THE COMBINED EFFECTS OF A TYPICAL AMERICAN-STYLE DIET AND CHRONIC SLEEP RESTRICTION ON ANXIETY-LIKE BEHAVIOR IN MICE

by

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ABSTRACT

Alzheimer's disease (AD) currently afflicts well over six million people in the United States, and this number is projected to increase exponentially in the coming years. While much remains to be understood about the causes and pathogenesis of AD, two potential risk factors are chronic insufficient sleep and long-term consumption of an unhealthy diet. Both of these lifestyle factors are often studied separately, and evidence suggests that each has negative impacts on brain health and cognitive function, perhaps due to increases in inflammation, which itself is associated with increased anxiety and cognitive dysfunction. The current study investigated the combined effects of long-term consumption of a typical American-style diet (TAD) and six weeks of chronic sleep restriction on locomotor activity and anxiety-like behavior in male and female wild-type mice not otherwise predisposed to disease pathology. Female mice that underwent sleep restriction and consumed the TAD displayed greater anxiety-like behavior compared to mice that the TAD and did not undergo sleep restriction. This difference was not observed in male mice. Furthermore, male mice that underwent chronic sleep restriction displayed greater locomotor activity compared to controls. These differences were not observed in females. Given the prevalence of AD and the projected rise in AD cases, understanding how controllable lifestyle or environmental factors can increase AD risk is essential. Importantly, as AD is more prevalent in women compared to men, it is imperative that research efforts utilize male and female animals seek to understand the mechanisms driving this phenomenon.

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INTRODUCTION

It has become common knowledge that "the mitochondria is the powerhouse of the cell," and the same could be said about the brain running the body. It is a complex and intriguing organ that requires adequate attention because it is the primary processor of memories, emotions, sensations, and bodily regulations. Nonetheless, recognition is not enough, and action is required because there are multifarious diseases associated with the brain, most notedly: Alzheimer's disease.

Alzheimer's disease is a devastating neurodegenerative disease that affects the majority of the world directly or by association. Approximately 44 million people worldwide have Alzheimer's disease, over 6 million of which are people living in America, and a third of affected Americans die with Alzheimer's (Alzheimer's News Today, 2023). The disease takes a devastating toll emotionally and monetarily. The money spent on long-term care facilities, medications, and treatments is outrageous. Furthermore, the actions taken are merely defensive rather than preventative. Therapeutic treatments and drugs are useful in alleviating symptoms, but there are not drugs to treat the pathology effectively.

There is a considerable lack of understanding about Alzheimer's disease, specifically its causes and pathology. Old age, genetics, chronic stress, unhealthy diet, and irregular/inadequate sleep are prominent factors discerned to be associated with the disease. Research surrounding the subject area has inquired whether these constituents promote Alzheimer's disease or if Alzheimer's gives rise to these factors. A bidirectional relationship was noted between the advancement of the disease and these influences. For example, specifically with sleep habits, a connection has been found. UK Biobank is a study that observed the correlation between genetics, sleep, and Alzheimer's. Through surveying 500,000 individuals, the study found a

higher Alzheimer's genetic risk score was associated with shorter sleep for an individual, especially when over 55 years old. A genetic risk, such as APOEε4, influences sleep patterns prior to the onset of dementia (Leng, 2020). Moreover, poor sleep quality is correlated with Alzheimer's disease, and it is often viewed as an early marker for the disease.

Early indicators are salient to distinguish to start treatments in efforts to slow down the onset of Alzheimer's disease. Alzheimer's is often found in those over 65 years old, after cognitive symptoms are present. It is often diagnosed after pathology is present, occurring as a late onset neurodegeneration (Lau, 2023). Memory loss and neurocognitive decline are often viewed as expected effects of older age. With dementia becoming normalized, people typically fail to recognize Alzheimer's affects more than memory; it slowly shuts the body down and takes away one's ability to control basic bodily functions even. It is not always the cause of death, but 44.1% of people at death are found with Alzheimer's dementia, which raises curiosity and urgency to observe the pathology further (Boyle, 2018).

Hallmarks of the disease are neurofibrillary tau tangles and amyloid-beta plaques. Neurofibrillary tau tangles are highly insoluble and proteolysis-resistant protein filaments. The tangles occur in neuron clusters and disturb the connection between the hippocampus and neocortex. In a healthy brain, tau is a microtubule association protein in neurons; it promotes microtubule formation and stability in mature neurons, contributing to neurite outgrowth. Tau's functions are regulated by site-specific phosphorylation. In neurodegenerative diseases, such as Alzheimer's disease, tau gets aberrantly phosphorylated. Hyperphosphorylation results in neurotoxic events: breakdown of microtubules/neurite outgrowth. Additionally, tau folds improperly and begins binding to other tau proteins or other cytosolic proteins, thereby inducing

neurofibrillary tangles (Johnson, 2004). Whereas neurofibrillary tangles form inside neurons, Amyloid beta plaques are protein aggregations between neurons.

The APP gene encodes an amyloid precursor protein, which is known to enhance neural growth and maturation. However, the complete physiological functions of APP are unknown. APP is involved in a cleavage cascade in which one of the products is amyloid beta. These amyloid beta peptides are secreted from the cell as fibrils, which oligomerize and aggregate into plaques. These plaques are known to induce neurotoxicity, neuroinflammation, and synaptic dysfunction. There is more to be understood about the plaques to help understand their role and role in contributing to Alzheimer's disease. Moreover, in a healthy brain, the protein fragments are degraded (Coronel, 2018). However, typically found in Alzheimer's disease patients are accumulations of these sticky protein particles.

Diet has significant effects on health by serving as the nutrient source fueling the body. Improper diet may have serious negative implications on the body, and eating poorly is common in America. Obesity, cardiovascular disease, hypertension, and diabetes are conditions often traced back to an improper diet. The adverse effects of poor nutrition on brain health are often overlooked. According to the National Library of Medicine, the typical American diet includes an excess in calories, saturated fats, sugars, and sodium (Wartella, 2010). These unhealthy foods often include high-fat content, specifically saturated fats, which induces insulin resistance and inflammation. The fats are more difficult to break down and cause inflammation in the body. Furthermore, saturated fats are TLR4 agonists. TLR4 is a member of the toll-like receptor family. When bound, it is activated and leads to an intracellular signaling pathway that results in inflammation. Unhealthy options including refined carbohydrates cause a spiking pattern in blood sugar. The rapid increase in blood sugar levels stresses the body as the body acts quickly

with insulin to normalize the blood sugar level, which increases cortisol released in the body. Cortisol is a hormone produced by the adrenal cortex in response to stress. Chronic cortisol is found to be associated with cognitive decline. The relationship between diet and glucocorticoid is bidirectional. Irregular cortisol concentrations can increase craving for foods rich in calories and quick energy: sugar and fat (Pistollato, 2016). Moreover, the vicious cycle leads to chronic inflammation in the body, along with other negative repercussions: microglia activation, cognitive deficits, and oxidative stress (Mazzei, 2021). High glucocorticoid and cortisol are observed to be positively correlated with amyloid formation by increasing amyloid precursor protein expression. Furthermore, an unhealthy diet is found to cause short and long-term memory impairment and decreased spatial memory with mice (Alzoubi, 2012).

Sleep is pivotal to health and homeostasis in a body, yet its importance is often neglected due to illness, environment, and other stressors. In America, over 50 million Americans report sleep-related problems (American Sleep Apnea Association, 2022). This is devastating, as sleep is salient for healing and protecting the body. Humans store energy during sleep, reverse damages caused while awake, and process learned information. It is important for synaptic plasticity, which when lost, impairs memory and learning. Sleep deficiency is found to be associated with increased oxidative stress, glucocorticoid levels, neuroinflammation, and Alzheimer's disease hallmarks. Moreover, with increasing age, the circadian rhythm is often gradually lost. A meta-analysis of observational studies discovered with increased age, came decreased NREM sleep, which resulted with increased risk for Alzheimer's disease. Decreased NREM sleep is correlated to an increase in amyloid-beta deposition and tau accumulation. There is a positive feedback loop because amyloid-beta plaques further negatively impact sleep quality. The long-term effects are baneful with sleep deficiency resulting in chronic inflammation. The

stress induced by poor quality sleep leads to excessive glucocorticoid levels, which advances neuronal loss and cognitive decline (Lv, 2022).

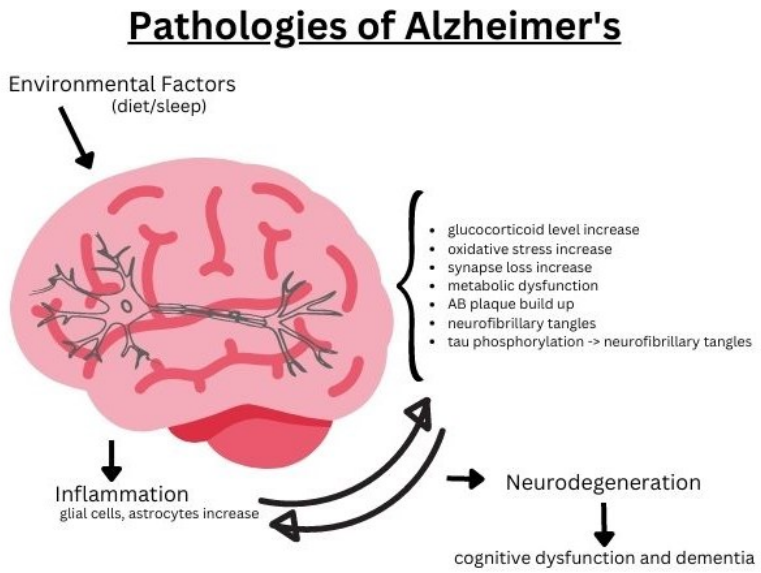

Figure 1: Pathologies of Alzheimer's

The two factors of diet and sleep heavily influence the body individually and collaboratively. Nutrients obtained from a diet impact the glucocorticoid and cortisol levels in the body. Specifically with an improper diet, the high cortisol level impacts one's circadian rhythm and sleep quality. This is a brutal bidirectional relationship with also poor sleep quality resulting in increased cortisol levels, which triggers cravings for low-quality foods (Pistollato, 2016). *Will elaborate on but need organization* The experimental paradigm for the current study was designed to combine the effects of poor sleep and diet in mice.

METHODS

Subjects

Male and female wild-type C57BL/6J mice were used in the experiments. Animals were cared for in accordance with the guidelines set by TCU Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The mice were bred, in the Texas Christian University vivarium from a breeding stock purchased from The Jackson Laboratories in Bar Harbor, Maine, and weaned at around three weeks of age. Groups of 3 to 4 mice were housed in each 12.5 cm x 15 cm x 25 cm polycarbonate cages and subjected to the same 12-hour light/dark cycle. Mice were assigned to standard rodent chow diet or typical American diet. Food hoppers were placed inside the cages filled with the experimental groups' respective diets.

Experimental Groups

This study incorporated a 2x2 design with the first independent variable concerning sleep (controls vs chronic sleep restriction) and the second pertaining to diet (normal rodent chow vs typical American diet).

Mice were randomly assigned as home cage control mice or chronic sleep restriction mice. During the chronic sleep restriction period of the experiment, the HCC mice were transferred to new normal cages daily. This served as a control against chronically sleep-deprived and accounted for daily stress from handling mice. During this time, the CSR mice were transferred to water cages daily, which provided a method of sleep restriction during the mice's normal sleeping hours.

The diets studied compared a standard diet against a typical American diet. The standard chow is one used throughout vivariums in the country. The typical American diet chow was formulated in the lab by graduate student, Paige Braden, in collaboration with the TCU nutrition department, Dr. Jada Willis. The two diets also have distinct sources and percentages of macronutrients. The main point of comparison is the fat percentage kcal. Most other studies present an exaggerated fat concentration in the experiment, but this diet was made to better reflect an average American's diet.

Macronutrients kcal%		
Macronutrients	Standard Chow (Std)	Typical American Diet Chow (TAD)
Protein	21.114%	15.000%
Fat	13.758%	35.000%
Carbohydrate	65.128%	50.000%

Experimental Set-Up

Figure 3: Modified Multiple Platforms

The Modified Multiple Platform Method, which is a widely accepted method of sleep disruption validated by rodent EEG methodology, was used for sleep manipulation. During the sleep restriction portion of the experiment, the experimental sleep-restricted test group was

placed in sleep restriction cages for ten hours a day from 08:00 to 18:00. The sleep restriction cage was a 26.67 cm x 48.26 cm x 15.56 cm polycarbonate box with 14 platforms inside. The platforms are PVC pipe plugs that are 3.3 cm in diameter and 3.2 cm tall. The platforms are glued to the floor of the box with aquarium sealant, roughly 4.5 cm apart from each other in a staggered pattern. The cages were filled with warm water up to 1 cm below the surface of the PVC platforms, and the water was consistently kept warm at 25°C with the cages placed on seedling heating mats (Vivosun, Los Angeles, CA) to prevent hypothermia. Four food hoppers and four water dispensers were allotted a cage to ensure every animal had fair access to both, and the supply was replenished daily to stay full. The platforms were big enough for mice to sit, stand, and move about on. However, the platforms were too small to support high-quality sleep. Every time the mice reached deeper sleep and were not actively working to stay upright, they fell into the water, which woke them up. The mice assigned to home-cage conditions served as a control group in contrast to the sleep-restricted group. These mice were transferred the same times as the experimental group but to clean dry cages. This accounted for potential stress that could have been attributed to handling the mice daily. These 12.5 cm x 15 cm x 25 cm polycarbonate cages were identical to the home cages with the same bedding and food/water sources.

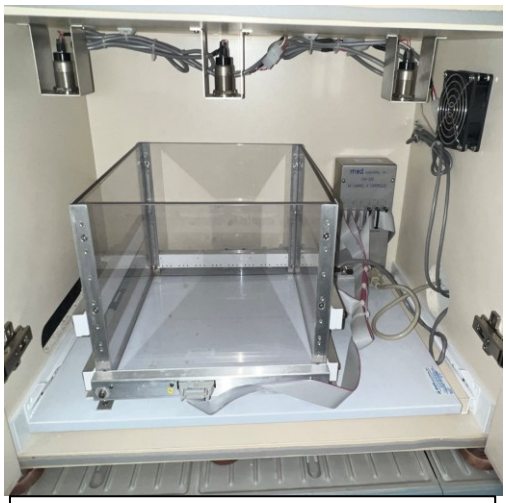

Figure 4: Open Field Arena

After 6 weeks of CSR, open field testing was conducted with the mice. An open field arena is a 27 cm x 27 cm completely enclosed and house light-illuminated box. Between 7:00 and 9:00, animals were each placed alone in the box and allowed to explore for 10 minutes. The Activity Monitor video computer-recording program (Med Associates, Inc., St. Albans, VT) tracked the locomotor activity and anxiety-like behavior using infrared beams. Locomotor activity was determined by average speed (cm/sec), distance traveled (cm), vertical counts, and resting time (sec). Anxiety-like behavior was directly correlated to time spent away from the center of the box. Parts of the arenas were assigned individual zones, which were calculated by dividing the area of the box into 16 squares. The four central squares represented the center zone, which accounted for 50% of the total area.

Experimental Timelines

To control against outliers and ensure soundness of the experiment, 20 mice were observed. About 21 days after birth, when the mice were weaned, they were put on their assigned diet (TAD or Std) and continued living in home cages but a different one separate from their mothers. Weekly, for four months, the mice's body weights and daily diet consumptions were measured

and recorded. Six weeks of chronic sleep restrictions ensued as the mice were continued on their assigned diets. Mice were continued to be weighed weekly. This led up to the testing session on the last day of the chronic sleep restriction period, when mice were put through an open field study to determine general/locomotor activity and exploration habits of the subject.

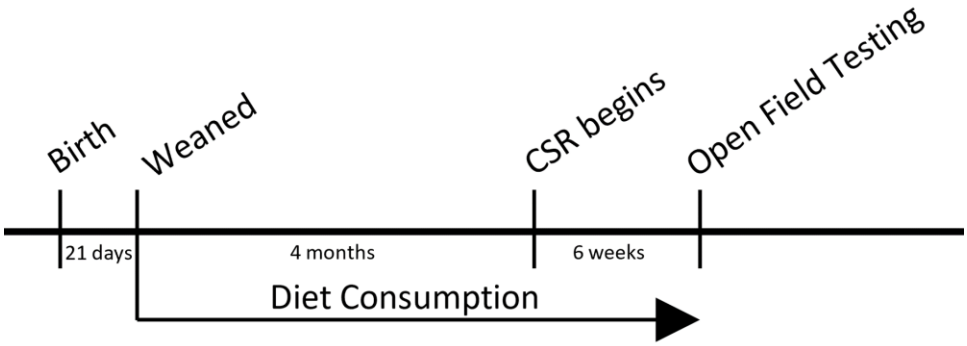

Figure 5: Experimental Timeline

Data Collection

The mice were weighed weekly on a scale. Mice were contained on the scale inside a graduated measuring bucket. The locomotor activity and anxiety-like behavior were tracked by the Activity Monitor video computer-recording program (Med Associates, Inc., St. Albans, VT). The technology emits infrared beams, and when broken, the beams record the data as motion. This allows tracking of the movements of the mouse for a 10-minute period.

RESULTS

Body Weight: There was no significant effect of diets for females, but the males revealed a significant main effect of diet.

Body weight was recorded weekly once mice were weaned onto their assigned diets. In examining body weights, a significant effect of weight gain over the months was observed in both male and female mice. The male mice additionally displayed a trend in increased weight disparity between the diet groups over time starting to exponentially increase at the second month. The TAD diet males gained more weight than the standard diet mice with a significant effect.

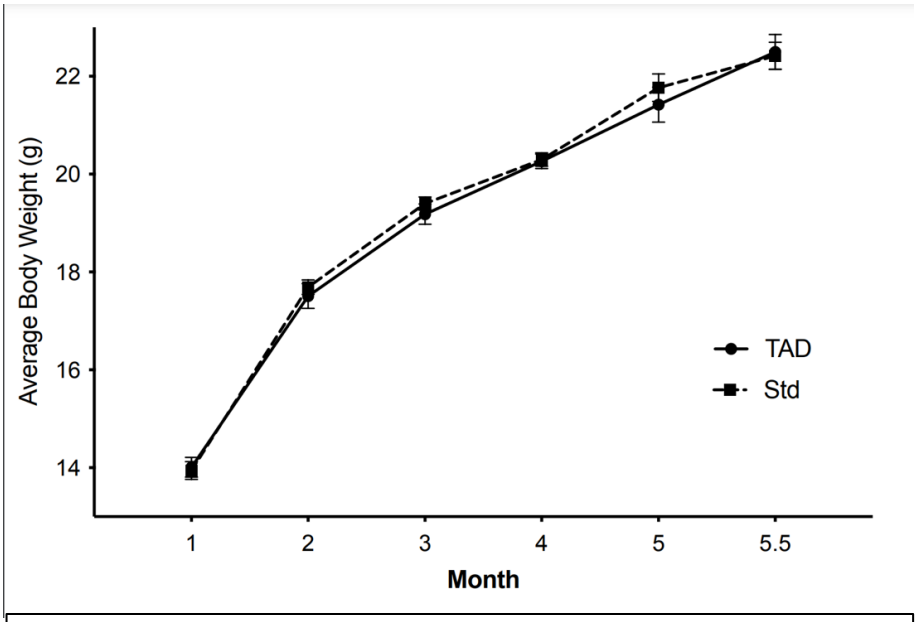

Figure 6: Female Body Weight

Repeated measures ANOVA revealed a significant effect of week such that all mice gained weight throughout the study. Bars represent +/- SEM. N's = 53-54.

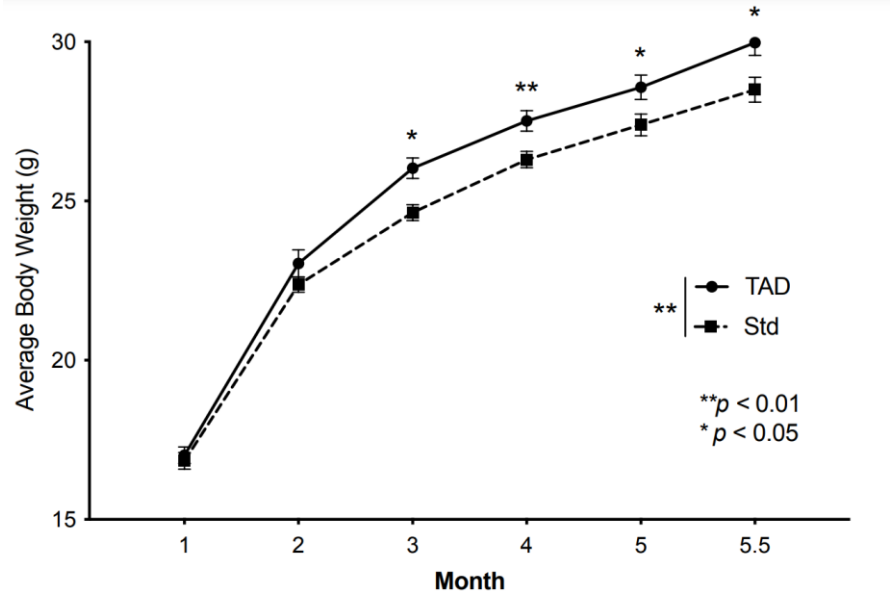

Figure 7: Male Body Weight

Repeated measures ANOVA revealed a significant main effect of diet such that the TAD mice gained more weight than Std mice. Bars represent +/- SEM. N's = 50-54.

Open Field Testing: There was a significant effect of sleep condition on male locomotor activity. There was a significant effect of sleep condition on female anxiety-like behavior.

All mice were allotted 10 minutes to explore in the open field paradigm. Distance traveled in centimeters is associated with locomotor activity. The female groups did not show significant differences. The impacts of sleep and diet in combination had a significant effect on the male mice groups. The male mice that were sleep restricted were recorded to travel greater distances. Time mice spent in the center is viewed as a greater tendency for exploratory behavior. Moreover, if the mice spent more time around the corners and edges, this is viewed to be associated with anxiety-like behavior. The male mice groups did not show significant effects from the assigned sleep and diet conditions. The female groups displayed a significant effect from the combined effects of sleep and diet conditions. The mice assigned to the home cage control spent more time in the center zone in contrast to the mice assigned to the sleep restricted conditions.

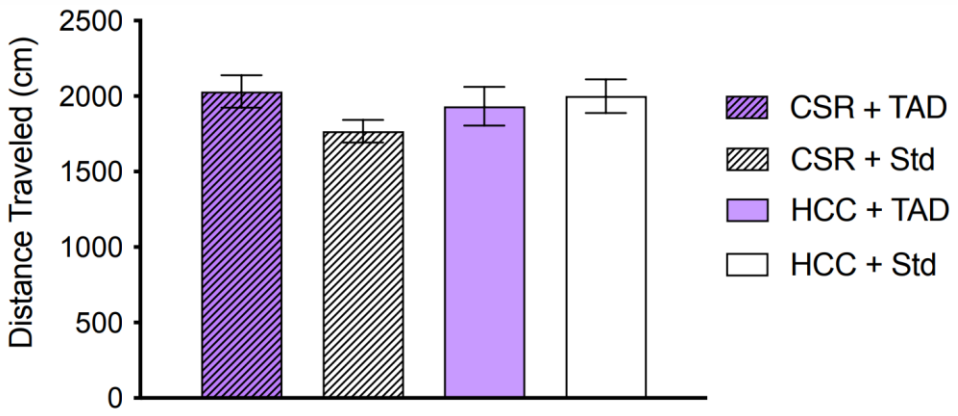

Figure 8: Female Open Field - Distance Traveled

Two-way ANOVAs revealed no significant effects. Bars represent +/- SEM. N's = 14-20.

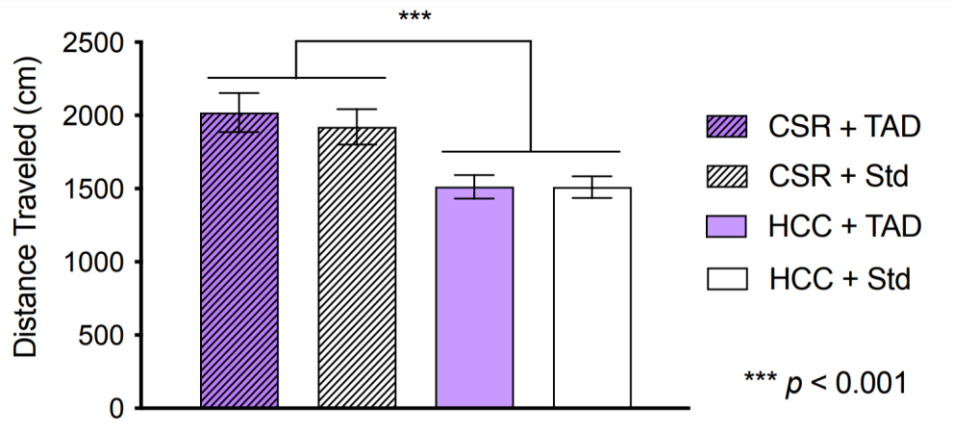

Figure 9: Open Field - Distance Traveled

Two-way ANOVAs revealed a significant main effect of sleep on distance. The CSR mice traveled farther than HCC mice. Bars represent +/- SEM. N's = 14-19.

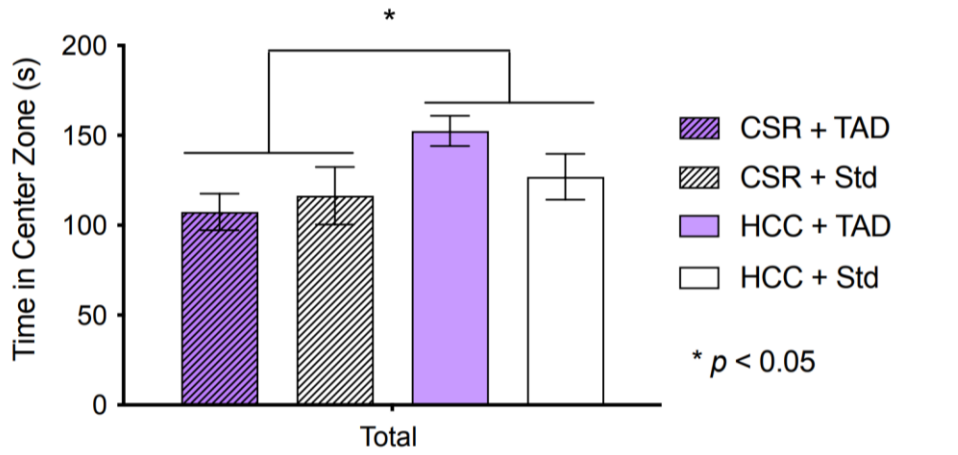

Figure 10: Female Open Field - Time in Center

Two-way ANOVAs revealed a significant main effect of sleep condition on time spent in the center zone. CSR mice spend less time in the center compared to HCC mice. Bars represent +/- SEM. N's = 14-20.

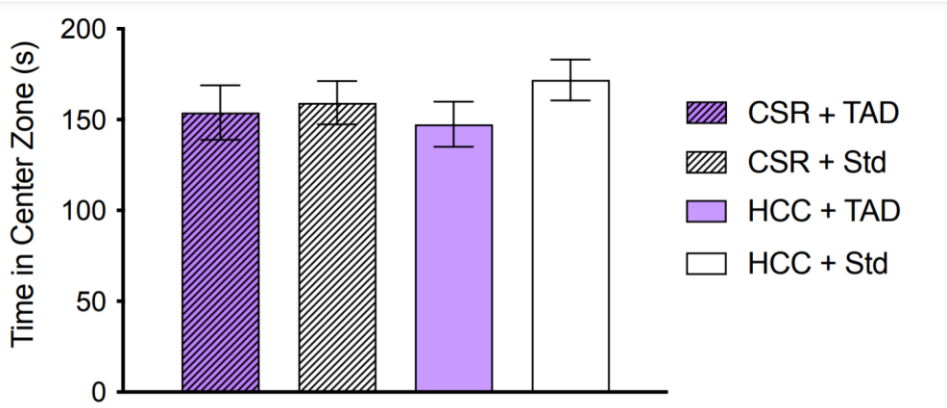

Figure 11: Male Open Field - Time in Center

Two-way ANOVAs revealed no significant effects. Bars represent +/- SEM. N's = 14-19.

DISCUSSION

The purpose of the study was to observe the combination of diet and sleep conditions on a mouse by observing body weight, locomotor activity, and anxiety-like behavior. The hallmark of a typical American diet is excess sodium, fat, and calories from sugar. The diets experimented with (Std and TAD) focused on comparing a difference in fat percentage kcal in the rodent chow. The TAD, which had a higher fat percentage kcal, was predicted to lead to weight gain. The data relayed a significant weight gain with the TAD male mice in comparison to Std male mice starting at the third month of assigned diet consumption. The female mice did not relay a similar significant effect of the TAD in comparison to the Std. This could be explained by the metabolic differences between genders. In another study, male mice were found to accumulate more body fat in comparison to females (Souza, 2022). Moreover, female weight gain might not be as obvious as male mice if they have less potential for a difference in weight gain. The sleep condition did not have a significant effect on weight, but time had a significant effect on weight. The effect of time was expected since it reflects natural growth over time, in which all mice gained weight as they aged.

There was a significant main effect of sleep condition on distance traveled in male mice and there was no significant effect in results for females. However, the male results contradicted the expected results. The hypothesis of sleep restricted mice traveling less distance during the test was based on the prediction of sleep restriction causing fatigue in mice. Nonetheless, a plausible reason could be the design of the sleep restriction paradigm. The modified multiple platforms method depends on mice muscles losing tone, which causes them to fall in the water and wake up. However, as mice repeatedly experience this, they may build muscle mass with time, which could lead to an increased endurance for motion and activity. Body composition was

not measured in the study to confirm the theory. Another possible theory is that the CSR mice feel constricted on the platforms, so when they are provided the chance to roam free in the open field paradigm, they release pent up energy and travel more. It is possible the CSR mice switched circadian rhythms, which would make them more active during the light cycle rather than dark cycle. There was not a means of monitoring the mice during the dark cycle, so this theory should be considered. These are merely potential theories for results unexpected for the lab. However, other research does not necessarily contradict the found data. Another study using the same modified multiple platforms method found that distance traveled among experimental groups was not affected by sleep deprivation (Yin, 2017). Another study found sleep fragmentation led to increased exploratory behavior in mice (Tartar et al., 2009). The literature has conflicting data, which points at more future research. Furthermore, the difference in diets did not result in significant findings, even when the lab predicted TAD mice to be less active. This raises concern to whether sleep may have a stronger effect on mice than sleep conditions.

There was a significant main effect of sleep condition on anxiety like behavior for females and no significant effects on male mice. This was the expected result such that sleep restricted mice exhibited more anxiety-like behavior (Simionato, 2022). An interesting point of discussion is that females rendered significant effects while male mice did not, which hints at a gender discrepancy. Another study found females are more sensitive to stress after sleep disruption in comparison to male mice. This translates to humans such that women are twice as likely to develop mental disorders than men during adolescence, which points at men and women brains being wired differently (Murack, 2021). Moreover, it reflects why only females in the current study portrayed significant effects. However, suspicion should not be eliminated. Future directions taken with this lab may be repeating the experiment to ensure no confounding

variables interfered with the data. Furthermore, other behavioral tests could be performed. An elevated plus or zero maze is a method of testing for behavior. This test may be optimal since it combines natural preferences to assess anxiety-like behavior. Mice naturally prefer darker spaces in contrast to illuminated/open spaces. Utilizing a mouse's natural preferences allows a different angle of analyzing anxiety-like behavior that is well-founded. Furthermore, the bounds of the test are clearer cut such that dark and light are opposites. In contrast, time spent in center or away from center are based on lines experimenters drew to form conclusions. They are more estimates than exact measures.

Other future directions could be experimenting with a different sleep restriction paradigm. The current method of sleep restriction may have led to increased muscle mass in CSR mice, which could have interfered with data. Other methods of sleep restriction create an uncomfortable underlying condition that disrupts sleep: aversive odors, cage exchange, etc. Another testing paradigm may include a forced treadmill during certain periods that keep the mice awake. This may still result in building muscle mass, but it may not be as great of a degree as formed with the modified multiple platforms method. Another approach to build on the current study could be adding a new layer of variables all together. Genetics are a risk factor for Alzheimer's disease, such as individuals expressing an allele that may predispose a mouse to Alzheimer's disease. Therefore, repeating the experiment using transgenic mice that can mimic Alzheimer's disease or related pathologies. Examples are rTg4510 and PS19 mice, which are mutated mice that express a repressible form of human tau (Jankowsky, 2017). The current study utilizes C57B6 mice, which do not display the exact symptoms as humans. Wild-type mice have three amino acid differences in the metal ion binding regions of murine AB, so the amyloid beta does not bind into plaques. Instead of amyloid beta plaques, they develop brain granules in the

hippocampus, especially in older mice (Jucker, 1992). The deposits mimic the plaques and tangles in an older human brain. It is salient to observe other symptoms of Alzheimer's disease, such as anxiety and locomotor activity. These aspects were examined in the current study using various tests. Regardless of the minor differences between mice and humans, the mice served as excellent models for observing the neurobiological effects of diet and sleep.

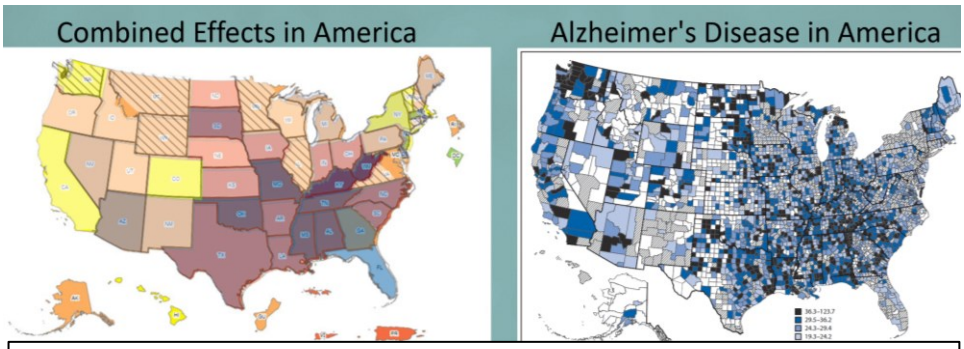

Figure 12: Obesity + Sleep Deprivation + Alzheimer's in America

In relation to humans, it is interesting to observe the overlay of obesity rates and sleep deprivation on the American map closely corresponds to a map representing the case per 1000 for Alzheimer's disease. The relation raises question and concern towards the subject, which makes the research relevant. The data overall hints at adverse effects of poor diet and sleep on Alzheimer's prevalence, and more so, continued research should be done on the matter to better understand the disease and work towards a more effective treatment.

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