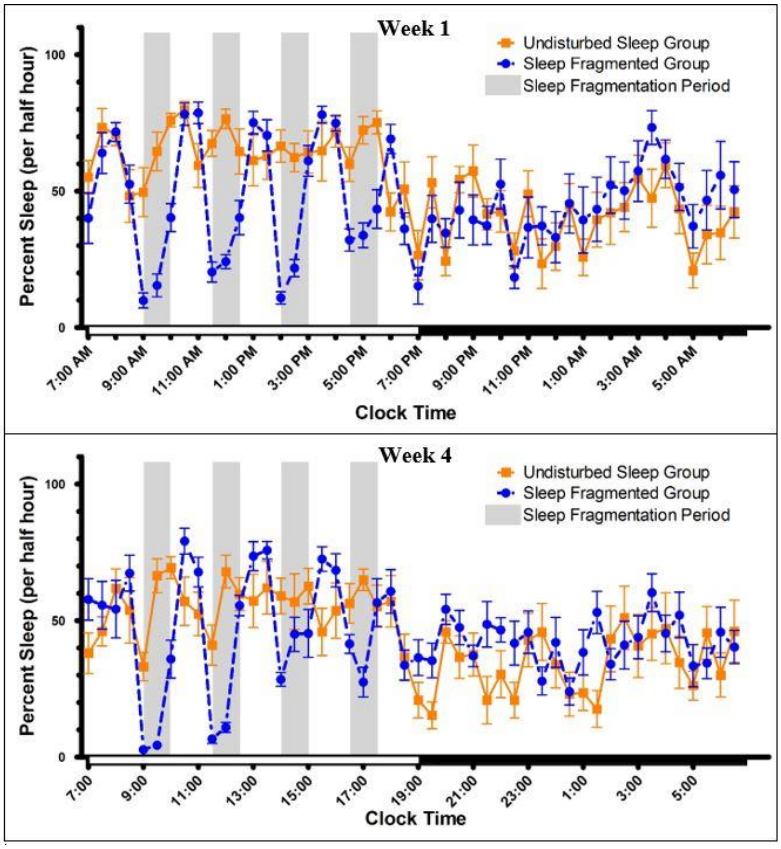

Figure 1. Chronic sleep fragmentation alters the daily sleep profile. Values represent the mean ± SEM sleep percentage for 30 min bins. This data was taken from SF 2 during weeks 1 and 4 as an average of mice in each group across the 5 days of the SF protocol. SF 3 and 4 graphs look very similar so are not shown here. N=8/group. Horizontal white and black bars at the bottom indicate the light and dark phases respectively.

hour day, with about 50-70% sleep during the light phase, or active phase, and about 30-40% sleep during the dark phase, or rest phase. Additionally, Figure 1 shows areas where the SF mice attempt to make up for the sleep loss they incurred from the SF intervals. Inter-fragmentation intervals represent the times during the light phase between the SF intervals, including 10:00-11:30 am, 12:30-2:00 pm, and 3:00-4:30 pm. SF mice experience an increase in sleep during these inter-fragmentation periods, especially by week 4 of SF (Figure 1, Table 1). Additionally, SF mice tend to sleep more than the US mice during the dark phase (when mice are normally active) to make up for their sleep loss. This shows the homeostatic control of sleep, or sleep rebound. The SF mice would like to sleep during the light phase, however our protocol forbids this during certain intervals; as a result, the SF mice have a build up of sleep need and compensate for this by sleeping more during inter-fragmentation intervals and during the dark phase by week 4 of the study.

Table 2 breaks down sleep percentages across 24 hours, dark phase, and light phase for all three experiments (SF 2, 3 and 4) for baseline, week 1 SF, recovery, and week 4 SF. During the first week of SF for all three experiments, SF mice had decreased sleep over 24 hours and during the light phase compared to US mice, but there was no significant change in dark phase sleep. The decrease in sleep was more dramatic during the light phase compared to the 24 hour sleep—for instance, in SF 2, SF mice had decreased sleep compared to US mice by 19.2% in the light phase and only 9.1% over 24 hours. This makes sense because the SF mice had four, one-hour SF intervals during the light phase across the 5 days of the week that greatly reduced their sleep. Any sleep made up during the dark phase would have caused the 24-hour sleep to not be quite as decreased in SF compared to US mice.

By week 4, slightly different sleep trends are seen. In SF 2 and 3, there is no significant change in sleep percentage during the light phase and 24 hours; instead, SF mice show a significant increase in sleep compared to US mice during the dark phase. This data specifically shows the power of sleep rebound. By week 4 of the experiment, SF mice are extremely sleep deprived and will try to make up sleep during the inter-fragmentation intervals and dark phase. This make-up sleep is why SF mice sleep 30.8% (SF 2) and 19.7% (SF 3) more in the dark phase compared to US mice in week 4. At first glance, it may appear strange that SF mice show no difference in sleep compared to US mice during the light phase even though sleep fragmentation intervals were still occurring. The reason behind this is that even though the SF mice had four, one-hour intervals of sleep deprivation during the light phase, they would immediately fall asleep during the other hours of the light phase, especially the inter-fragmentation intervals (Table 1). As stated above, these inter-fragmentation intervals occur between SF sessions, Monday through Friday from 10:00-11:30 am, 12:30-2:00 pm, and 3:00-4:30 pm. Specifically in SF 3, the SF mice showed a 21.4% increase in sleep compared to US mice during the inter-fragmentation intervals of week 4 ($p=0.0009$). This resulted in the lack of significant difference between light phase sleep in SF and US mice in SF 2 and 3. These younger SF mice (8 months old and 11 months old) changed their distribution of sleep across the light and dark phases while keeping their total, 24-hour amount of sleep similar, in response to the SF protocol.

However, the older 14-month-old mice of SF 4 had difficulty in adjusting their distribution of daily sleep in response to the SF protocol. By week 4 of SF 4, the SF mice still had decreased light phase sleep and only showed an insignificant increase in dark phase sleep. This means that the SF mice were kept awake during the SF intervals in the light phase and couldn't recover this sleep during the inter-fragmentation intervals. In fact, there was only a 1.9% increase in sleep in the SF mice compared to US mice during the inter-fragmentation intervals of week 4 for SF 4, and this was insignificant, proving the lack of rebound sleep in SF mice during these time intervals (Table 1). Moreover, the insignificant increase in dark phase sleep in SF mice of SF 4 compared to US mice shows that SF mice had trouble adjusting their daily sleep distribution to recover lost sleep from the SF sessions, and this could be due to their increased age.

Table 1. Sleep Percentage Data during the Inter-fragmentation Intervals. Inter-fragmentation intervals occur between SF sessions, Monday through Friday from 10:00-11:30 am, 12:30-2:00 pm, and 3:00-4:30 pm. Data is shown for each pilot study on weeks 1 and 4 when piezoelectric sleep was recorded, N=8/group.

Sleep %	SF 2				SF 3				SF 4			
Week	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)
1	SF US	63.0 (1.9) 62.8 (1.3)	NS	0.3%	SF US	49.2 (2.5) 51.8 (2.2)	NS	-5.0%	SF US	55.4 (3.4) 61.5 (1.3)	NS	-9.9%
4	SF US	57.5 (1.3) 54.1 (1.4)	NS	6.3%	SF US	63.0 (1.9) 51.9 (1.9)	0.0009	21.4%	SF US	60.4 (2.4) 59.3 (1.5)	NS	1.9%

The youngest mice at 8 months old (SF 2) may have adjusted their sleep best in response to the SF protocol compared to the others. This is evident when looking at the recovery weekend between weeks 3 and 4 of the SF protocol (Table 2). The SF mice from SF 2 were the only SF mice out of all the experiments that showed a significant increase in dark phase sleep and 24-hour sleep compared to US mice. This proves that they had already adjusted their sleep by the recovery weekend instead of waiting until week 4 to adjust (like in SF 3) or not adjusting well at all (like in SF 4).

Table 2. Sleep Percentage Data for SF 2, 3 and 4.
Values represent mean ± SEM. N=8/group.

SF 2 (8-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep %	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**
24-hours	SF US	48.3 (1.9) 51.7 (2.0)	NS	-6.6%	SF US	46.6 (1.4) 51.2 (1.1)	0.0016	-9.1%
Dark phase	SF US	33.5 (1/7) 36.8 (2.0)	NS	-8.8%	SF US	42.8 (1.3) 39.1 (1.1)	NS	9.4%
Light phase	SF US	63.1 (1.6) 66.7 (1.4)	NS	-5.5%	SF US	49.8 (2.3) 61.6 (1.1)	<0.0001	-19.2%
	Recovery				Week 4 Fragmentation			
24-hours	SF US	49.7 (1.6) 44.7 (1.8)	0.034	-11.2%	SF US	45.4 (1.2) 43.7 (1.1)	NS	3.8%
Dark phase	SF US	38.0 (1.4) 30.4 (1.4)	0.0009	25.3%	SF US	42.8 (0.9) 32.8 (1.0)	<0.0001	30.8%
Light phase	SF US	61.4 (1.6) 59.1 (1.4)	NS	3.9%	SF US	47.5 (1/9) 52.5 (1/5)	NS	-9.7%
SF 3 (11-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep %	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**
24-hours	SF US	48.9 (1.5) 47.1 (1.7)	NS	3.8%	SF US	40.8 (1.4) 47.3 (1.3)	0.008	-13.7%
Dark phase	SF US	39.9 (1.6) 37.5 (1.6)	NS	6.4%	SF US	41.8 (1.3) 40.6 (1.4)	NS	3.0%
Light phase	SF US	58.6 (1.8) 57.3 (2.2)	NS	2.3%	SF US	40.0 (2.2) 52.8 (1.8)	0.0002	-24.2%
	Recovery				Week 4 Fragmentation			
24-hours	SF US	52.8 (1.5) 45.7 (1.8)	NS	15.5%	SF US	46.5 (1.4) 45.4 (1.2)	NS	2.4%
Dark phase	SF US	42.7 (1.6) 34.4 (1.6)	NS	24.1%	SF US	42.5 (1.1) 35.5 (1.2)	0.03	19.7%
Light phase	SF US	62.9 (1.6) 57.1 (2.2)	NS	10.2%	SF US	49.9 (2.3) 53.6 (1.5)	NS	-6.9%
SF 4 (14-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep %	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**	**Expt. Group**	**Mean (SEM)**	**P value**	**Av. % change (SF vs US)**
24-hours	SF US	50.5 (1.8) 53.3 (1.9)	NS	-5.3%	SF US	35.5 (3.2) 51.4 (4.2)	<0.0001	-30.9%
Dark phase	SF US	40.5 (1.7) 42.2 (1.6)	NS	-4.0%	SF US	35.4 (1.9) 43.2 (2.9)	NS	-18.1%
Light phase	SF US	60.5 (1.4) 64.3 (1.3)	0.0330	-5.9%	SF US	36.8 (4.2) 60.2 (2.1)	<0.0001	-38.9%
	Recovery				Week 4 Fragmentation			
24-hours	SF US	54.7 (4.9) 53.2 (5.1)	NS	2.8%	SF US	46.3 (5.3) 49.4 (3.8)	NS	-6.3%
Dark phase	SF US	42.4 (1.8) 41.0 (2.8)	NS	3.4%	SF US	43.8 (2.2) 41.0 (2.8)	NS	6.8%
Light phase	SF US	67.0 (2.5) 65.4 (2.6)	NS	2.4%	SF US	48.9 (7.2) 57.7 (1.8)	<0.0001	-15.3%

Sleep percentages were not the only sleep measure affected by the SF protocol—sleep bout duration was altered as well (Table 3), and it showed similar trends to the sleep percentage data. Remember that rodents sleep in shorter sleep cycles called sleep bouts, and the duration of these sleep bouts can change based on sleep need. Longer sleep bouts indicate less sleep fragmentation while shorter sleep bouts indicate more sleep fragmentation. During week 1 of the SF protocol, sleep bout duration was reduced in SF compared to US mice (significant reductions in SF 3 and 4, non-significant reduction in SF 2). Again, this is expected as mice are introduced to the SF intervals where their sleep is being disrupted for four, one-hour intervals during the light phase. Shorter sleep bout durations indicate that sleep is more fragmented and there are more frequent awakenings. Again, by week 4 of SF, SF mice are experiencing longer sleep bouts compared to US mice during the dark phase to make up for their lost sleep during the SF intervals. However, this is only significant in the youngest SF mice (8 months old, SF 2) and not significant in the older SF mice (SF 3 and 4). Therefore, younger mice may be better at adjusting their sleep and increasing their sleep bout lengths during the dark phase in response to the SF protocol to minimize the amount of fragmentation they are being exposed to. We measured sleep bout duration and not the number of sleep bouts, so it is possible that the older mice increased the number of sleep bouts during the dark phase without increasing each sleep bout's length in order to make up for their lost sleep, which is supported by the sleep percentage data.

Furthermore, there are interesting results when looking at how the mice's sleep was altered during the recovery weekend of rest between weeks 3 and 4 of SF. There appears to be a trend towards an increase in sleep bout length in the SF mice during the dark phase, however this is only significant in the 11-month-old mice (SF 3). Additionally, there is a trend towards an increase in sleep bout length during the light phase in the SF mice, however this is only significant in the 14-month-old mice (SF 4). Overall, this again shows that during the recovery weekend, SF mice are attempting to recover their lost sleep through increasing their sleep bout lengths and possibly the number of sleep bouts (not measured).

Table 3. Sleep Bout Duration for SF 2, 3, and 4.
Values represent mean ± SEM. N=8/group

SF 2 (8-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep Bout Length	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)
24-hours	SF	374.7 (25.8)	NS	-14.9%	SF	328.5 (21.3)	NS	-7.5%
	US	440.1 (29.6)			US	355.3 (18.6)		
Dark phase	SF	230.7 (14.4)	NS	-14.3%	SF	309.4 (15.9)	NS	11.7%
	US	269.3 (17.1)			US	277.0 (11.2)		
Light phase	SF	518.7 (26.5)	NS	-15.1%	SF	347.5 (39.6)	NS	-19.9%
	US	610.8 (27.3)			US	433.6 (27.4)		
	Recovery				Week 4 Fragmentation			
24-hours	SF	384.9 (22.1)	NS	21.3%	SF	297.0 (14.5)	NS	4.4%
	US	317.2 (21.0)			US	284.5 (13.5)		
Dark phase	SF	265.1 (13.1)	NS	30.5%	SF	282.4 (11.9)	0.0434	34.2%
	US	203.1 (10.5)			US	210.4 (8.3)		
Light phase	SF	504.6 (24.2)	NS	17.0%	SF	311.5 (26.5)	NS	-13.1%
	US	431.3 (23.5)			US	358.6 (14.1)		
SF 3 (11-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep Bout Length	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)
24-hours	SF	376.0 (18.3)	NS	3.2%	SF	273.3 (15.5)	NS	-10.5%
	US	364.2 (21.7)			US	305.4 (12.2)		
Dark phase	SF	305.0 (15.1)	NS	16.0%	SF	289.2 (9.8)	NS	4.6%
	US	263.0 (15.0)			US	276.5 (16.7)		
Light phase	SF	446.9 (26.5)	NS	-4.0%	SF	257.5 (29.4)	0.0193	-23.0%
	US	465.3 (28.5)			US	334.3 (15.9)		
	Recovery				Week 4 Fragmentation			
24-hours	SF	406.3 (21.7)	0.0471	29.6%	SF	323.9 (22.8)	NS	13.4%
	US	313.5 (21.8)			US	285.7 (15.3)		
Dark phase	SF	315.6 (19.8)	0.0153	48.3%	SF	298.7 (13.0)	NS	28.7%
	US	212.8 (12.5)			US	232.1 (13.3)		
Light phase	SF	496.9 (28.6)	NS	20.0%	SF	349.2 (43.6)	NS	2.9%
	US	414.2 (30.0)			US	339.4 (22.9)		
SF 4 (14-month-old mice)								
	Baseline				Week 1 Fragmentation			
Sleep Bout Length	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)	Expt. Group	Mean (SEM)	P value	Av. % change (SF vs US)
24-hours	SF	362.5 (19.6)	NS	-12.4%	SF	250.5 (13.4)	<0.0001	-30.4%
	US	413.6 (23.1)			US	359.9 (15.3)		
Dark phase	SF	254.1 (13.3)	NS	-13.9%	SF	242.4 (8.7)	NS	-14.3%
	US	295.2 (15.8)			US	282.7 (14.4)		
Light phase	SF	470.9 (19.4)	NS	-11.5%	SF	258.6 (25.5)	<0.0001	-40.8%
	US	531.9 (26.5)			US	437.0 (15.3)		
	Recovery				Week 4 Fragmentation			
24-hours	SF	424.4 (25.9)	NS	7.0%	SF	279.3 (45.5)	NS	-9.1%
	US	396.6 (60.9)			US	307.1 (42.0)		
Dark phase	SF	278.4 (30.4)	NS	5.8%	SF	257.4 (11.6)	NS	11.1%
	US	263.1 (35.4)			US	231.6 (10.3)		
Light phase	SF	563.5 (57.9)	0.009	6.8%	SF	321.3 (66.0)	NS	-22.8%
	US	527.5 (50.8)			US	416.1 (101.2)		

Effects of SF on Aβ:

SF led to increased levels of Aβ in the hippocampus. In the 8-month-old mice of SF 2, sleep fragmentation increased the DEA-soluble and RIPA-soluble levels of Aβ40 in the hippocampus (Figure 2, 3). Also, SF mice had 64% increased levels of RIPA-soluble Aβ42 in the hippocampus compared to US mice (Figure 2, 3). In the cortex, the only significant result found was a small increase in the levels of DEA-soluble Aβ40 in the SF compared to US mice—everything else was insignificant (Figure 3).

In the 11-month-old mice of SF 3, sleep fragmentation increased the RIPA-soluble levels of Aβ42 in the hippocampus. There was no difference in Aβ40 levels in the hippocampus and no significant effect in the cortex of these mice (Figure 3).

Finally, in the 14-month-old mice of SF 4, there were no significant differences in the amount of Aβ found in the hippocampus or cortex in SF compared to US mice. These mice were very old and had much higher levels of Aβ compared to the younger mice of SF 2 and 3 (Figure 4). There may have been a ceiling effect where Aβ levels were already at their maximum and could not be further increased by sleep fragmentation.

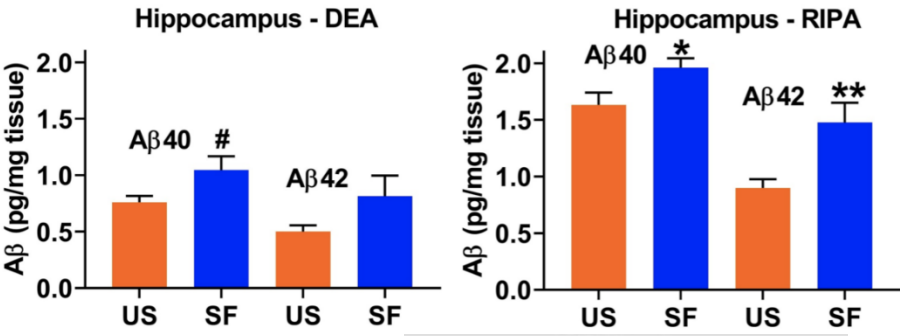

Figure 2. Chronic sleep fragmentation increases Aβ levels in the hippocampus of 8-month-old SF mice. Data show mean ± SEM for N=8/group. #p=0.05, *p<0.05, **p<0.01, unpaired t-test.

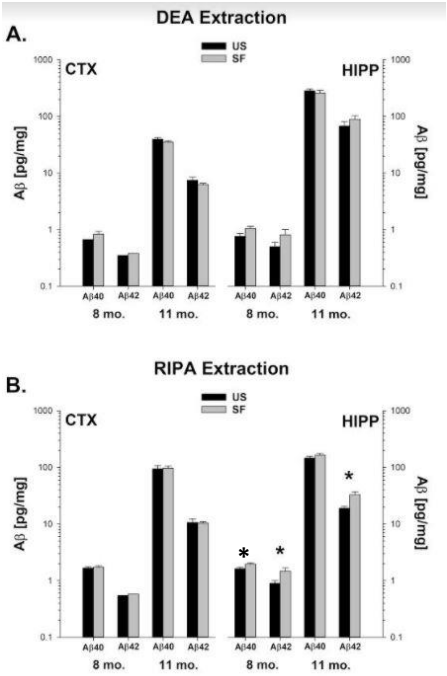

Figure 3. DEA- and RIPA-soluble Aβ levels for SF vs. US mice in SF 2 (8 months old) and SF 3 (11 months old) in the hippocampus (HIPP) and cortex (CTX). Data show mean ± SEM for N=8/group. *p<0.05, unpaired t-test.

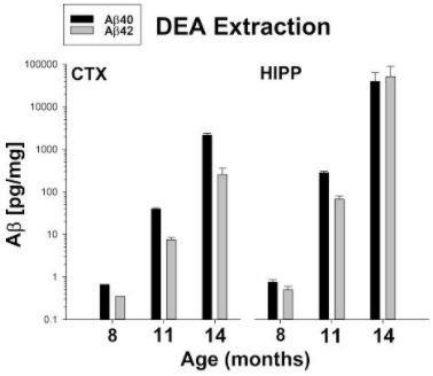

Figure 4. DEA-soluble Aβ40 and Aβ42 levels in mice aged 8 months (SF 2), 11 months (SF 3), and 14 months (SF 4) in the hippocampus (HIPP) and cortex (CTX). Data show mean ± SEM for N=16/age group.

Effects of SF on Neuroinflammation:

Sleep fragmentation caused a significant increase in levels of markers of reactive microglia and trends towards increases in the other markers of neuroinflammation—reactive astrocytes, inflammatory cytokines, and inflammatory chemokines—in the hippocampus of SF mice of 8 months of age (SF 2) and 11 months of age (SF 3). 14-month-old mice from SF 4 were not analyzed for gene expression markers of neuroinflammation. Figure 5A shows Z-scores for the SF versus US mice's expression of each specific marker in the four classes of neuroinflammatory markers measured—a Z-score of 0 represents the mean, or average, levels of neuroinflammatory markers in all SF 2 and 3 mice analyzed (N=30); therefore, a positive Z-score indicates that the mice are expressing higher than average levels of neuroinflammatory markers while a negative Z-score indicates that the mice are expressing lower than average levels of neuroinflammatory markers. Consequently, one can tell from Figure 5A that SF mice are expressing higher levels of each marker of all four classes of neuroinflammatory markers (positive Z-scores) in comparison to the US mice (negative Z-scores). A composite Z-score of the three inflammatory markers in each class uncovered a significant effect of SF on increased expression of reactive microglia (Figure 5B). There was also a trend towards an increase in the three kinds of proinflammatory cytokines expressed in the SF compared to US groups, however this was not significant at a p-value of 0.058 (Figure 5B). Finally, there were no significant differences in any of the four classes of gene expression markers of neuroinflammation in the cortex between SF and US mice at 8 months and 11 months of age.

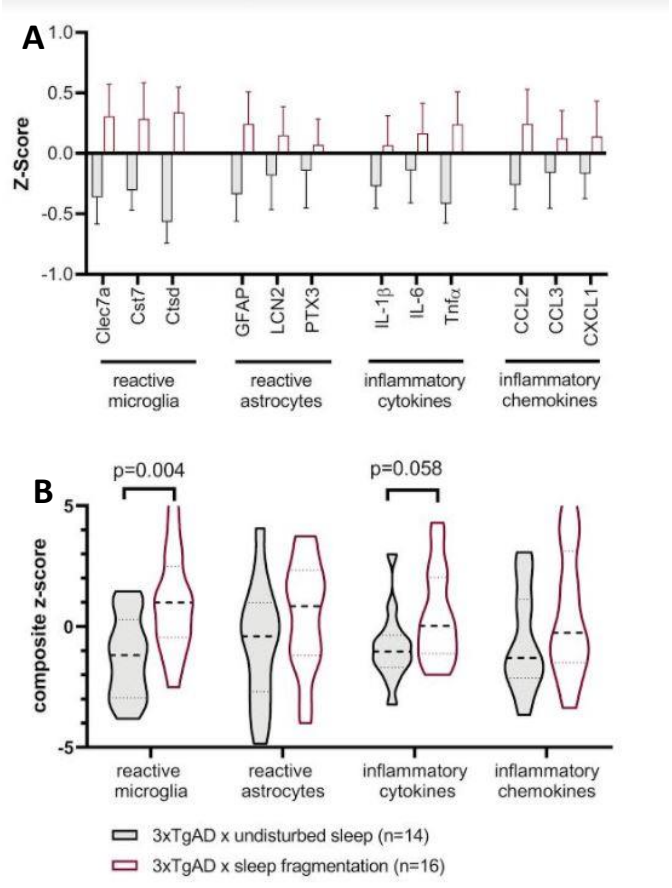

Figure 5. Effects of SF on mRNA neuroinflammatory marker expression in the hippocampus. 4 classes of neuroinflammatory markers were measured in the SF (N=16) and US (N=14) groups. **(A)** shows Z-scores for each of the markers while **(B)** shows composite Z-scores of each of the three neuroinflammatory markers in its class (4 total classes).

Discussion:

This study aimed to determine the effects of fragmentation of the daily sleep-wake rhythm on levels of Aβ and gene expression markers of neuroinflammation in the brains of female 3xTg-AD mice. The 3xTg-AD mice model AD in humans, and they have three mutations that result in the formation of Aβ plaques and neurofibrillary tangles (Oddo et al., 2003). Many humans suffering from Alzheimer's disease have sleep disorders and disrupted sleep-wake cycles, resulting in fragmented sleep during the night and sleepiness during the day (Ju et al., 2013; Peter-Derex et al., 2015; Holth et al., 2017). We completed three pilot studies that aimed to explore a possible causal relationship between sleep and AD in greater detail and see if disruption of the daily sleep-wake cycle via sleep fragmentation can actually lead to the progression of AD-like neuropathological changes. To disrupt the daily sleep-wake cycle of the mice, we subjected them to a SF protocol that involved 4 one-hour-long SF sessions during the light phase, 5 days a week, for 4 weeks. The SF mice were kept awake during these SF intervals by placing novel toys in their cages and lightly tapping the mice with paintbrushes while leaving the US mice undisturbed and allowed to sleep normally. We decided to disrupt their sleep in this manner as opposed to sleep depriving the mice for several straight hours because our protocol more closely mirrors the fragmentation of sleep in humans with AD. Mice normally sleep more during the light phase and less during the dark phase, so our SF intervals during the light phase in mice closely match the frequent awakenings that human AD patients have throughout the night.

The SF protocol altered the daily distribution of sleep and wakefulness. SF mice slept significantly less during the SF intervals from 9:00-10:00 am, 11:30 am-12:30 pm, 2:00-3:00 pm, and 4:30-5:30 pm compared to US mice that were free to sleep as expected during these intervals. In the first week of SF, the SF intervals caused SF mice to have significantly less sleep in the light phase compared to US mice. As a result, the SF mice slept less across 24 hours compared to US mice. This mimics how human AD patients often exhibit less total daily sleep due to frequent nighttime awakenings (Peter-Derex et al., 2015). However, by the recovery weekend and fourth week of each study, the SF mice had adapted to the protocol by altering the daily distribution of their sleep and wakefulness. The SF mice slept more than the US mice during the inter-fragmentation intervals, which were the times during the light phase between the SF intervals. This increase in sleep immediately following SF intervals was the SF mice's way of making up for that lost sleep, which resulted in total light phase sleep between SF and US mice to be not significantly different by week 4, especially in the younger mice of SF 2 and 3. Older mice of SF 4 exhibited this trend, but couldn't quite adjust their sleep schedules as successfully, resulting in some data that was not significant. Additionally, by the recovery weekend and fourth week of each study, the SF mice were sleeping more than the US mice during the dark phase; again, this increase in dark phase sleep was a way for SF mice to make up for their lost sleep incurred from the SF protocol. This mirrors human AD patients experiencing daytime sleepiness and taking naps during their active period to make up for lost sleep at night (Ju et al., 2013). Thus, our experiments illustrate that AD-like neuropathological changes occurred due to altered sleep patterns from the sleep fragmentation protocol, and not because of mice getting less total 24-hour sleep. This is interesting because previous studies have shown that fragmentation of the

daily sleep-wake rhythm even without loss of total sleep is associated with increased risk of AD (Lim et al., 2013). Furthermore, it was interesting that the youngest SF mice of SF 2 even had increased sleep bout lengths in the dark phase by week 4 of the study, indicating that they were sleeping for longer periods of time with less fragmentation during their normally active phase to make up for lost sleep. Overall, the younger SF mice of SF 2 and 3 were better able to alter their daily distribution of sleep and wakefulness in response to the SF protocol compared to the older mice of SF 4 experiencing sleep fragmentation. This mirrors how as AD progresses in older human patients, sleep disturbances get worse (Ju et al., 2013). On the other hand, the undisturbed mice of each study continued sleeping "normally," with about 60-70% of their sleep occurring in the light phase and 30-40% occurring during the dark phase.

Furthermore, 3xTg-AD mice (of SF 2 and 3) exposed to sleep fragmentation exhibited significantly higher levels of Aβ in the hippocampus. SF especially induced large increases in the amount of RIPA-soluble Aβ in the hippocampus, which is the less soluble kind of Aβ often responsible for plaques. This suggests that Aβ is building up in the hippocampus and aggregating in response to the SF protocol, increasing the amount of RIPA-soluble Aβ more than DEA-soluble Aβ, which measures smaller Aβ monomers. Likewise, Aβ42, which is the more neurotoxic form of Aβ, increased even more than Aβ40 in SF mice. Older mice of SF 4 did not show significant increases in Aβ in response to sleep fragmentation; however, these older mice already exhibited such high levels of Aβ by the time they were euthanized that a ceiling effect is likely. Moreover, the hippocampus plays a large role in learning and memory, so it makes sense that Aβ would accumulate in the hippocampus in AD, eventually leading to the characteristic symptoms of memory loss and cognitive decline. It is also possible that the hippocampus is very sensitive to altered sleep-wake rhythms, resulting in the higher levels of Aβ found in the hippocampus after SF. There were no changes in the amount of Aβ found in the cortex following sleep fragmentation in all three pilot studies. Perhaps the cortex is not as sensitive to sleep fragmentation as the hippocampus is, or maybe the studies needed to last longer to see an effect in the cortex. The cortex is responsible for many functions, including storing long-term memories after the hippocampus creates these memories. The cortex has been shown to atrophy in AD patients at later stages than the hippocampus, which shows neuropathological changes much earlier (Schachter and Davis, 2000). Because the hippocampus is responsible for short-term memory formation, this also may explain why many AD patients show short-term memory impairment early in their disease progression and lose the ability to remember what day it is or what they ate for breakfast, for example. On the other hand, many early-stage AD patients still have intact long-term memories from their childhoods since this information is stored in the cortex. Therefore, our studies suggest that SF will interfere with short-term memory while having less effect on long-term memory.

3xTg-AD mice (of SF 2 and 3) exposed to sleep fragmentation exhibited significantly higher levels of neuroinflammatory markers of reactive microglia, and increasing trends in the other 3 classes—reactive astrocytes, proinflammatory cytokines, and inflammatory chemokines—in the hippocampus. Similar to Aβ, this effect was not seen in the cortex. It is possible that the increased levels of Aβ caused overactivation and increased expression of

microglia in the hippocampus, resulting in increased neuroinflammation. In humans, this cascade would lead to cell death, synapse loss, and neurodegeneration.

In conclusion, these findings that disruption of the daily sleep-wake cycle with sleep fragmentation increases Aβ levels and expression of neuroinflammatory markers in the hippocampus of 3xTg-AD mice have many important implications. Increases in Aβ levels and expression of neuroinflammatory markers can lead to the progression of AD and worsening of symptoms as more cells die and synapses degrade. While some studies have looked at prolonged sleep deprivation (Qiu et al., 2016; Kang et al., 2009; Kincheski et al., 2017), our version of sleep fragmentation more closely mirrors the altered sleep-wake cycles of human AD patients, and may serve as a better model for sleep disruption in the disease. These findings indicate a strong link between sleep and AD in that sleep can have causal effects on the progression of AD-like neuropathological changes. Future studies should investigate whether sleep consolidation could be a potential therapeutic strategy for reducing the progression of AD neuropathology in humans.

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