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An Exploration of Sleep Fragmentation and Sleep Enhancement in Mice

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Introduction

What is sleep, and why do we do it? Surprisingly, some of the main functions of sleep are relatively unknown. One thing that is known about sleep is that it is vitally important; this can be assumed from its highly conserved nature across the entire animal kingdom. Proper sleep is essential for optimal physical and mental health. Sleep disorders impact the brain as well as overall health in many ways (Capezuti, 2016). Disrupted sleep can be caused by multiple different diseases and disorders. In today's 24/7 society, access to technology and availability of entertainment at all times has contributed to a significant decrease in average sleep time over the past 30 years (Ferrie *et al.*, 2011). Along with this decrease in average night's sleep, the prevalence of sleep disorders in the general population has grown and is continuing to grow at an alarming rate (Ferrie *et al.*, 2011). As important as sleep is, it is surprising that the purpose and mechanisms behind sleep are widely unknown. It is imperative to study sleep disturbances and the diseases that are often related to these disturbances in order to further the understanding of the mechanism of sleep as well as emphasize the importance of sleep in order to improve the health of society.

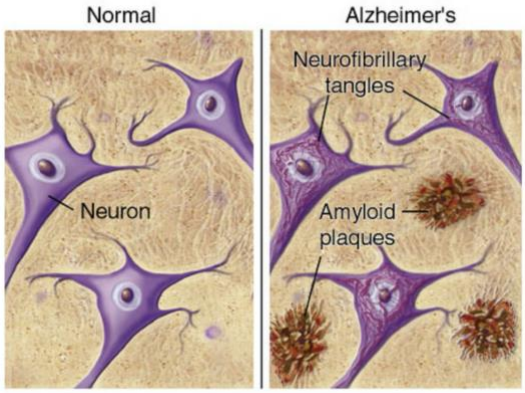

Figure 1. Illistrational example of amyloid beta plaques and neurofibrillary tangles in an AD brain (Amyloid plaques and neurofibrillary tangles).

One disease that has shown to have consistent sleep disruptions alongside its main symptoms is Alzheimer's Disease. Alzheimer's Disease (AD) is a devastating neurodegenerative disease that is defined by memory and cognition impairments, and it affects nearly 5.7 million people in the United States alone (Alzheimer's Disease: Get The Facts 2020). AD cases have grown over 145% in the past 20 years, and the disease is projected to affect 3 times as many people by the year 2050 (Alz.org: Facts and Figures). Although the disease is growing in prevalence, little is known about the cause and cure for AD. In an AD brain, the development of large plaques composed in part of protein fragments and tangles composed largely of hyperphosphorylated tau protein are seen, which form the hallmarks of AD – Amyloid plaques (Aβ) and neurofibrillary tangles. The Aβ protein found in plaques is the result of abnormal cleavage of a normal, healthy amyloid precursor protein by specific secretases. The Aβ cleavage can result in different sizes of the protein, including Aβ 40 and Aβ 42. The Aβ peptides can form soluble oligomers or misfold and aggregate as insoluble plaques that stick to the brain and may cause a lot of damage to the nerves surrounding them (Schmidt *et al.*, 2009), while other evidence suggests the soluble oligomers are much more toxic (Yang *et al.*, 2017). These processes additionally cause neuroinflammation throughout the brain. Figure 1 shows an image of what an AD brain with Aβ plaques and neurofibrillary tangles looks like compared to a normal, healthy brain. Nerve damage due to this Aβ plaque build-up as well as neuroinflammation may be a cause of the severe cognitive decline seen with the development of AD (Schmidt *et al.*, 2009).

The Aβ protein is produced in healthy brains as well, however it is often cleared out of the brain as a waste product (Yoon *et al.*, 2021). Waste clearance in the brain is believed to occur during sleep via the glymphatic system; this system's purpose is to clear soluble proteins and metabolites from the central nervous system using a network of tunnels within the vascular system

(Jessen *et al*., 2015). Lack of sleep could therefore influence Aβ clearance in the brain. AD patients have often been shown to have disrupted sleep symptoms associated with their disease. Previous research has shown up to 40% of people suffering from AD also show sleep disruption along with the hallmark cognitive symptoms (Lloret *et al*., 2020). The sleep pattern of an AD patient is often fragmented with frequent nighttime awakening and daytimes naps (Lloret *et al*., 2020). Due to this disrupted sleep and decreased availability for waste clearance from the brain, these sleep symptoms may be a contributing factor in the progression of AD. Previous studies have indicated that alterations in the daily sleep-wake cycle precede the diagnosis of AD by many years (Bliwise, 2004). A role for sleep disruption in AD pathology has also been supporting sleep mouse models of AD, which accelerates various pathological markers. However, these studies did not mimic the sleep disruption patterns found in people with AD. Therefore, the first goal of this research was to investigate whether the disrupting sleep patterns 4 times/day would provide a better model of AD patients, and the role disrupted sleep might play in the progression of disease itself. This experiment investigated whether chronic disruption of daily sleep-wake cycles with sleep fragmentation increased Aβ 40 and 42 levels and neuroinflammation in the brains of transgenic AD mice (3xTgAD). The 3xTgAD mice express 3 mutations, the $PS1m_{146v}$, APP_{Swe}, and tau_{P301L} transgenes, that are associated with familial, early-onset AD, causing expression of Aβ plaques and tau pathology (Sterniczuk *et al*., 2010). While mice sleep differently than people, with highly polyphasic sleep, and a majority of sleep during the light period, they are still good models for sleep studies because sleep disruptions in rodents show comparable cognitive impairments to humans after sleep disruption, and the basic neurophysiology of sleep is very similar across all mammals. The hypothesis for this study was that sleep fragmentation will increase Aβ 40 and 42 levels as well as neuroinflammation in the brain when compared to the non-sleep fragmented group of transgenic 3xTgAD mice.

If sleep fragmentation may lead to greater progression of AD, then increasing sleep amount or quality may help slow the progression of AD. Often times pharmaceuticals are used to help patients who suffer from sleep disorders, but those come along with undesirable side effects and have not been shown to treat the underlying sleep disorder (Pagel & Parnes, 2001). Another option for sleep enhancement is using mechanical movement. This idea stems from the common way that parents help their babies go to sleep with gentle rocking. Similarly, many people tend to doze off in a car or on a boat due to the nature of slight, consistent movement. Is this phenomenon of rocking inducing sleep scientifically sound and significant? This phenomenon has only just recently been observed in rodents; rodents do not typically rock their young to sleep, but the idea is that the movement could mimic movements of the mother when the babies are in utero. Due to the high similarities between mouse and human sleep and overall physiology, could a mouse model show improved sleep after mechanical rocking? These are the questions that inspired the next research question of this project.

The scientific basis behind gentle rocking and sleep lies in the vestibular system, which is located in the ear with the main purpose of detecting linear and angular motion of the head as well as orientation and perception (Besnard *et al*., 2018). The vestibular organs have direct inputs into the suprachiasmatic nucleus (SCN), which is known as the master circadian clock in the brain with large influences on sleep/wake cycles of both humans and mice (Besnard *et al*., 2018). Additionally, the vestibular organs play an important role in synchronizing the fluctuations of body temperature seen among biological rhythms (Besnard *et al*., 2018). So, when the vestibular organs sense a consistent, light movement, these signals are believed to be transmitted to the SCN and influence sleep (Besnard *et al*., 2018). A previous study found that gentle rocking using a

mechanical rocking platform may help consolidate sleep using a mouse model (Kompotis *et al*., 2019). The study by Kompotis and colleagues used a rocking platform at multiple different frequencies to investigate how this method could be used to improve sleep in mice. This current study aimed to investigate whether mechanical rocking could be used to improve sleep using a mouse model, and whether this method would be successful in helping slow the progression of AD. The hypothesis of this experiment was that slight rocking using a shaker platform will improve sleep in mice, and that improved sleep will slow the progression of AD pathology in the brain.

Methods

Sleep Fragmentation Study

For this experiment, 8-month-old female 3xTgAD mice were used as the model. Only female mice were used in this study due to females expressing a more consistent phenotype as well as the ease of being able to re-group house females without fear of injury from fighting. Mice were kept in standard group housing with four mice to a cage during the protocol, except for weeks 1 and 4 when sleep monitoring required each mouse to be housed individually. The light conditions were standard 12:12, with lights on at 7:00 AM and lights off at 7 PM. All mice were given standard mouse chow and water *ad libitum* throughout the entire protocol.

Sleep was monitored using the PiezoSleep System (Signal Solutions LLC). The PiezoSleep system consists of piezoelectric films placed underneath the floor of the mouse cage (Figure 2). These films detect changes in pressure caused by movement and change these pressures into electrical signals that can be analyzed to detect sleep. The system is precise enough to detect breathing and has been shown to have a classification accuracy of >90% (Mang *et al*., 2014). This option was chosen as it is less invasive than the standard surgery to insert EEG connection in the brain.

Figure 2. The piezoelectric films used to record sleep for both the sleep fragmentation and sleep enhancement studies (www.sigsoln.com).

Mice were sorted into two separated groups: a sleep fragmentation group (SF) and an undisturbed sleep group (US). Both groups consisted of 8 mice each. The protocol was 4 weeks long. During the first and fourth weeks, mice were kept in individual cages equipped with piezoelectric sensors in order to monitor sleep and sleep disruption. During the second and third weeks, mice were group housed with no sleep monitoring.

The sleep fragmentation group underwent four one-hour sleep fragmentation sessions each day, five days a week, for four weeks. During weeks two and three, SF mice were removed from group housing and placed in novel, individual cages for the sleep fragmentation sessions and then placed back into group housing immediately after the sleep fragmentation sessions concluded. The one-hour sleep fragmentation sessions consisted of keeping the mice awake by introducing novel toys such as Legos or cardboard tubes and using a paintbrush to lightly disturb them. The US group mice were left undisturbed during the sleep fragmentation intervals. After the four-week protocol, all mice were anesthetized using CO_2 asphyxiation and brains were harvested after decapitation. The hippocampus and cortex of the brains were extracted and stored frozen at -80°C.

Brain matter was analyzed for Aβ 40 and 42 levels and neuroinflammatory markers (reactive microglial, reactive astrocytes, inflammatory cytokines, and inflammatory chemokines). Aβ 40

and 42 levels were quantified using sandwich ELISAs, using two different assay buffers, radioimmunoprecipitation assay buffer (RIPA) and 0.2% diethylamine (DEA). RIPA buffer was used to measure aggregated and protofibrillar Aβ, while the DEA buffer measured diffusible Aβ monomers and oligomers. Neuroinflammatory markers were quantified using RNeasy mini columns as well as High-Capacity cDNA Reverse Transcription Kits. Sleep data was collected from the piezoelectric system in 30-minute bins. For each 30-minute interval, the percent sleep was recorded as an average of that 30-minute bin. Data was analyzed using GraphPad Prism and 2-way repeated measures ANOVA by group and time.

Sleep Enhancement Study

For this experiment, three seven-month-old C57/BL6 (commonly referred to as black 6) mice were used as the model. One mouse that was used was a male and the other two were female. All mice were kept on a 12:12 light dark schedule (lights on 7:00 AM – 7:00 PM) in standard group housing with standard mouse chow and water *ad libitum.* The protocol was performed on the mice one at a time. During the four-day experiment, the mouse was housed individually in a cage equipped with piezoelectric system film (Signal Solutions LLC) and placed upon the MouseQwake platform (Signal Solutions LLC). The set-up of the cages was as follows: the MouseQwake platform rested on a shelf, with a piezoelectric film directly on top of it, and then the standard mouse cage with food and water placed on top of the film. A large rubber band was used to secure the cage to the MouseQwake platform to ensure that nothing fell out of place while the platform was rocking. Additionally, a metal water bottle stand was screwed into the base of the MouseQwake platform to ensure that the rocking wouldn't cause the water bottle to leak.

For the four-day protocol, the first day was an acclimation day where the mouse was placed into the experimental cage set-up and no data was analyzed. The second day was a baseline day, where no rocking occurred, and the recording began at lights on (7:00 AM). Day three was the experimental day. The MouseQwake platform rocked from 7:00 AM-7:00 PM at 2 Hz frequency and 30 m amplitude. The last day, day four, was a recovery day where no rocking occurred, and the data analysis ended at 7:00 AM the next morning. Each mouse's tail was then marked using sharpie before returning it to group housing after the four-day protocol was complete. Sleep data was analyzed in 60-minute bins, with average total percent sleep for the 60-minute interval recorded. Sleep stats and GraphPad Prism were used to statistically analyze data (2-way ANOVA) and create graphs.

Results

Sleep Fragmentation Study

Results from the piezoelectric monitoring showed that sleep/wake patterns were successfully altered using the sleep fragmentation sessions. Total sleep percent was significantly reduced during the one-hour sleep fragmentation sessions ($p<0.01$). Figure 3 illustrates the average percent sleep for each hour on week one and week four. During week one, light phase sleep for the SF group was significantly decreased compared to US; however, week one dark phase sleep was not significantly affected. In contrast, during week four, light phase sleep as well as dark phase sleep was significantly altered for the SF group compared to the US group. Additionally, during week four, mice had a higher percent sleep during the sleep fragmentation intervals. Sleep bout duration was also affected by the sleep fragmentation intervals. During both week one and week four, sleep bout durations were significantly decreased when compared to the US decreased. Sleep

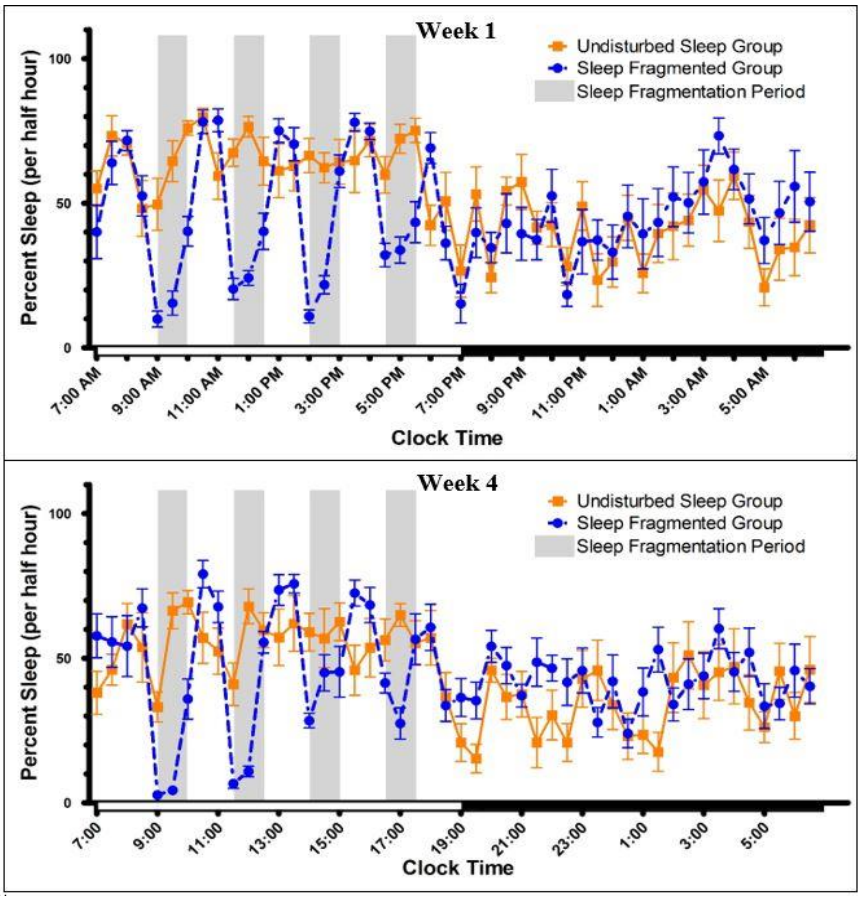

Figure 3. Chronic sleep fragmentation alters the daily sleep profile. Values represent the mean + S.E.M. sleep percentage for 30 min bins, N=8/group, for piezoelectric recordings during weeks 1 and 4. Horizontal white and black bars at the bottom indicate the light and dark phases.

bout length as well as sleep percent was increased during the dark phase for the SF group compared to US.

Results from the ELISA analysis showed that the SF mice trended to having higher levels of both protein sizes in the hippocampus. However, only the Aβ 42 in the RIPA buffer produced significant results in the hippocampus. Chronic SF significantly increased the hippocampal levels of RIPA-soluble Aβ 40 by 20% and Aβ 42 by 64% (significance $p<0.05$) when compared to US. The DEA-soluble Aβ 40 and 42 did not produce significance. This is shown in Figure 4. For the cortex, the data trended higher levels in the SF group however none of the results were significant.

Analysis of neuroinflammation markers showed higher levels in the hippocampus of the SF group compared to US, however this result was not statistically significant. Figure 5 shows the composite z-score for reactive microglial, reactive astrocytes, inflammatory cytokines, and inflammatory chemokines in the hippocampus and neocortex.

Sleep Enhancement Study

Results from the three trials of sleep enhancement of the MouseQwake platform showed that slight rocking did not increase percent sleep or sleep bout lengths. Table 1 shows the average percent sleep values for baseline, experimental, and recovery days. A small trend can be seen that shows slightly less percent sleep during the light phase on the experimental day (when the shaker was rocking the mouse) and then slightly more sleep during the dark phase after the shaker is turned off. Figure 6 shows the total sleep across all three days as well as the average light and dark phase sleep percent averaged over the three days. There was no significant difference between the percent sleep for baseline, experimental, and recovery. There was a trend towards a greater amount of dark phase sleep during the experimental day.

Discussion

Sleep Fragmentation Study

This study established that the 3xTgAD mouse provides a good model for the disrupted sleep/wake patterns demonstrated in AD patients. The findings of this study show that the sleep fragmentation sessions successfully reduced sleep percent during the SF intervals. Overall, it was found that protein levels of Aβ 40 and 42 as well as neuroinflammation markers in the hippocampus were significantly higher in SF mice, leading to the progression of AD neuropathology. Decreased sleep and shortened/fragmented sleep cycles may play a role in limiting the amount of Aβ 40 and 42 cleared from the brain, resulting in increased Aβ deposition and neuroinflammation in the brain, especially in the hippocampus.

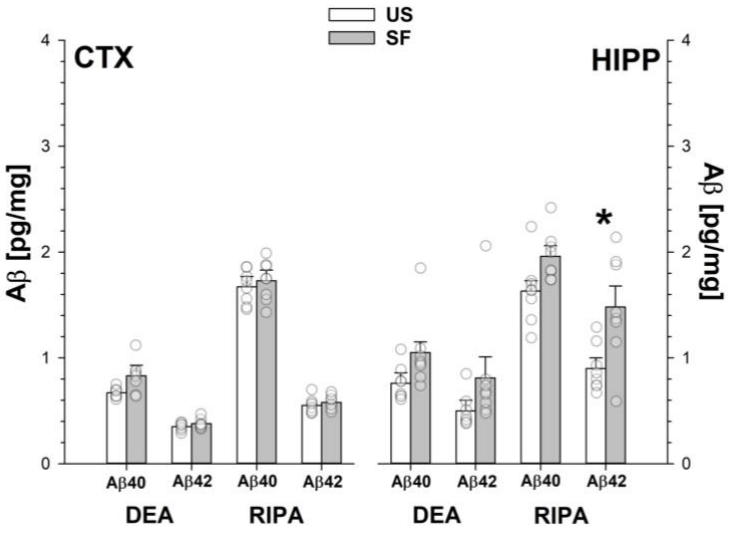

Figure 4. Aβ 40 and 42 levels in RIPA and DEA buffers for the cortex and hippocampus. Approximately a 100-fold difference was found between the cortex and hippocampus. Vertical bars represent mean +/-SEM. Significance was found for Aβ 42 in RIPA buffer ($p<0.05$).

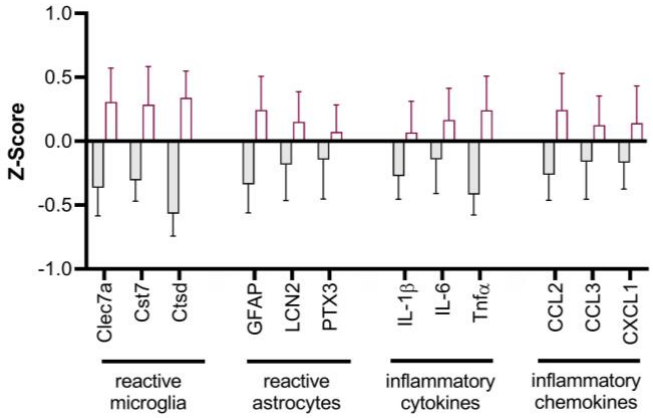

Figure 5. Relative change (z-score) of neuroinflammatory markers. Red is SF and black in US. A significant result was found for reactive microglial signature ($p=0.0461$).

Few previous studies have investigated how sleep fragmentation alters daily sleep patterns. The one-hour sleep fragmentation intervals used in this study are comparable to the frequent nighttime awakenings experienced by AD patients, more so than previous sleep deprivation studies. The results from the sleep data showed that by week four, SF mice had altered sleep in the dark phase as well as light phase, indicating that the sleep fragmentation sessions altered the sleep/wake patterns. These findings also suggested that the SF group experienced longer sleep bouts during the dark phase during recovery sleep in response to chronic sleep fragmentation. During week four, a greater percent sleep was observed during the sleep fragmentation session when compared to week one. This indicated that the SF mice were having a harder time staying awake during the sleep fragmentation sessions, especially towards the end of the light phase, demonstrating the cumulative effects of four weeks of chronic sleep fragmentation. Although the overall reduced total sleep that AD patients often experience was not seen in this mouse model, the sleep fragmentation protocol did mimic the fragmented nature of the human AD patient's sleep pattern.

Analysis of Aβ 40 and 42 showed increases from both buffers in the hippocampus of the SF mice compared to the US mice. However, only the RIPA soluble Aβ 42 in the hippocampus

was significant. A longer sleep fragmentation protocol may be useful to produce significant results for the DEA buffer and RIPA Aβ 40, as AD is a disease that accumulates over many years. These results suggest that continuing the study for a longer period of time may cause additional Aβ build-up and therefore produce more significant results. Additionally, the Aβ 42 protein has been shown to have greater neurotoxicity in the brain, so it may be more influential in the progression of cognitive symptoms associated with AD (Schmidt *et al*., 2009).

The neuroinflammatory markers were found in higher levels in the brains of SF mice, indicating that more damage to neurons were causing greater inflammation as a result of chronic sleep fragmentation. These results suggest that chronic sleep fragmentation as well as greater levels of Aβ drive inflammation. Although results showed trends toward higher levels of neuroinflammation markers, there was a large amount of variability between subjects within both groups. So, more trials with larger sample sizes may help make stronger conclusions about neuroinflammatory markers and AD pathology.

This study had some limitations. Only female mice were used, and this could be an issue because male and female mice tend to have different sleep profiles. Another limitation is that only the 3xTgAD transgenic model was investigated; other transgenic AD mouse models, such as the APP mouse model would be useful to investigate using this same protocol. Additionally, only hippocampal and cortex Aβ and neuroinflammatory levels were measured. AD pathology comes along with other defects in the brain such as neurofibrillary tangles, neuronal death, and cognitive impairments. These hallmarks of AD were not studied in this experiment, so the effect of chronic sleep fragmentation on these facets remain unknown. Another potential limitation is the use of the piezoelectric system. Although this system has the advantage of being non-invasive, the ability to study different phases of sleep such as REM and slow wave sleep are lost by using this method.

Altogether, the 3xTgAD mouse model provides a model that mimics AD patient when exposed to chronic sleep fragmentation. Chronic sleep fragmentation, as seen in AD patients, stimulates greater accumulation of Aβ as well as neuroinflammation in the hippocampus of the brain. Future rodent studies could utilize both sexes and other AD models, with larger sample sizes, and analyzing other aspects of AD such as memory and cognition as well as neuronal death would be beneficial to furthering the understanding of the relationship between sleep and AD. If these future rodent studies continue to support the findings from this experiment, then sleep enhancement and consolidation may be a potential therapeutic method for slowing the progression of AD pathology.

Sleep Enhancement Study

Since it was found that sleep fragmentation expedited the development of AD pathologies in the brain, this study aimed to explore whether sleep enhancement could be used to slow the progression of AD pathology in the brain. First, a sleep enhancement method must be determined. This experiment intended to determine if slight rocking using a mechanical shaker platform, MouseQwake, could improve the sleep of C57/BL6 mice. Significant alterations of sleep were not found when using the mechanical shaker. Due to the very small sample size (n=3) used for this experiment, limited conclusions can be made. The continuation of this experiment with a larger sample size is crucial in order to fully understand the relationship between slight rocking and sleep

	Baseline	Experimental	Recovery
Light phase sleep	53.8%	52.9%	56.4%
Dark phase sleep	31.0%	32.2%	27.9%
Total sleep	42.4%	42.6%	42.1%

Table 1. This table shows the average percent sleep values for all three days of the protocol (n=3).

improvement. A very small trend can be observed in the data, showing that the recovery day had slightly increased amounts of light phase sleep and slightly less dark phase sleep. This may indicate that the slight rocking from the MouseQwake platform caused a slight sleep disruption for the mouse, leading to greater amounts of light phase sleep on the third recovery day. The slight increase in dark phase sleep after the 12 hours of rocking may also indicate sleep recovery from disruption due to the shaker.

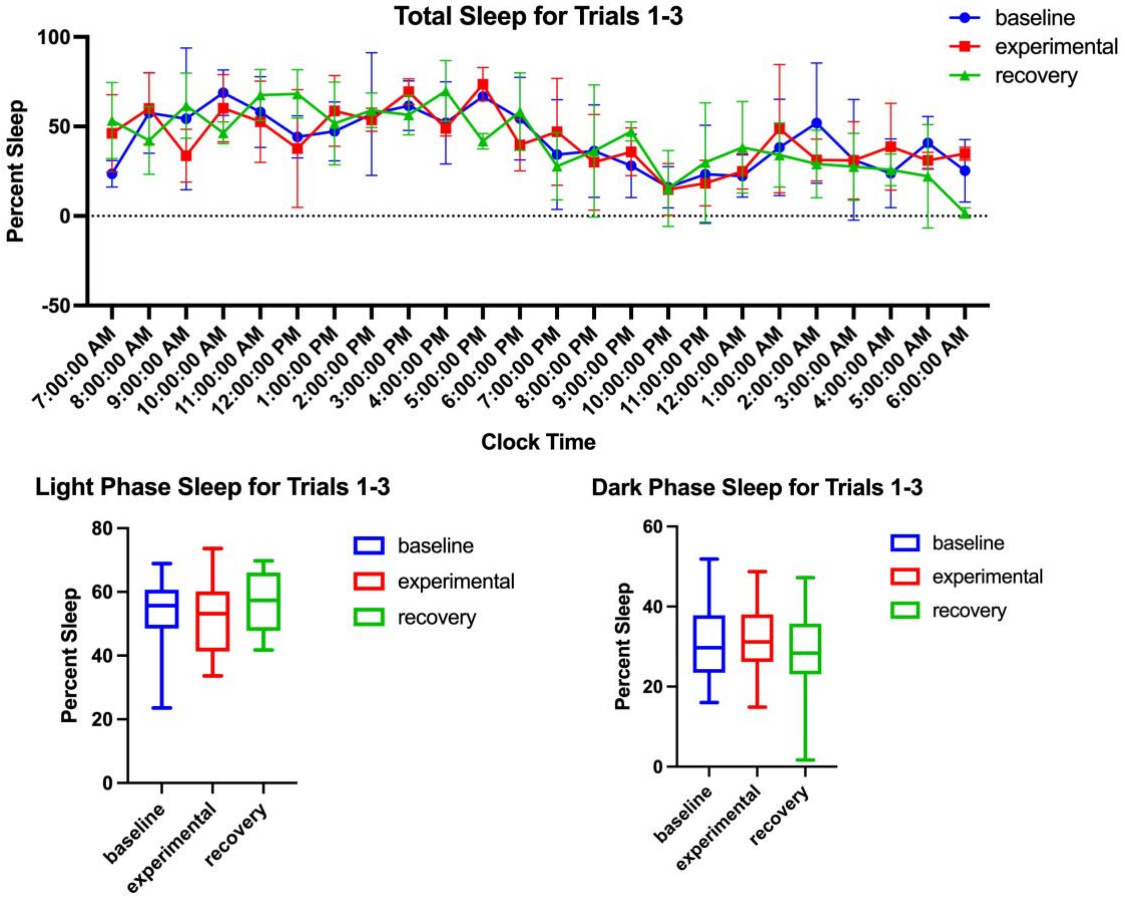

Figure 6. This figure shows the percent sleep values across the three days. No significant results were found. Although, the recovery day trended to have greater light phase sleep and less dark phase sleep.

A previous study, by Kompotis *et al*. in 2019, found significant improvement in sleep when using mechanical rocking on mice. This studied used lateral mechanical rocking of twenty-three C57BL/6J mice monitored using EEG. This studied explored multiple different frequencies but found that 1 Hz and greater had the most significant impact on sleep structure. This study found that slight rocking, which influences the body's vestibular system, has a significant impact on improving NREM sleep. The vestibular system is known to project directly to the brain's central

circadian clock, the SCN. So, this mechanism of action should have a huge influence on the body's daily sleep/wake cycles.

The failure to find significant results for this study may be attributed to the fact that this experiment did not look at NREM and REM sleep cycles, but only sleep percent and mean sleep bout length as a whole. Perhaps the total sleep time and sleep bouts were unaffected, however the theta waves in NREM may have increased. This was not measured using the piezo system, so this would be something important to investigate with future studies. Another limitation to this study was that mice with sleep disorders to begin with were not studied. The sleep percentages recorded for the mice were relatively average for a normal, healthy mouse. Unfortunately, a good mouse model for insomnia or any other sleep disorder has not yet been discovered. A mouse that already experiences healthy sleep may be difficult to try to use when trying to improve sleep. If the mouse is already obtaining normal levels of sleep, then increasing that sleep amount may be difficult.

Another drawback to this experiment was the equipment used. This MouseQwake system was a prototype, and this was the first time it was used for this application. The exact frequency was slightly off, so the desired frequency had to be felt by hand which may be off due to human error. Additionally, every 30 seconds, the shaker "hiccupped" slightly, causing a very slight inconsistency in its rocking. This slight blip may have been enough to disturb a resting mouse's sleep. Additionally, the equipment made a slight humming noise that may have disturbed the mouse in an otherwise quiet environment.

Additional trials will be conducted in order to further the results of this study. Since the current protocol did not successfully show sleep enhancement, future plans to alter the frequency (2 Hz) and amplitude (30 m) settings used are in place in order to investigate whether different frequencies/amplitudes may help improve sleep. Slight rocking may be sleep enhancing, but only at the correct settings. Studies that have found successful sleep enhancement with a shaking device (ex. Kompotis *et al.*, 2019) have used a platform that uses lateral movement in order to have a more "true rocking" motion, whereas the platform used in this current experiment used vibrations to induce the simulation of shaking. So, it is important to continue this experiment using different settings as well as reducing the noise of the equipment and any other confounding variables that may be affecting sleep. If changing the amplitude and frequency of the shaker is not proven successful, then possibly introducing a new type of mechanical device that allows for horizontal and vertical movement, in order to create a true rocking motion, would be beneficial. The future goal of this study is to successfully enhance sleep using mechanical rocking and test to see if enhanced sleep from mechanical rocking can slow the progression of AD pathology using transgenic 3xTgAD mouse models.

Reflection

Participation in this research project has been a huge learning experience for me. First and foremost, I have learned about the process of real-life science: trial and error, success and failure. Not that I would consider my sleep enhancement trials a failure, but I did learn how to adapt and ask new questions when the expected or desired results did not occur. I have learned to critically think and create my own protocol rather than simply follow one that already existed. The lessons I have learned from conducting research are ones I will carry with me throughout my future education and career. I want to thank all of my professors and advisors that have helped me achieve my goals throughout my undergraduate career. I want to specially thank my research mentors, Dr. Bruce O'Hara, Dr. Marilyn Duncan, and (soon to be Dr.) Ren Guerriero, for all the time, effort, and advice they have given me over the past 3 years.

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