CHRONIC SLEEP RESTRICTION EXACERBATES ALZHEIMER'S DISEASE-LIKE PATHOLOGY AND ALTERS PERIPHERAL IL-1β EXPRESSION IN C57BL/6 MICE

by

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1. Introduction

Alzheimer's disease (AD) is the sixth-leading cause of death in the United States and the most common cause of dementia among the elderly (Tarawneh & Holtzman, 2012). More than 44 million people worldwide, including an estimated 5.8 million Americans, are living with this neurodegenerative disease. One in ten people aged 65 or older and nearly one in three people over the age of 85 are diagnosed with AD (Alzheimer's Association, 2019). Further, AD is currently the recorded cause of death for one out of every three elderly people. While deaths from most other diseases have been steadily decreasing since 2000, the number of deaths from AD have increased by 145 percent from 2000 to 2017 (Alzheimer's Association, 2019). Exacerbating an already bad situation, the number of people diagnosed with AD is expected to increase greatly in the coming years, with an estimated 13.8 million people living with AD by 2050 (Alzheimer's Association, 2019). Economically, this malady is nothing short of a disaster. It is estimated that the total cost of care for patients with AD will total $290 billion in 2019 alone (Alzheimer's Association, 2019). Individuals who suffer from AD experience a multitude of symptoms, including memory loss, changes in personality, difficulty executing basic daily tasks, and general cognitive decline. As the disease progresses, these symptoms worsen, and the individual's physical health deteriorates until death eventually results (Alzheimer's Association, 2019). Although specific genetic mutations have been identified as risk factors for developing AD, these account for less than six percent of diagnosed cases of AD (Tarawneh & Holtzman, 2012; Alzheimer's Association, 2019). In most instances, AD is classified as late-onset with no known cause (i.e., sporadic), and no available cure currently exists. Therefore, given the enormous tragedy of this devastating disease in the lives of sufferers and their families, it is

imperative that we seek better understanding of and remediation for the neuropathological mechanisms that underlie the development of this disease.

AD Pathology. The two primary histopathological aspects of AD are the formation of extracellular amyloid-beta plaques and intracellular neurofibrillary tau tangles that both lead to impairments in normal neuronal function. Amyloid-beta, a protein fragment that is produced by amyloid precursor protein (APP), is subsequently processed in one of two pathways. In the nonamyloidogenic pathway, α-secretase cleaves APP into sAPPα and C-terminal fragment (CTF)-83 that is then released into the extracellular space and cleaved by γ-secretase into smaller fragments, preventing the formation of toxic species of amyloid-beta. Alternatively, in the amyloidogenic pathway, APP is cleaved by β-secretase (BACE1) into sAPPβ and CTF-99, which is subsequently cleaved by γ-secretase into the amyloid-beta peptides $A\beta_{40}$ or $A\beta_{42}$ (LaFerla, Green, & Oddo, 2007). In AD, $A\beta_{40}$ and $A\beta_{42}$ peptides are generated more rapidly than they are cleared, initially disrupting synaptic function as oligomers (Cleary et al., 2005; Haass & Selkoe, 2007), and leading to their accumulation outside of neurons and aggregation into extracellular plaques that disrupt neural communication and appear to engender cell death (Sanabria-Castro, Alvarado-Echeverria, & Monge-Bonilla, 2017).

The second pathogenic event that occurs is the increased phosphorylation of tau, a protein that supports and stabilizes microtubules in a normal, functioning neuron. In patients with AD, hyperphosphorylated tau tangles form inside neurons and obstruct the axoplasmic transport of nutrients and proteins within the neurons, thereby contributing to cell death and brain atrophy (Sanabria-Castro et al., 2017). Evidence shows that these changes in the brain may begin over 20 years before symptoms of AD manifest themselves (Alzheimer's Association, 2019).

Risk Factors for AD. Although overt causes remain elusive, many risk factors for developing sporadic AD have been identified. Indeed, considerable evidence exists linking controllable components of lifestyle at mid-life and beyond to an increased risk of developing sporadic AD in older age, including poor cardiovascular health, unhealthy or high-fat diets, smoking, lack of physical activity, and low educational attainment (Xu et al., 2015; Kivipelto, Mangialasche, & Ngandu, 2018). More recently, evidence has also emerged to suggest that chronic sleep loss could potentially act as yet another risk factor for developing AD (Sharma, Sharma, Deshmukh, & Singh, 2015; Spira & Gottesman, 2017). Lending credence to this idea, one of the features observed in many patients with AD is a disrupted sleep/wake cycle (Lucey & Bateman, 2014). Further, regularly getting insufficient sleep is known to be associated with several health risks including cardiovascular disease, obesity, high blood pressure, stroke, depression, anxiety, and impaired immune function (Watson et al., 2015; Frenda & Fenn, 2016). Sleep loss over time is also associated with diminished cognitive ability, including impaired performance on vigilance, alertness, memory consolidation, executive function, and working memory tasks (Van Dongen, Maislin, Mullington, & Dinges, 2003; Frenda & Fenn, 2016). Furthermore, evidence demonstrates that sleep loss over several consecutive nights results in performance decrements equal to one night of total sleep deprivation, suggesting that sleep loss may compound over multiple consecutive nights to cause impairments in aspects of cognitive function (Frenda & Fenn, 2016). Chronic insomnia is a risk factor for accelerated cognitive decline during aging (Cricco, Simonsick, & Foley, 2002). Perhaps most damningly, healthy older adults who experienced greater levels of sleep disturbance exhibited faster cognitive decline and were more likely to develop AD than were older adults with less sleep disturbance (Irvin & Vitiello, 2019). Additionally, sleep decrements due to sleep disordered breathing are

associated with increased risk for developing AD and other dementias (Irvin & Vitiello, 2019). Still other research indicates that disruptions in sleep and circadian function often precede the hallmark symptoms of AD such as cognitive impairments and memory loss, and these sleep disruptions worsen as a potentially destructive feed-forward clockwork of pathology progresses (Lucey & Bateman, 2014; Sharma et al., 2015; Irvin & Vitiello, 2019).

Sleep. Over the past 100 years, people have decreased their sleep time by 20 percent (Sharma et al., 2015). The American Academy of Sleep Medicine and the Sleep Research Society both recommend that adults get 7 to 9 hours of sleep per night (Watson et al., 2015). However, over one third of the U.S. population reports getting less than the recommended minimum 7 hours per night (CDC Data and Statistics, 2017). Furthermore, the number of American adults reporting less than 6 hours of sleep per night has been increasing over the last fifty years (Frenda & Fenn, 2016). This trend also affects younger individuals; although the recommended amount of sleep for adolescents is 8 to 10 hours per night (Paruthi et al., 2016), over 50 percent of individuals between 15 and 17 years of age report getting less than 7 hours of sleep per night (Engle-Friedman, 2014). Unsurprisingly, however, individuals often sacrifice sleep time to accommodate the demands of daily responsibilities that must be completed during waking hours. In addition, the introduction of always-accessible backlit smartphones and tablets and the subsequent rise in internet use throughout society have led to changes in sleep patterns that have likely contributed to the decline in total sleep time among both adults and adolescents (Sharma et al., 2015).

Sleep and Amyloid-beta Pathology. Increasing evidence suggests that there may be a bidirectional relationship between Alzheimer's Disease and disrupted circadian rhythms (Bedrosian & Nelson, 2012; Lucey & Bateman, 2014; Irvin & Vitiello, 2019). However, the

mechanisms underlying this association remain unclear. This point noted, amyloid-beta expression levels themselves may provide a clue to this relationship, in that fluctuation of amyloid-beta levels in the brain follows a diurnal pattern, with higher levels present during wakefulness and lower levels present during sleep (Cedernaes et al., 2017). Sleep apnea, a disorder common among AD patients and related to increased disruptions in sleep, is associated with increases in serum amyloid-beta (Bu et al., 2015). Furthermore, cognitively healthy older adults reporting excessive daytime sleepiness or experiencing reduced sleep efficiency (the amount of time spent asleep during the time spent attempting to sleep) are more likely to have increased amyloid-beta deposition (Lucey et al., 2019). Ju et al. (2017) found that disrupting slow wave sleep in healthy human adults for one night led to increased amyloid-beta in spinal CSF the following morning. Indeed, not only has sleep loss been associated with increased amyloid-beta, but also with elevations in insoluble tau proteins in the human brain (Lucey et al., 2019). In rats, Chen et al. (2017) found that, although 2 and 4 days of sleep deprivation increased levels of amyloid-beta in the cerebral cortex, there was no compensatory upregulation in either neprilysin or insulin-degrading enzyme, two enzymes that are important in the degradation of amyloid-beta, suggesting the possibility that amyloid-beta was being broken down more slowly than it was being produced in animals undergoing sleep deprivation. Additionally, this buildup of amyloid-beta after sleep loss may partially arise due to a dysfunction in clearance. Amyloid-beta is normally cleared via the brain's glymphatic system, which is hypothesized to be most active during sleep (Rainy-Smith et al., 2018). Rainy-Smith and colleagues (2018) found that genetic polymorphisms in Aquaporin-4 (AQP4), a water-channel protein hypothesized to be involved in clearing amyloid-beta from the brain, may moderate the interaction between sleep loss and amyloid-beta increase. However, particularly

germane to the current research, inflammation may also play a significant role in the association between sleep loss and AD-like pathology.

Inflammation and AD. Inflammation increases cognitive deficits and triggers amyloid-beta deposition (Mandrekar & Landreth, 2010). As extracellular amyloid-beta increases, microglia become activated to clear these toxic proteins, along with debris from dead neurons. This triggers an increased release of the proinflammatory cytokines TNFα, IL-1β, and IL-6. Although inflammation is essential for proper immune functioning and defense against and destruction of pathogens, and proinflammatory cytokines also produce some beneficial effects in ameliorating amyloid burden in the short term (Mandrekar & Landreth, 2010), evidence demonstrates that repeated activation of the immune system and extended release of these proinflammatory cytokines can be detrimental to neuronal functioning and lead to increases in amyloid-beta in the hippocampus and cerebral cortex (Lee et al., 2008; Kahn et al., 2012; Weintraub et al., 2014). Converging evidence shows that overexpression of TNFα triggers decreased levels of insulin degrading enzyme, an enzyme that degrades amyloid-beta, leading to exacerbated amyloid-beta deposition (Mandrekar & Landreth, 2010). Furthermore, evidence suggests that chronic inflammation inhibits the ability of microglia to ameliorate amyloid-beta deposition. In turn, increases in amyloid-beta trigger heightened production and release of proinflammatory cytokines, leading to a cyclical feedforward pattern of inflammation and amyloid-beta production and deposition (Mandrekar & Landreth, 2010).

Sleep Loss and Inflammation. Findings have shown that chronic inflammation can result from an extended period of stress. Sleep loss over time is inherently stressful for the body, leading to decreases in parasympathetic activity and increases in blood pressure, evening cortisol, and proinflammatory cytokine production (McEwen, 2006). Moreover, chronic stress

can lead to an exacerbated immune response and increased proinflammatory cytokine production in the hippocampus in response to an acute immune challenge (Johnson, Connor, Deak, Stark, Watkins, & Maier, 2002). Furthermore, proinflammatory cytokines are demonstrated modulators of sleep/wake behaviors (Imeri & Opp, 2010), and TNFα and IL-1β receptors are present throughout the brain, including the hippocampus. In humans, plasma IL-1β concentrations peak during sleep onset, indicating that IL-1β may be important in initiating sleep onset (Imeri & Opp, 2010). Increases in proinflammatory cytokines TNFα and IL-1β lead to increases in non-REM sleep and diminished REM sleep (Opp, 2004). However, this relationship seems to be very complex, as high doses of IL-1β administered at different times during the day have different effects on non-REM sleep onset and duration, and suppressing the release of these cytokines decreases non-REM sleep (Imeri & Opp, 2010). Conversely, mild chronic sleep restriction studies in humans have shown that sleep loss leads to increases in TNFα and IL-6 (Mullington, Simpson, Meier-Ewert, & Haack, 2010). Additionally, rats that underwent 18 hours of sleep restriction every day for 21 days had elevated levels of plasma TNFα and IL-6 compared to controls (Venacio & Suchecki, 2015). Utilizing mice, Kincheski et al. (2017) found that 3 hours of sleep restriction performed five days a week for four weeks increased mRNA levels of IL-1β, but not TNFα in the hippocampus. However, Zielinski et al. (2014) found that, in rats, 5 days of sleep restriction led to increased mRNA for IL-1β and TNFα in the hippocampus and frontal cortex. Based on the evidence that sleep loss is linked to increases in proinflammatory cytokines, and that sustained increased release of proinflammatory cytokines is associated with increased accumulation in amyloid-beta and cognitive impairments, it follows that sleep loss may exacerbate AD-like pathology through its effects upon proinflammatory cytokine production.

At present, a gap exists in the literature regarding the potential link between sleep loss, inflammation, and amyloid-beta pathology. The current research aimed to investigate the effects of chronic sleep loss and increased proinflammatory cytokine expression (following intraperitoneal LPS administration) on neurobiological outcomes and behavioral outcomes, such as learning and memory. Previous work in our laboratory has demonstrated that intraperitoneal administration of LPS (lipopolysaccharide, an endotoxin isolated from a gram-negative bacterial cell wall) leads to increased proinflammatory cytokine action in the brain (Kahn et al., 2012). Furthermore, seven consecutive days of LPS administration was associated with increased levels of amyloid-beta in the hippocampus, as well as deficits in learning and memory (Kahn et al., 2012). In the present research, a chronic sleep restriction protocol was implemented to model sleep loss over time, as most people who suffer from sleep loss typically obtain less than optimal amounts of sleep per night, rather than skipping entire nights of sleep altogether (Frenda & Fenn, 2016). The focus was primarily on examining these effects on the hippocampus, a part of the brain severely impacted by AD that is important in learning and memory and is replete with cytokine receptors (Gadient & Otten, 1994; Parnet, Kelley, Bluthé, & Dantzer, 2002; Sparkman et al., 2006). Investigating the possible interactions between increased proinflammatory cytokines and chronic sleep loss is important, as evidence shows that both may exacerbate neurodegeneration and Alzheimer's disease. Given the large percentage of adults reporting getting less than the minimum recommended 7 hours of sleep per night, combined with the alarming climb in rates of sporadic AD and a growing body of work suggesting a link between these factors, investigating the detrimental effects of not getting enough sleep is an increasingly important area of scientific inquiry.

Hypotheses. We hypothesized that chronic sleep restriction would accelerate amyloid-beta pathology, and that animals that underwent the sleep restriction protocol would have significantly higher levels of soluble amyloid-beta and deficits in contextual fear conditioning (CFC) compared to large cage and home cage control animals. Furthermore, we expected to observe a main effect of LPS injection on amyloid-beta levels and on contextual fear acquisition, with animals receiving seven days of LPS injections showing increased hippocampal amyloid-beta and less freezing behavior (indicative of a corresponding learning deficit) during CFC, compared to conspecifics receiving saline, as these effects have previously been established (Lee et al., 2008; Kahn et al., 2012; White et al., 2016). Finally, we hypothesized a significant interaction effect between sleep loss and inflammation such that hippocampal amyloid-beta and learning deficits due to sleep restriction were further exacerbated by immune stimulation through LPS injections. We hypothesized that hippocampal amyloid-beta and cognitive deficits would prove greatest among the sleep-restricted animals receiving LPS injections compared to all other groups.

2. Materials and Methods

2.1 Subjects

Experimentally-naïve, non-transgenic C57BL/6J mice bred in the Texas Christian University vivarium from a breeding stock acquired from Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME) were utilized in all experiments. In the first two experiments, male mice that were 4–5 months of age at the beginning of the sleep restriction protocol were utilized. Animals used in the third experiment were males that were 14–16 months of age at the beginning of the sleep restriction protocol. All animals received care in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and all studies were

approved by the Texas Christian University Institutional Animal Care and Use Committee (IACUC). Mice were housed in groups of 3–4 in standard polycarbonate mouse cages (30 x 20 x16 cm), under a 12-hour light/dark cycle (lights on from 7:00 am to 7:00 pm), with food and water available *ad libitum*.

2.2 Chronic Sleep Restriction

Animals were assigned to one of three sleep conditions – chronic sleep restriction, large cage control, or home cage control. The modified multiple platform method – a frequently utilized and widely-accepted method of sleep restriction (Colavito et al., 2013; Chen et al., 2017; Yin et al., 2017) that has been validated by rodent EEG methodology (Machado, Hipolide, Benedito-Silva, & Tufik, 2004; Machado, Suchecki, & Tufik, 2006) – was used for the sleep restriction manipulations. Each polycarbonate sleep restriction cage was 26.67 cm X 48.26 cm X 15.56 cm and contained 14 small platforms (PVC pipe plugs glued to the bottom with aquarium sealant) 3.3 cm in diameter, 3.2 cm tall, and placed roughly 4.5 cm apart in a staggered placement pattern. The cages were filled with water, up to 1 cm below the surface of the platforms and were placed on heating mats (Vivosun, Los Angeles, California) that kept the water temperature constant at 25ºC (Yin et al., 2017) and thus prevented subject hypothermia. Animals in the chronic sleep restriction condition were placed with their home cage groups into the sleep restriction cages for 10 hours, from 8:00 am to 6:00 pm. At the end of this 10-hour period, they were returned to their regular home cages for 14 hours, with normal bedding, food, and water. Animals were kept in their group housing assignments to avoid the considerable stress from social instability or isolation (Suchecki & Tufik, 2000). The mice were moved to a new study room for the sleep restriction procedure. The mice undergoing chronic sleep restriction were placed on the platforms at the beginning of the sleep restriction period and were able to

readily move from platform to platform, and easily able to stand. However, when the animals reached the paradoxical stage of sleep (REM) and lost muscle tone, they fell from the platforms into the lukewarm water, causing them to wake, but not undergo hypothermia. Food and water were available *ad libitum* (there were four separate platforms with access to food and four separate platforms with access to water bottles, so no one animal was able to restrict the other animals' access to food or drinking water). The animals in the large cage control group were placed with their cage mates into large platform cages identical to the sleep restriction cages, but with no water below the platforms, to control for the change of environment and aspects of the sleep restriction cage (Ashley, Sams, Brown, & Dumaine, 2016). These large cage control cages were not heated by a heating pad, as this would have caused the control cages to become warmer than the sleep restriction cages. Animals in the home cage control group were placed with their home cage mates into cages identical to their home cages, complete with food, water, and bedding, at the same time and in the same room as the chronic sleep restriction and large cage control animals. In Experiment 1, all animals were weighed daily throughout the protocol to monitor any possible weight loss due to stress. In Experiments 2 and 3, animals were weighed once weekly.

2.3 Intraperitoneal Injections

Previous work has shown that inflammation leads to repeated activation of the immune system and can lead to increases in amyloid-beta and proinflammatory cytokines in the brain, as well as cognitive deficits. This has been accomplished by administering repeated bouts of LPS intraperitoneally (Kahn et al., 2012). LPS elicits an immune response that is similar to that following a bacterial infection. When it enters the organism, LPS binds to CD14 receptors, activating Toll-like receptor 4 (TLR4), which causes a cascade of events that lead to increased

production and release of proinflammatory cytokines from immune cells (Pålsson-McDermott & O'Neill, 2004). After peripheral immune stimulation, proinflammatory cytokines are upregulated in the hippocampus (Tarr et al., 2011; Kahn et al., 2012). Further, our lab has demonstrated that these effects can be exacerbated in the presence of a stressor (Peterman, Eimerbrink, White, Boehm, & Chumley, 2017). The present research aimed to extend the current literature by demonstrating that this exacerbation would also occur in the animals that undergo chronic sleep restriction. On the final seven days of the sleep restriction protocol, all animals received one of two injection treatments – LPS or sterile saline. In Experiments 1 and 2, 4–5-month-old adults received a 250µg/kg dose of LPS (*Escherichia coli* serotype O55:B5; Sigma, St. Louis, MO) injected in the peritoneal cavity or were given 200µL injections of sterile saline (Dulbecco's PBS; Caisson Laboratories, Smithfield, UT). In Experiment 3, 14–16-month-old adults received a smaller dose of LPS in order to avoid sepsis (175µg/kg; *Escherichia coli* serotype O55:B5; Sigma, St. Louis, MO) injected into the peritoneal cavity, or were given 200µL injections of sterile saline. Injections were administered around 7:00 am each morning of the sixth week of the study, just prior to the start of the daily 10-hour period of sleep restiction.

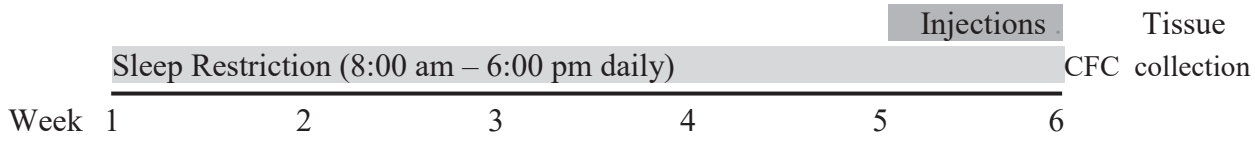

Figure 1. Experimental timeline. Adult C57BL/6 mice underwent a sleep restriction protocol for 10 hours per day for 6 weeks. During the final week of the sleep restriction protocol, all mice received LPS or saline injections. The day following the completion of the protocol, mice underwent contextual fear conditioning, which consisted of a training day and a testing day, with tissue collection occurring the day after the testing session.

2.4 Contextual Fear Conditioning

At the conclusion of the 6-week sleep restriction protocol, including the seven days of LPS or saline injections, all of the animals were trained in a contextual fear conditioning

paradigm. In the training phase, which took place on the day following the seventh injection of LPS or saline and final day of sleep restriction, the animals were placed into automated conditioning chambers with an electified grid floor (Coulborn Instruments, Whitehall, Pennsylvania, 7W x 7D x 12H). A specific visual (polka-dot walls) and olfactory (peppermint oil in a 1:10 dilution in water) context were paired with one or two 2-second mild (0.5mA) footshocks. Animals in Experiments 1 and 3 remained in the conditioning chamber for 180 seconds and received the footshock at 120 seconds. Animals in Experiment 2 remained in the conditioning chamber for 274 seconds and received footshocks at 180 and 242 seconds. On the next day, 24 hours later (between 8:00 am and 10:00 am), the animals were returned to the chambers for the testing phase. The visual and olfactory context remained unchanged, but the animals did not receive a footshock. Freezing behavior was measured and calculated using FreezeFrame™ software (ActiMetrics Software, Wilmette, Illinois) to assess the strength of the learned association between the visual and olfactory context and the aversive footshock. In this behavioral paradigm, animals that learn the association between the context and footshock freeze more than do animals that do not learn the association as well. This paradigm allows for the assessment of amyloid-beta-related learning deficits, as hippocampal impairment diminishes performance in this task.

2.5 Tissue and serum collection

Following the testing session of CFC, the animals were euthanized via rapid decapitation. Trunk blood was obtained and allowed to sit on ice for 15 minutes, followed by incubation at room temperature for 30 minutes. Blood samples were then centrifuged (2,000 x g) for 10 minutes, and serum was isolated and immediately stored at -80º C. Whole hippocampus from both hemispheres of the brain was extracted, preserved in a protein extraction solution containing

protease inhibitors (PRO-PREP, Bulldog Bio, Portsmouth, NH) and snap-frozen on dry ice. Samples were stored at -80º C until processing and analysis. Lysates were centrifuged (16,820 x g) for 20 minutes, and clear lysate was collected.

2.6 Amyloid-beta analysis

A *DC* Protein Assay (Bio-Rad Laboratories, Hercules, CA) was used to assess protein concentrations in hippocampal lysates. After protein quantification, a Mouse Aβ42 ELISA (Invitrogen, ThermoFisher Scientific, Waltham, MA) was performed to measure amyloid-beta. The samples were diluted 1:2 with incubation buffer, loaded onto a precoated microplate in duplicate wells, and allowed to incubate for 2 hours at room temperature. The wells were washed four times, Aβ42 Detection Antibody solution was added to each well, and the plate was allowed to incubate for 1 hour at room temperature. Wells were then washed, exposed to an HRP-tagged detection antibody, and allowed to incubate for 30 minutes at room temperature. Wells were washed, and a stabilized chromogen was added to each well. The plate was allowed to incubate in the dark at room temperature for 30 minutes before a stop solution was added and the optical density was read at 450nm (BMG LabTech FLUOstar Omega, Cary, North Carolina).

2.7 BACE1 Western Blotting

Remaining hippocampal lysates from the first group of experiments were analyzed for BACE1 using Western blotting. Proteins were subjected to SDS-PAGE in a 4–20% Mini-PROTEAN TGX polyacrylamide gel (BioRad, Hercules, CA) and were transferred to 0.45µm Hybond PVDF membranes (Genesee Scientific, San Diego, CA). The membranes were blocked with 5% BSA (albumin, bovine fraction) in Tris-buffered saline with Tween-20 (BSA; Research Products International, Mount Prospect, IL) and were incubated at 4º C with BACE mouse monoclonal primary antibody (Santa Cruz Biotechnology, Dallas, TX) at a 1:750 dilution in

TBST overnight. The following morning, the membranes were washed and incubated at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in a 1:50,000 dilution in TBST for two hours. The membrane was then washed, coated in a chemiluminescent solution (SuperSignal West Pico PLUS Chemiluminescent Substrate, ThermoScientific,Waltham, MA), and imaged (G:Box Chemi ST 4, Syngene, Frederick, MD). B-actin (1:500 in TBST; Santa Cruz Biotechnology, Dallas, TX) was used as the internal reference protein, and band signal intensity was compared using densitometry (GeneTools from Syngene, Frederick, MD).

2.8 MSD Cytokine Quantification

Serum from the animals in the first experiment was assayed for proinflammatory cytokines IL-1β and TNFα and anti-inflammatory cytokine IL-10 using MesoScale Discovery Proinflammatory Panel 1 (mouse) multiplexing kits (Meso Scale Diagnostics, Rockville, MD). Wells were washed three times, and serum was diluted 1:1 in the plates with a proprietary diluent, and allowed to incubate at room temperature with shaking for 2 hours. The wells were then washed three times, and detection antibody solution (for TNFα, IL-1β, and IL-10 antibodies in 2,820 µL of diluent) was added to each well and allowed to incubate at room temperature with shaking for two hours. The wells were washed three more times with wash buffer, after which read buffer was added to each well, and the electrochemiluminescent signal read using a QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD).

2.9 Statistical Analyses

Statistical analyses utilized were 3 (Sleep Condition: chronic sleep restriction, large cage control, vs. home cage control) x 2 (Injection Treatment: LPS vs. saline) analyses of variance (ANOVA), 2 (Sleep Condition: chronic sleep restriction vs. home cage control) x 2 (Injection

Treatment: LPS vs. saline) ANOVA, and repeated-measures ANOVA in SPSS (Version 23.0, IBM, Armonk, NY). The dependent variables assessed were freezing behavior during training and testing days of contextual fear conditioning (there should be no effect of the IVs during training), hippocampal amyloid-beta, hippocampal BACE1, serum levels of IL-10, TNFα, IL-1β, and body weight. Outliers were determined via SPSS's use of the interquartile range rule. All statistical analyses were performed with alpha levels of 0.05, and any significant interactions were unpacked using pairwise comparisons.

3. Results

3.1 Experiment 1A: Body Weight in 4–5-Month-Old Mice

All mice were weighed daily for signs of weight gain or loss. There were no significant changes in weight between the chronic sleep restriction, large cage control, and home cage control groups. A repeated-measures ANOVA (Sleep Condition [chronic sleep restriction vs. large cage control vs. home cage control] x Week [week 1 vs. week 2 vs. week 3 vs. week 4 vs. week 5 vs. week 6]) revealed a significant linear contrast effect of Week such that all animals across groups gained a significant amount of weight throughout the experiment ($F(1,82) = 101.231$, $p < .001$). However, this increase in weight was consistent across all three conditions, and there was no significant main effect of Sleep Condition ($F(2,82) = .057$, $p = .945$), nor any Week x Sleep Condition interaction ($F(2,82) = .675$, $p = .512$).

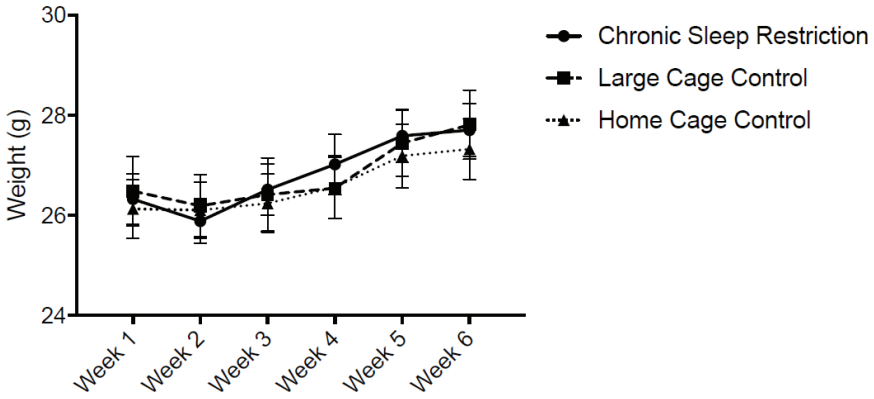

Figure 2. Chronic sleep restriction has no effect on body weight in adult mice. Repeated-measures ANOVA reveals no significant effect of sleep condition on body weight and a significant linear contrast such that animals in all groups gained a significant amount of weight during the six weeks of the experiment. Bars represent ± SEM. N's = 27–30.

3.2 Experiment 1B: Contextual Fear Conditioning in 4–5-Month-Old Mice That Received LPS with Less Than 3 Million Endotoxin Units

A 2 (Injection Treatment: LPS vs. saline) x 3 (Sleep Condition: chronic sleep restriction vs. large cage control vs. home cage control) ANOVA was performed to assess whether chronic sleep restriction and/or LPS injections affected learning in the contextual fear conditioning paradigm in adult mice. Twelve mice that were statistical outliers during the training session were excluded from analysis, as these animals exhibited freezing behaviors before the shock was presented. An additional five animals were excluded due to loud noises outside of the behavior room during the training or testing session or the shock box interface turning off unexpectedly in the middle of the training session before the shocks were presented. The results revealed a significant main effect of Condition ($F(2,53) = 5.793$, $p = .005$) in which the mice that underwent chronic sleep restriction exhibited learning deficits compared to the mice in the large cage control condition ($p = .011$) and home cage control condition ($p = .002$). There was no observed effect of Injection Treatment ($F(1, 53) = .881$, $p = .352$) and no significant Sleep Condition x Injection Treatment interaction ($F(2, 53) = 2.214$, $p = .119$).

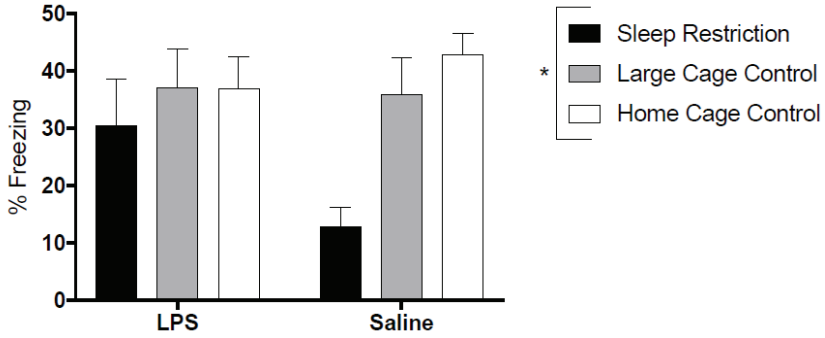

Figure 3. Chronic sleep restriction leads to impaired cognition in adult mice. A 2X3 ANOVA revealed a main effect of Condition, such that animals that underwent chronic sleep restriction froze significantly less than both control groups. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 8–11.

3.3 Experiment 1C: Hippocampal amyloid-beta in 4–5-Month-Old Mice That Received LPS with Less Than 3 Million Endotoxin Units

A 2 (Injection Treatment: LPS vs. saline) x 3 (Sleep Condition: chronic sleep restriction vs. large cage control vs. home cage control) ANOVA was performed to assess whether chronic sleep restriction and LPS injections impacted hippocampal amyloid-beta levels in adult mice. A natural log transformation was performed due to a violation in the assumption of homogeneity of variance. The results revealed a significant main effect of Sleep Condition ($F(2, 59) = 3.954$, $p = .024$) such that the mice that underwent chronic sleep restriction had higher levels of hippocampal amyloid-beta compared to the mice in the large cage control condition ($p = .037$) and home cage control condition ($p = .013$). There was no main effect of Injection Treatment ($F(1, 59) = .103$, $p = .749$) nor a significant Condition x Injection Treatment interaction ($F(2, 59) = .260$, $p = .772$).

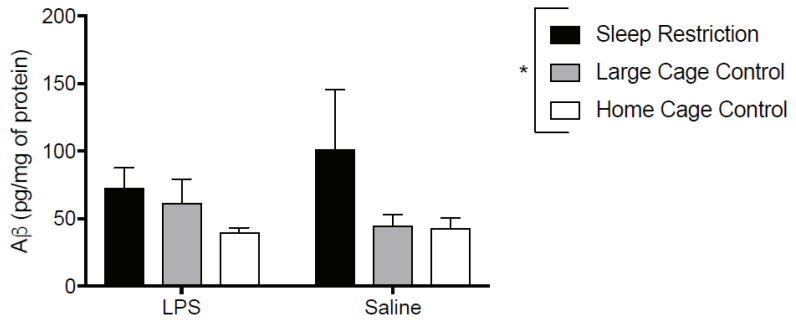

Figure 4. Chronic sleep restriction leads to increased amyloid-beta in the hippocampus. A 2X3 ANOVA revealed a main effect of sleep condition, such that animals that underwent chronic sleep restriction had higher levels of hippocampal amyloid-beta compared to both control groups. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 11–13.

3.4 Experiment 1D: BACE1 in 4–5-Month-Old Mice That Received LPS with Less Than 3 Million Endotoxin Units

A 2 (Injection Treatment: LPS vs. saline) x 3 (Sleep Condition: chronic sleep restriction vs. large cage control vs. home cage control) ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered BACE1 levels in the hippocampus. Results revealed a significant main effect of Sleep Condition ($F(2, 65) = 3.411$, $p = .039$), such that the home cage control mice showed significantly higher levels of BACE1 compared to large cage control mice ($p = .013$), and were trending towards having higher levels of BACE1 compared to mice that underwent chronic sleep restriction ($p = .072$). BACE1 levels did not differ between the chronic sleep restriction group and the large cage control group ($p = .424$). There was no main effect of Injection Treatment ($F(1, 65) = .001$, $p = .976$) and no significant Sleep Condition x Injection Treatment interaction ($F(2, 65) = .132$, $p = .876$).

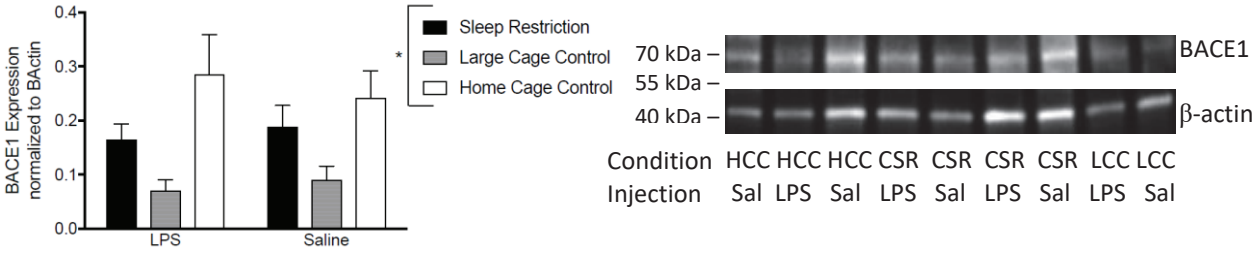

Figure 5. Chronic sleep restriction has no effect on BACE1 in the hippocampus. A 2X3 ANOVA revealed a main effect of condition, such that home cage control animals had higher levels of hippocampal BACE1 compared to chronic sleep restriction and large cage control animals. However, there was no significant difference between the home cage control and chronic sleep restriction groups. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 11–13.

However, some of the samples were rerun in attempt to obtain clearer images. These results revealed no main effects of Sleep Condition ($F(2, 65) = 2.125$, $p = .128$) or Injection Treatment ($F(1, 65) = .009$, $p = .924$) and no Sleep Condition x Injection Treatment interaction ($F(2, 65) = .225$, p = .799).

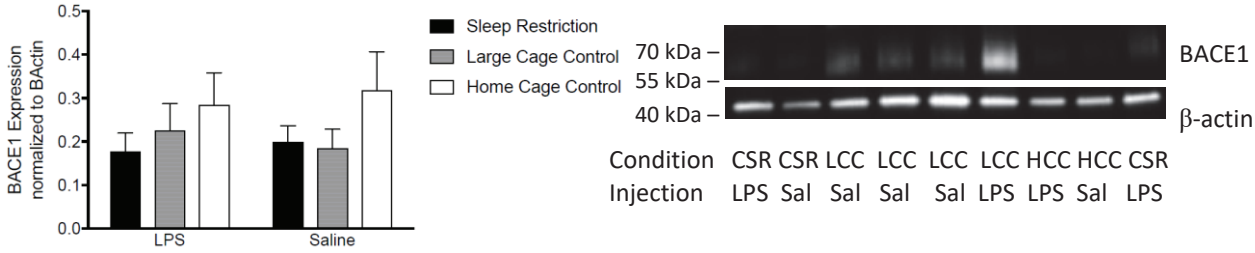

Figure 6. Chronic sleep restriction has no effect on BACE1 in the hippocampus. A 2X3 ANOVA revealed no significant effects. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 11–13.

As there were no significant differences between the large cage control and home cage control groups in freezing behavior during contextual fear conditioning and hippocampal amyloid-beta, the two control groups were combined for the cytokine analyses. We did this to increase the sample size of our control group, as we lost five samples in the home cage and large cage control groups due to the amount of IL-1β in the samples falling below the range of the

standard curve. In this experiment, there were two separate batches of animals used, and blood and tissue collection occurred at two different times during the day for each batch. Batch 1 was collected at 9:00 am (74 hours after the final injection of LPS or saline and 2 hours after the onset of the light period), and Batch 3 was collected at 1:00 pm (78 hours after the final injection of LPS or saline and 6 hours after the onset of the light period). (Batch 2 was excluded from all analyses due to reasons explained in the Discussion section.) As there is a diurnal fluctuation in proinflammatory cytokines in the serum with higher levels just before sleep onset and lower levels throughout the day (Opp, 2005), these two batches of animals were analyzed separately to investigate whether chronic sleep loss and/or LPS affected serum cytokine levels differently at the two time points.

3.5 Experiment 1E: Serum IL-1β Expression 2 and 6 Hours After Onset of the Light Period in 4–5 Month-Old Mice That Received LPS with Less Than 3 Million Endotoxin Units

A 2 (Injection Treatment: LPS vs. saline) x 2 (Sleep Condition: chronic sleep restriction vs. controls) ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered IL-1 levels in the periphery 2 hours after the onset of the light period. There was a marginally significant main effect of Sleep Condition ($F(1, 28) = 3.291$, $p = .080$), such that the animals that underwent chronic sleep restriction had lower levels of serum IL-1β compared to controls. There was no main effect of Injection Treatment ($F(1, 28) = .007$, $p = .932$), and no Sleep Condition x Injection Treatment interaction ($F(1, 28) = .018$, $p = .893$).

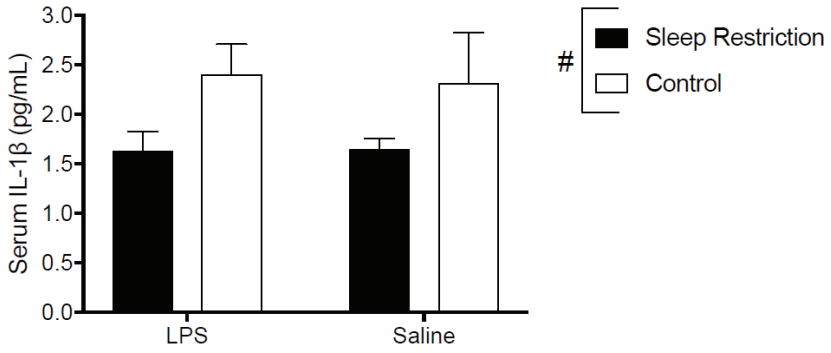

Figure 7. Chronic sleep restriction alters serum IL-1β levels 2 hours after the onset of the light period. A 2X2 ANOVA revealed a marginally significant main effect of condition, such that animals that underwent chronic sleep restriction had lower serum levels of IL-1β compared to control animals 2 hours after the onset of the light period. Bars represent ± SEM. Marginally significant differences are designated by #. N's = 13–20.

A second 2X2 ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered IL-1β levels in the periphery at six hours after the onset of the light period. Five samples that fell below the detectable standard range were excluded from analysis. (Of note, these were all control animals – 3 large cage control animals and 2 home cage control animals.) Results revealed a significant main effect of Sleep Condition ($F(1, 30) = 4.723$, $p = .038$) such that the animals that underwent chronic sleep restriction showed higher levels of serum IL-1β compared to control animals. There was no main effect of Injection Treatment ($F(1, 30) = .503$, $p = .484$), and no Sleep Condition x Injection Treatment interaction ($F(1, 30) = .267$, $p = .609$).

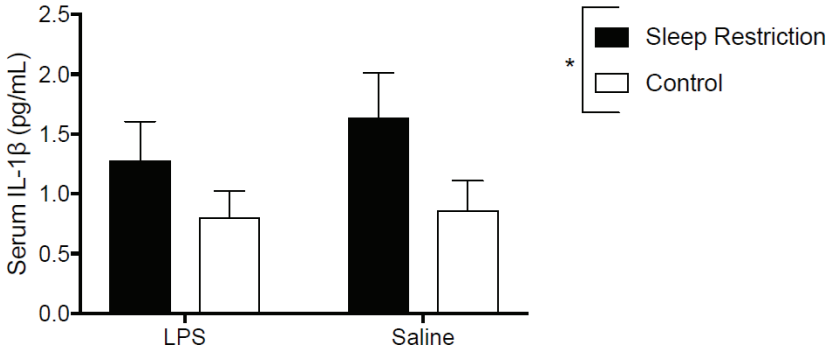

Figure 8. Chronic sleep restriction alters serum IL-1β levels 6 hours after the onset of the light period. A 2X2 ANOVA revealed a main effect of condition, such that animals that underwent chronic sleep restriction had higher serum levels of IL-1β compared to control animals six hours after the onset of the light period. Bars represent ± SEM. Significant differences are designated by *. N's = 13–20.

3.6 Experiment 1F: Serum TNFα Expression 2 and 6 Hours After Onset of the Light Period in 4–5-Month-Old Mice That Received LPS with Less Than 3 Million Endotoxin Units

A 2 (Injection Treatment: LPS vs. saline) x 2 (Sleep Condition: chronic sleep restriction vs. controls) ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered TNFα levels in the periphery two hours after the onset of the light period. There was no main effect of Sleep Condition ($F(1, 28) = .740$, $p = .397$), no main effect of Injection Treatment ($F(1, 28) = .068$, $p = .796$), and no Sleep Condition x Injection Treatment interaction ($F(1, 28) = .032$, $p = .860$).

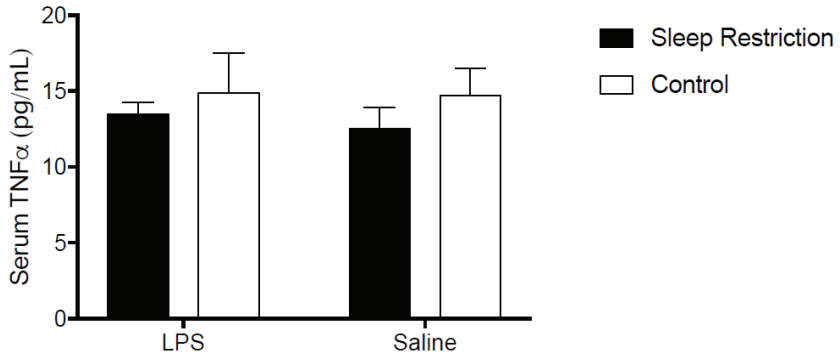

Figure 9. Chronic sleep restriction does not affect serum levels of TNFα 2 hours after the onset of the light period. A 2X2 ANOVA revealed no significant effects. Bars represent ± SEM. N's = 13–20.

A second 2X2 ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered TNFα levels in the periphery six hours after the onset of the light period. Again, there was no main effect of Sleep Condition ($F(1, 35) = .851$, $p = .363$), no main effect of Injection Treatment ($F(1, 35) = 1.331$, $p = .257$), and no Sleep Condition x Injection Treatment interaction ($F(1, 35) = 2.823$, $p = .102$).

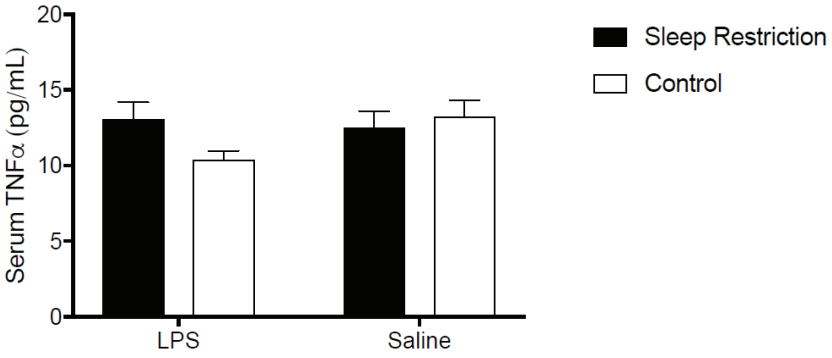

Figure 10. Chronic sleep restriction does not affect serum levels of TNFα 6 hours after the onset of the light period. A 2X2 ANOVA revealed no significant effects. Bars represent ± SEM. N's = 13–20.

3.7 Experiment 1G: Serum IL-10 Expression at 2 and 6 hours after the onset of the light period

A 2 (Injection Treatment: LPS vs. saline) x 2 (Sleep Condition: chronic sleep restriction vs. controls) ANOVA was performed to assess whether chronic sleep restriction and LPS

injections altered IL-10 levels in the periphery two hours after the onset of the light period. Results revealed a significant main effect of Injection Treatment ($F(1, 28) = 27.452$, $p < .001$), such that the animals that received LPS injections had higher levels of serum IL-10 compared to animals that received saline. There was no main effect of Sleep Condition ($F(1, 28) = .326$, $p = .572$), and no Sleep Condition x Injection Treatment interaction ($F(1, 28) = .288$, $p = .596$).

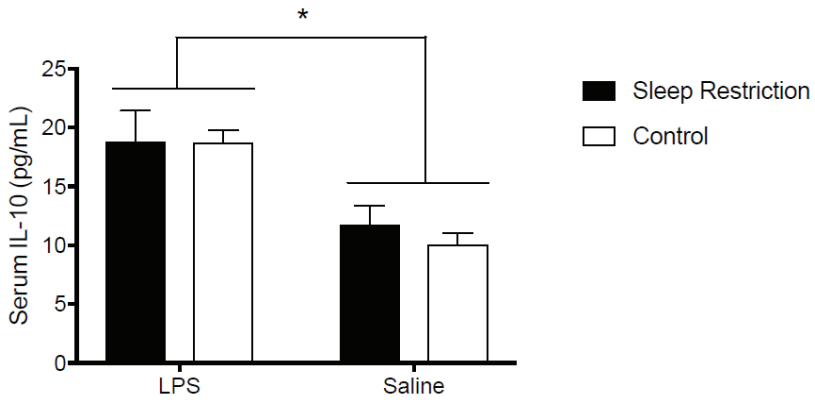

Figure 11. Serum IL-10 is elevated 2 hours after the onset of the light period. A 2X2 ANOVA revealed a main effect of injection treatment such that animals that that received LPS injections had higher serum levels of IL-10 compared to animals that received saline injections. Bars represent ± SEM. Significant differences are designated by *. N's = 13–20.

A 2 (Injection Treatment: LPS vs. saline) x 2 (Sleep Condition: chronic sleep restriction vs. controls) ANOVA was performed to assess whether chronic sleep restriction and LPS injections altered IL-10 levels in the periphery six hours after the onset of the light period. Results revealed a marginally significant effect of Injection Treatment ($F(1, 35) = 3.175$, $p = .083$), where the animals that received LPS injections had higher serum levels of IL-10 compared to animals that received saline injections. There was no main effect of Sleep Condition ($F(1, 35) = .154$, $p = .698$), and no Sleep Condition x Injection Treatment interaction ($F(1, 35) = 1.070$, $p = .308$).

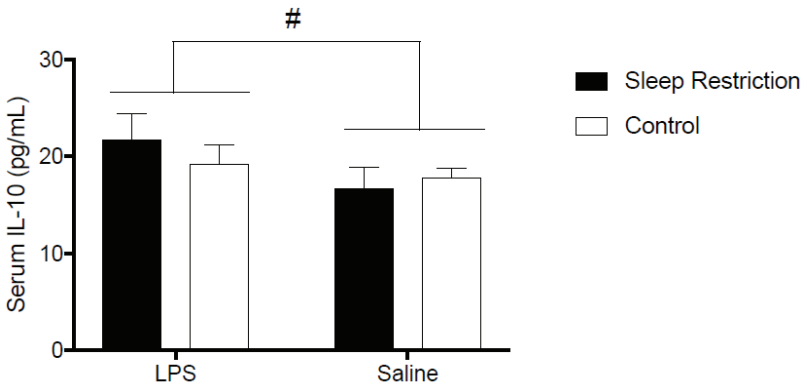

Figure 12. Serum IL-10 is elevated 6 hours after the onset of the light period. A 2X2 ANOVA revealed a marginally significant main effect of injection treatment such that animals that that received LPS injections had higher serum levels of IL-10 compared to animals that received saline injections. Bars represent ± SEM. Marginally significant differences are designated by #. N's = 13–20.

Experiment 2. The first set of experiments aimed to explore whether chronic sleep restriction and LPS administration would impact freezing behavior in CFC and hippocampal amyloid-beta levels in 4–5-month-old adult mice. Previously, research has shown that administering 250µg/kg of LPS via intraperitoneal LPS injections for seven consecutive days is associated with elevated amyloid-beta levels in the hippocampus, as well as a corresponding learning deficit in contextual fear conditioning (Lee et al., 2008; Kahn et al., 2012). However, these effects were not observed in the first experiment discussed in the present research. This is potentially explained by a difference in potency of the LPS used in the current experiments compared to previous work. The LPS used in the first set of experiments described herein had a potency of 2.1 million endotoxin units, whereas the LPS used in preceding studies had a potency that exceeded 3 million endotoxin units. It is possible that the LPS administered in Experiment 1 was potent enough to induce an increase in the anti-inflammatory cytokine IL-10 and sickness behavior marked by a decrease in weight during the first three days of injections but was not strong enough to cause observable elevations in hippocampal amyloid-beta levels and the

corresponding learning deficits in the contextual fear conditioning paradigm. Thus, this experiment was repeated in Experiment 2, again utilizing 4–5-month-old adult C57BL/6 mice with a different lot of LPS that was more potent than the LPS used in the first experiments to examine the effect that the additional inflammatory insult might have on the variables measured previously. Further, as there were no differences between the large cage control group and home cage control group in these measures, the large cage control group was eliminated from Experiment 2 to minimize the number of animals used.

3.8 Experiment 2A: Body Weight in 4–5-Month-Old Mice

All mice were weighed once weekly to monitor for signs of significant weight changes, beginning on the first morning of the protocol before the animals were moved to their respective experiment cages. A repeated-measures ANOVA (Sleep Condition [chronic sleep restriction vs. large cage control vs. home cage control] x Time [week 1 vs. week 2 vs. week 3 vs. week 4 vs. week 5 vs. week 6]) revealed a significant main effect of Sleep Condition such that the animals in the home cage control group were heavier than the animals in the chronic sleep restriction group ($F(1,46) = 4.478$, $p = .040$). Additionally, there was a significant linear trend of Week such that all mice regardless of group gained a significant amount of weight during the experiment ($F(1,46) = 29.078$, $p < .001$). However, there was no significant Weight by Week interaction, ($F(1,46) = .776$, $p = .991$).

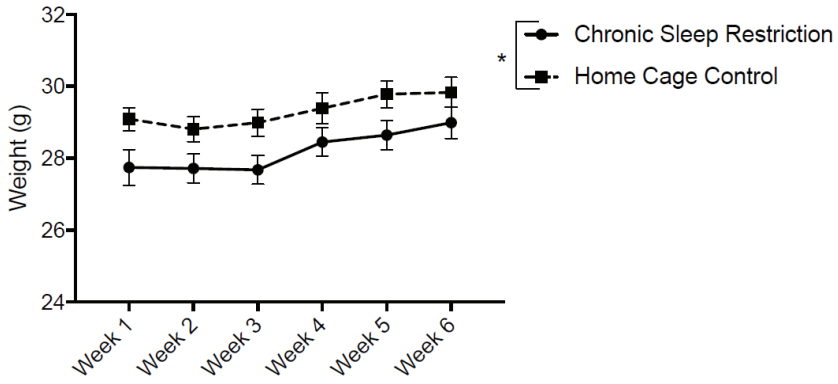

Figure 13. Chronic sleep restriction is associated with decreased body weight in adult mice. A repeated-measures ANOVA revealed a significant effect of Sleep Condition such that the chronic sleep restriction animals weighed less than the controls and a significant linear contrast such that animals in all groups gained a significant amount of weight during the six weeks of the experiment. Bars represent ± SEM. N's = 24.

3.9 Experiment 2B: Contextual Fear Conditioning in 4–5-Month-Old Mice That Received LPS with More Than 3 Million Endotoxin Units

A 2 (Sleep Condition: chronic sleep restriction vs. home cage control) x 2 (Injection Treatment: LPS vs. saline) ANOVA was performed to assess whether chronic sleep restriction and LPS injections affected learning in the contextual fear conditioning paradigm in adult mice. Five mice that were statistical outliers during the training session were excluded from analysis, as these animals exhibited freezing behaviors before the shock was presented. An additional three animals were excluded due to experimenter error during the training session. The results revealed a significant Sleep Condition x Injection Treatment interaction ($F(1,35) = 4.747$, $p = .036$) such that within the animals that received LPS, the animals that underwent chronic sleep restriction froze significantly less than did the home cage control mice ($p = .037$). There were no significant main effects of Sleep Condition ($F(1,35) = .600$, $p = .444$) or Injection Treatment ($F(1,35) = .252$, $p = .691$).

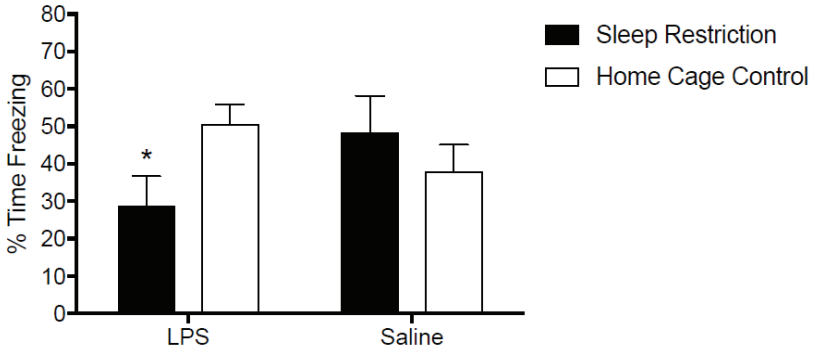

Figure 14. Chronic sleep restriction paired with LPS leads to impaired cognition in adult mice. A 2X2 ANOVA revealed a significant interaction such that, among the animals that received LPS, those that underwent chronic sleep restriction froze significantly less than those in the control condition. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 8–12.

3.10 Experiment 2C: Hippocampal Amyloid-beta in 4–5-Month-Old Mice That Received LPS with More Than 3 Million Endotoxin Units

A 2 (Sleep Condition: chronic sleep restriction vs. home cage control) x 2 (Injection Treatment: LPS vs. saline) ANOVA was performed to assess whether chronic sleep restriction and LPS injections impacted hippocampal amyloid-beta levels in adult mice. One animal was excluded from analysis because it was a statistical outlier among all other animals during the training session, and eight animals were excluded from analysis as they did not gain or lose weight in the expected pattern during the week of LPS injections. Specifically, if they received saline but lost more than 2 grams, or if they received LPS and did not lose more than 2 grams, they were excluded from amyloid-beta analysis. Of those excluded, seven were saline animals that lost more than 2 grams, and one was an LPS animal that did not lose more than 2 grams. The results revealed a significant main effect of Sleep Condition ($F(1, 34) = 5.427$, $p = .026$) such that the mice that underwent chronic sleep restriction had higher levels of hippocampal amyloid-beta compared to the mice in the home cage control condition. There was no main effect of

Injection Treatment ($F(1, 34) = 1.134$, $p = .294$) and no significant Sleep Condition x Injection Treatment interaction ($F(1,34) = .803$, $p = .377$).

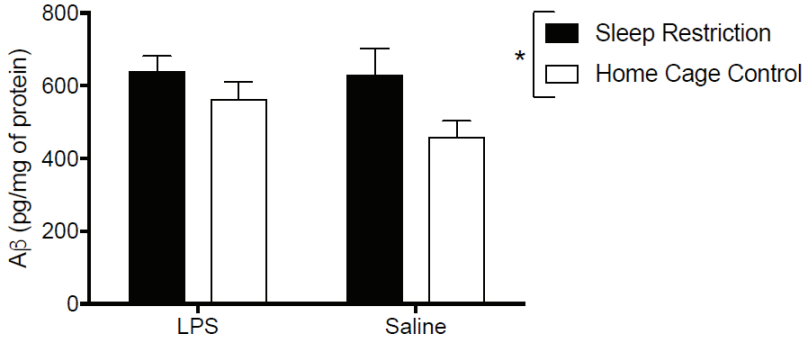

Figure 15. Chronic sleep restriction leads to increased hippocampal amyloid-beta in adult mice. A 2X2 ANOVA revealed a significant main effect of Sleep Condition such that the animals that underwent chronic sleep restriction had higher levels of hippocampal amyloid-beta than the animals in the control condition. Bars represent ± SEM. Significant differences ($p < .05$) are designated by *. N's = 7–12.

Experiment 3. As the first two sets of experiments revealed that chronic sleep restriction was associated with deficits in contextual fear conditioning and increased hippocampal amyloid-beta, as well as a compounding effect of LPS and chronic sleep restriction in Experiment 2, Experiment 3 aimed to examine whether similar effects would be observed in aged animals. For Experiment 3, 14 –16-month-old mice were utilized.

3.11 Experiment 3A: Body Weight in 14–16-Month-Old Mice

All mice were weighed once weekly to monitor for signs of significant weight changes beginning on the first morning of the protocol before the animals were placed into their respective experiment cages. A repeated-measures ANOVA (Sleep Condition [chronic sleep restriction vs. large cage control vs. home cage control] x Time [week 1 vs. week 2 vs. week 3 vs. week 4 vs. week 5 vs. week 6]) revealed a significant interaction such that the animals in the chronic sleep restriction condition weighed less compared to animals in either control condition during the second week of the protocol ($F(10,195) = 1.951$, $ps \leq .006$).

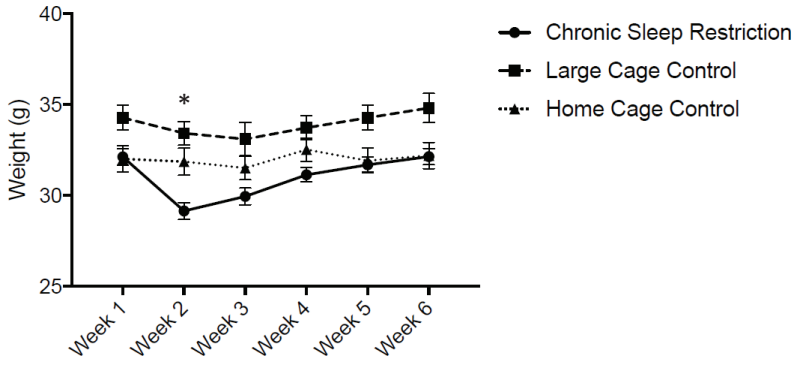

Figure 16. Chronic sleep restriction leads to short-term decreases in weight in aged mice. A repeated-measures ANOVA revealed a significant interaction such that the animals that underwent chronic sleep restriction weighed less than animals in either control group lost weight after the first week of the experiment. Bars represent ± SEM. Significant differences are represented by *. N's = 14.

3.12 Experiment 3B: Contextual Fear Conditioning in 14–16-Month-Old Mice

A 2 (Treatment: LPS vs. saline) x 3 (Sleep Condition: chronic sleep restriction vs. large cage control vs. home cage control) ANOVA was performed to assess whether chronic sleep restriction and LPS injections impacted learning in the contextual fear conditioning paradigm in 14–16-month-old mice. Seven mice that were statistical outliers during the training session were excluded from analysis, as these animals exhibited freezing behaviors before the shock was presented. One animal died after the training session and was therefore excluded from behavior analysis. The results revealed no effect of Sleep Condition ($F(2, 27) = .236$, $p = .791$), no effect of Treatment ($F(1, 27) = 2.392$, $p = .134$), and no significant Sleep Condition x Treatment interaction ($F(2, 27) = 1.818$, $p = .182$).

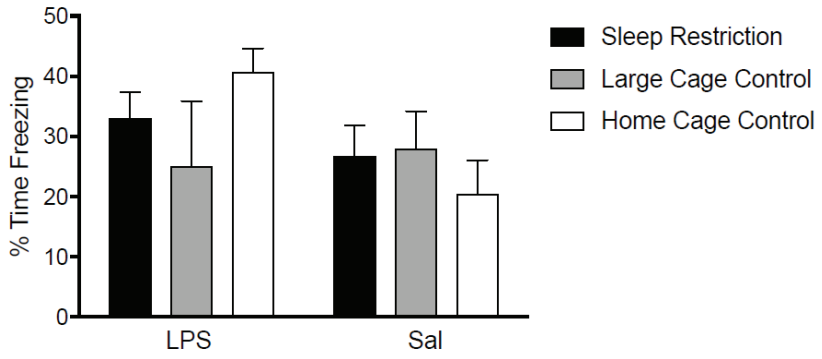

Figure 17. Chronic sleep restriction does not affect contextual fear conditioning in aged mice. A 2X3 ANOVA revealed no significant main effects and no significant interaction. Bars represent ± SEM. N's = 5–6.

3.13 Experiment 3C: Hippocampal Amyloid-beta in 14–16-Month-Old Mice

A 2 (Injection Treatment: LPS vs. saline) x 3 (Sleep Condition: chronic sleep restriction vs. large cage control vs. home cage control) ANOVA was performed to assess whether chronic sleep restriction and LPS injections elevated hippocampal amyloid-beta levels in aged mice. There was no main effect of Sleep Condition ($F(2, 31) = 1.956$, $p = .158$), no main effect of Treatment ($F(1, 31) = .620$, $p = .437$), and no Sleep Condition x Treatment interaction ($F(2, 31) = .390$, $p = .680$).

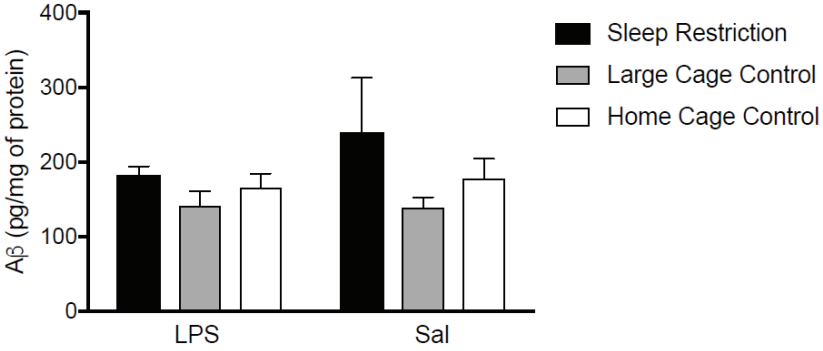

Figure 18. Chronic sleep restriction does not affect hippocampal amyloid-beta in aged mice. A 2X3 ANOVA revealed no significant main effects and no significant interaction. Bars represent ± SEM. N's = 5–7.

4. Discussion

The present research sought to explore the relationship between chronic sleep restriction, inflammation, and Alzheimer's-like pathology. Specifically, these experiments examined the effects of chronic sleep restriction and intraperitoneal LPS administration on cognition, hippocampal amyloid-beta, and peripheral levels of IL-1β, TNFα, and IL-10. We hypothesized that mice that underwent chronic sleep restriction would exhibit deficits in CFC and have significantly higher levels of soluble amyloid-beta in the hippocampus and compared to control mice, and that LPS injections would be associated with cognitive deficits and increased amyloid-beta. Moreover, we hypothesized that LPS administration would further exacerbate the harmful effects of chronic sleep restriction, and we explored inflammation as a possible mechanism driving this exacerbation. We expected to observe these hypothesized effects in aged animals as well; indeed, possibly to an even greater degree. Our results partially confirmed these hypotheses, as chronic sleep restriction was associated with deficits in CFC, increased amyloid-beta, and differences in serum IL-1β compared to control animals. In Experiment 2, LPS administration and chronic sleep restriction together led to exacerbated deficits in contextual fear conditioning, Additionally, chronic sleep restriction alone was again associated with increased amyloid-beta in Experiment 2. However, contrary to our original hypotheses, LPS administration alone had no effect on CFC or amyloid-beta in Experiment 1 or Experiment 2, and none of the effects discussed above were observed with the aged animals utilized in Experiment 3.

Consistent with the hypothesis that chronic sleep restriction alone would lead to increased hippocampal amyloid-beta in 4–5-month-old mice, chronic sleep restriction significantly elevated hippocampal amyloid-beta levels compared to both control conditions in Experiment 1. These results were replicated in adult mice in Experiment 2. Further, as hypothesized, chronic

sleep restriction alone significantly impaired freezing behavior in contextual fear conditioning in adult mice in Experiment 1, indicating cognitive deficits in these animals compared to animals in either control condition. In Experiment 2, using LPS with greater than 3 million endotoxin units, a significant interaction was observed such that, among the animals that underwent chronic sleep restriction, the animals that received LPS injections exhibited deficits in contextual fear conditioning as compared to the animals that received saline injections. This indicates a relationship between chronic sleep restriction and LPS such that an inflammatory event in the periphery combined with chronic sleep restriction could be more detrimental to cognitive ability and learning than chronic sleep restriction alone. We did not observe the main effect of Sleep Condition on CFC in adults in Experiment 2B as we did in Experiment 1B, but it is difficult to directly compare the two experiments. First, animals were only administered one shock in Experiment 1B, remaining in the CFC chamber for a total of 3 minutes during the training session, whereas animals received two shocks and remained in the chamber for nearly 5 minutes in Experiment 2B. This change was made in an attempt to increase the salience of the context, as it was expected that doing so would increase differences observed between groups in Experiment 1B. However, it is possible that the additional shock and increased time in the chamber increased the salience of the context, and rendered the task easier, such that chronic sleep restriction alone was not enough to elicit a significant learning deficit, and the main effect of Sleep Condition was eliminated. Furthermore, the animals were housed differently in the two experiments. In Experiment 1, the animals were housed in room 322 in the TCU vivarium between the hours of 6:00 pm and 8:00 am, in which they were not in their respective experiment cages. During Experiment 2, the animals were housed at all hours in room 319 in the TCU vivarium for the

duration of the experiment. Additionally, the use of a different, more potent lot of LPS increases the difficulty in comparing the two experiments.

Importantly, the main effect of chronic sleep restriction on amyloid-beta in adults observed in Experiment 1C was replicated in Experiment 2C, with chronic sleep restriction associated with higher levels of hippocampal amyloid-beta. Together, these findings are consistent with the literature regarding the effects of sleep loss on Alzhiemer's-like pathology in animal models. Zhao et al. (2016) found that amyloid-beta was increased in the prefrontal cortex and temporal lobe following 8 weeks of chronic sleep restriction via the rotating drum method in nine-month-old C57BL/6 mice. Moreover, Chen et al. (2017) demonstrated that cognitively normal rats that underwent 2 and 4 days of sleep deprivation showed cognitive disfunction and elevated amyloid-beta in the cerebral cortex. Closely related to the current research, it has also been shown that chronic sleep restriction for 3 hours per day, 5 days per week, for 4 weeks resulted in learning deficits in contextual fear conditioning in non-transgenic mice (Kincheski et al., 2017). To our knowledge, the current studies are the first to demonstrate an increase in soluble amyloid-beta in the hippocampus with cognitive deficits, in adult, non-transgenic mice following a period of chronic sleep restriction.

Additionally, our findings are generally consistent with those of studies using transgenic AD mouse models that demonstrate cognitive disfunction and elevated amyloid-beta in the brain following chronic sleep loss. For example, in adult APP/PS1 transgenic mice, 21 days and 27 days of chronic sleep fragmentation have been associated with increased amyloid-beta plaque load in the brain (Kang et al., 2009; Minakawa et al., 2017). Conversely, while Di Meco, Joshi, and Praticò (2014) found that implementing a 20/4-hour light/dark cycle for 8 weeks to induce chronic sleep restriction was associated with cognitive disfunction in the Morris water maze,

decreased post synaptic density protein 95 (PSD-95), and increased insoluble tau in the brain in eight-month-old 3xTg mice, but did not induce deficits in contextual fear conditioning or increased soluble amyloid-beta in the brain. This suggests that the effects of sleep loss on amyloid pathology are complex and likely differ based upon background strain of the animals tested, age at testing, and the distinct transgenic model of Alzheimer's-like pathology, among a myriad of other experimental parameters.

In an attempt to explore the mechanisms through which the observed effects on contextual fear conditioning and amyloid-beta occurred in the adult animals, the amount of BACE1 in the hippocampus was quantified using Western blots. Evidence suggests that BACE1 is increased, although not specifically in the hippocampus, following chronic sleep restriction (Zhao et al., 2016) and acute sleep deprivation (Chen et al., 2017). Chen et al. (2017) found that both 2 and 4 days of total sleep deprivation was associated with increased BACE1 and BACE1 mRNA in the cortex following in rats. However, while Zhao et al. (2016) found increased BACE1 levels and BACE1 mRNA in the prefrontal cortex and temporal lobe cortex after 2 months of chronic sleep restriction in non-transgenic mice, but not in the hippocampus. Somewhat inconsistent with this literature, the current work found that BACE1 expression in the hippocampus appeared to be the highest in the home cage control animals, while there were no differences between the chronic sleep restriction and large cage control groups. However, after repeating BACE1 Westerns for some of the samples, no significant effects were observed, indicating that the initial findings were potentially inaccurate. It is possible that the increase in hippocampal amyloid-beta following chronic sleep loss is not due to an upregulation in BACE1 activity or expression and the activation of the amyloidogenic pathway. Westerns for C terminal fragment 99 could be performed to further explore this question. However, some key technical

issues may have influenced measures of BACE enzymes in the current study. For example, it is necessary to consider the integrity of the lysates themselves. Most of the samples were over eight months old at the time that Western blots were performed. They were preserved in ProPrep in -20ºC, which is designed to preserve proteins up to around 6 months. Thus, it is likely that some of the protein had degraded, potentially to varying degrees between batches. Additionally, these samples had undergone several freeze-thaws due to freezer malfunctions and unexpected power outages. To address these caveats, Western blots will be performed on the samples from Experiment 2 to explore the potential effects on BACE1 in the hippocampus due to chronic sleep restriction and LPS administration.

To assess another potential mechanism through which amyloid-beta may be elevated, serum levels of proinflammatory cytokines TNFα and IL-1β and anti-inflammatory cytokine IL-10 were measured. While evidence shows that levels of TNFα and IL-1β mRNA are elevated in the brain following acute and chronic sleep loss in rats (Zielinski et al., 2014), serum levels of TNFα did not differ significantly between sleep restricted animals and controls in the current research. Conversely, differences were observed in serum IL-1β. Oddly, 2 hours after the onset of the light period, serum levels of IL-1β were higher in the control animals compared to the animals that underwent chronic sleep restriction. However, there was somewhat of a reversal of these effects 6 hours after the onset of the light period, with the animals that underwent chronic sleep restriction demonstrating higher levels of serum IL-1β compared to control animals. When examining the means across the two time points, it appears that the serum levels of IL-1β in the animals that underwent chronic sleep restriction did not change much between the two time points. (Independent samples *t* tests confirmed that the means from the two time points did not differ significantly among the animals that underwent chronic sleep restriction.) Instead, serum

IL-1β levels were (significantly, by independent samples *t* test and by 2-way ANOVA) different among the controls between 2 and 6 hours after the onset of the light period. The significance of these unanticipated findings, if any, is currently unclear. Taishi, Bredow, Guha-Thakurta, Obál, and Krueger (1996) found that IL-1β expression follows a diurnal pattern in the brain, with the highest levels of mRNA being expressed at the onset of the light period and decreasing throughout the day. These findings are consistent with the trend found in the current research among the control animals, but fail to explain the findings for the animals that underwent sleep restriction. This difference in IL-1β expression pattern between experimental conditions may potentially arise from a perturbation in the normal diurnal expression pattern of serum IL-1β among the sleep-restricted animals. This suggests that restricting sleep time could be associated with an impaired inflammatory response. Interestingly, however, there were no differences observed in serum TNFα between the animals that underwent chronic sleep restriction and controls at the different time points, although evidence shows a diurnal fluctuation in TNFα mRNA in the brain (Bredow, Guha-Thakurta, Taishi, Obál, & Krueger, 1997).

In further examining key cytokine expression profiles, the current work found that, as hypothesized, the animals that received seven days of LPS injections had higher levels of serum IL-10 at both time-points, compared to controls. IL-10 is an anti-inflammatory cytokine which acts in response to increased inflammation to modulate the inflammatory response and decrease the release of proinflammatory cytokines such as TNFα and IL-1β (Fiorentino, Zlotnik, Mosmann, Howard, & O'Garra, 1991). IL-10 reduces non-REM sleep (Opp, 2005), although evidence suggests that 72 hours of REM sleep deprivation is not associated with changes in plasma IL-10 in rats immediately following sleep deprivation or after a period of 7 days of recovery (Yehuda, Sredni, Carasso, & Kenigsbuch-Sredni, 2009). Consistent with this literature,

there were no differences between the animals that underwent chronic sleep restriction and the control animals in terms of serum IL-10 production. However, the animals that received LPS injections had higher levels of serum IL-10 at both time points compared to animals that received saline. While serum IL-1β and hippocampal IL-1β mRNA return nearly to baseline following seven days of LPS injections in mice (Kahn et al., 2012), this observed increase in IL-10 in response to LPS is potentially a negative feedback mechanism in the initiation of endotoxin tolerance and downregulates the production of proinflammatory cytokines (Frankenberger, Pechumer, & Ziegler-Heitbrock, 1995; Byrne & Reen, 2002),

As all animals predictably demonstrated an increase in body weight throughout the six weeks of the protocol regardless of condition or injection treatment in Experiment 1, it can be inferred that there was no weight difference due to the stress of the sleep restriction paradigm in that experiment. In Experiment 2, the animals that underwent chronic sleep restriction weighed less than did the control animals. However, this difference was observable at the first timepoint, which was before the beginning of the sleep restriction protocol. Thus, it is possible that the observed effect of sleep restriction on weight was not due to the sleep restriction procedure. The increase in weight across groups was likely due to normal growth, as these animals were just 4–5 months of age at the beginning of the experiment. Furthermore, as there were no observed statistical differences between the mice in the large cage control and home cage control conditions in Experiment 1, it could be inferred that any stress induced by the novelty of the platform cages was not sufficient to affect the body weight of the animals in adult mice. Interestingly, this is inconsistent with findings suggesting that chronic sleep restriction leads to increased weight gain and predisposition to obesity in mice (Wang et al., 2014; Mendes de Oliveira et al., 2015), as well as literature linking chronic sleep loss to increased risk for obesity

and diabetes in humans (Watson et al., 2015), although adipose tissue was not quantified in the current research. On the other hand, in Experiment 3, which utilized 14–16-month-old mice, the animals that underwent chronic sleep restriction lost a significant amount of weight during the first week of the protocol compared to the animals in the control conditions. These animals were fully grown, potentially making it easier to observe more subtle changes in weight during the experiment. As the animals' weight did not increase significantly from the Week 1 to Week 6, perhaps the observed interaction was due to the initial stress of being placed in the multiple platform water cage environment; i.e., the aged animals were simply more sensitive to the initial change in environment and therefore more susceptible to weight change due to stress.

Interestingly, the effects on CFC and amyloid-beta found in Experiments 1 and 2 with adult mice were not observed in Experiment 3 with aged mice. There were no group differences in contextual fear conditioning or hippocampal amyloid-beta in the 14–16-month-old animals, although the means for amyloid-beta trended in the hypothesized direction, with the chronic sleep restriction groups showing slightly higher levels of amyloid-beta to controls. While, to our knowledge, this was the first attempt to examine the effects of chronic sleep loss during old age in non-transgenic mice, these results are inconsistent with the literature describing exacerbated effects of stress and peripheral immune stimulation on cognition and inflammation in aged mice compared to adults (Buchanan, Sparkman, Chen, & Johnson, 2008; Chen et al., 2008; Tarr et al., 2011). Utilizing 14-month-old 3xTg mice, Rothman, Herdener, Frankola, Mughal, and Mattson (2013) found that 6 weeks of sleep restriction for 6 hours per day by the multiple platform method was associated with learning deficits in contextual fear conditioning and increases in phosphorylated tau and soluble amyloid-beta in the cortex, but not in the hippocampus. Thus, as the present research utilized C57BL/6 mice, a period of chronic sleep loss during old age may

not be sufficient to elicit observable learning deficits in contextual fear conditioning or increases in hippocampal amyloid-beta in non-transgenic animals. Furthermore, although the LPS used in Experiment 3 had greater than 3 million endotoxin units (though the aged adults received 175μg/kg doses), there were no observable effects of LPS on CFC or amyloid-beta in the aged animals. The reasons for these unexpected findings are currently unclear.

From the first set of experiments with 4–5-month-old adult mice, the second batch of animals was excluded from analysis, as there were multiple temperature fluctuations in the vivarium during this six-week period. Furthermore, during the week before behavioral testing, a power outage occurred during the light phase, causing the lights to turn off when they should have been on. This could have disrupted the animals' circadian rhythmicity, and acted as an additional stressor, thus confounding the integrity of the results of this batch. However, it is important to note that, even with batch two included in analysis, both of the observed main effects of chronic sleep restriction (deficits in CFC and increased hippocampal amyloid-beta) in Experiment 1B and 1C persist.

Future research efforts in our lab will explore the effects of chronic sleep restriction on amyloid-beta plaque deposition in 5xFAD animals, a transgenic mouse model of early onset Alzheimer's-like pathology. Further, given the propensity for females to develop AD more frequently than males (Mielke, Vemuri, & Rocca, 2014), this work must also examine the effects of these manipulations on females, to examine whether they will be affected in the same way as males. It may also prove interesting to examine whether a recovery period would ameliorate the effects demonstrated in the current research. Similarly, it may prove useful to implement a sleep restriction protocol that includes performing multiple bouts of sleep restriction throughout adulthood, to better model human chronic sleep loss and its impact as individuals age.

To further explore the effects of chronic sleep loss on cytokine expression, particularly IL-1β, blood could be collected at different time points to further examine the impact sleep loss may have on the normal diurnal fluctuation in IL-1β in the serum. Likewise, it could prove useful to extend the investigation of alterations in cytokines to the brain as well by administering one LPS injection following a period of chronic sleep loss and measuring hippocampal mRNA for IL-1β, IL-10, TNFα, IL-6, and HMGB1. Furthermore, other mechanisms driving cognitive deficits should be explored, such as hippocampal BDNF mRNA, markers of synaptic integrity such as synaptophysin and PSD-95, and dendritic spine density in the hippocampus.

In summary, this research illustrates the impact that chronic sleep loss has on the development of Alzhiemer's-like pathology in otherwise cognitively healthy mice. Our findings were consistent with previous literature demonstrating that sleep loss can exacerbate Alzheimer's-like pathology in transgenic animal models of the disease as well as non-transgenic animals. This work extends prior research in demonstrating that chronic sleep loss may have detrimental effects upon cognitive function through increasing amyloid-beta levels in the hippocampus, as well as potentially disrupting the diurnal fluctuation in the proinflammatory cytokine IL-1β, which may play a role in the differences found in amyloid-beta levels. The current research, like studies done before it, provides additional support for chronic sleep loss as an important risk factor for Alzhiemer's pathogenesis and raises a number of important questions to be addressed in future research.

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ABSTRACT

CHRONIC SLEEP RESTRICTION EXACERBATES ALZHEIMER'S DISEASE-LIKE PATHOLOGY AND ALTERS PERIPHERAL IL-1β EXPRESSION IN C57BL/6 MICE

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Alzheimer's Disease, a devastating neurodegenerative disease characterized by amyloid-beta plaques and neurofibrillary tangles, is the 6th leading cause of death in the U.S., and its prevalence is increasing. A common feature of Alzheimer's is a disrupted sleep/wake cycle. 35.3% of adults in the U.S. get less than the minimum 7 hours of sleep per night recommended by the National Sleep Foundation. Interestingly, evidence suggests a bidirectional relationship between sleep loss and Alzheimer's. We hypothesized that six weeks of sleep restriction would lead to increased amyloid-beta in the hippocampus and cognitive deficits in C57BL/6 mice. Further, we hypothesized that these effects would be exacerbated by intraperitoneal LPS administration. Chronic sleep restriction itself was associated with cognitive deficits in contextual fear conditioning, increased hippocampal amyloid-beta, and alteration in the circadian fluctuation in serum IL-1β. Furthermore, LPS administration coupled with chronic sleep restriction led to exacerbated cognitive dysfunction.