EXPLORING CHRONIC SLEEP LOSS AND TYPICAL AMERICAN-STYLE DIET AS RISK FACTORS FOR ALZHEIMER'S DISEASE

by

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CHAPTER 1: GENERAL INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease marked by severe cognitive decline and is the most common cause of dementia (Tarawneh & Holtzman, 2012; Alzheimer's Association, 2023). Sadly, it was the fifth-leading cause of death in individuals 65 and older in 2019 in the U.S. (Alzheimer's Association, 2023). Currently, over 6 million people are estimated to be suffering from AD in the U.S. alone, and this number is projected to increase dramatically in the coming years, with an estimated 13.8 million Americans living with the disease by 2060 (Alzheimer's Association, 2023). At present, it is estimated that more than one in nine people over the age of 64 (roughly 11 percent of the population aged 65 and older) and around one in three individuals over the age of 84 are living with AD. While deaths due to other leading causes of death in the U.S. have declined in the past two decades, deaths due to AD have increased by over 145 percent in the past 20 years (Alzheimer's Association, 2023).

The personal impact of AD on its victims is devastating. Individuals with AD experience numerous debilitating symptoms, including progressive memory loss, changes in personality, substantial cognitive decline, difficulty executing daily tasks, and physical decline, until death ultimately results from AD (Tarawneh and Holtzman, 2012; Huang & Mucke, 2012). In addition to the devastating impact of AD on the patient, loved ones, and caregivers, this disease is also a massive economic burden. Patients with AD can cost the healthcare system approximately three times the amount compared to age-matched elderly individuals who are cognitively healthy (Grossberg et al., 2019). It is estimated that the cost of care for AD patients in the U.S. totaled nearly $340 billion in 2022 alone (Alzheimer's Association, 2023). Alarmingly, with the aging of the "baby boom" generation, these costs are expected to rise significantly to almost $1 trillion per year by 2050 (Alzheimer's Association, 2023). Therefore, given the destructive effect of AD

on the lives of sufferers and their loved ones, as well as the tremendous economic burden that it imposes, it is crucial that we pursue research to further our understanding of and seek remedies for the neuropathological mechanisms underlying development and progression of this disease.

Despite ongoing research efforts to elucidate the mechanisms of AD, much remains unknown regarding the root causes of the disease (Selkoe et al., 2012; Alzheimer's Association, 2023). The first documented case of Alzheimer's disease was published in 1907 by Dr. Alois Alzheimer, who noticed rapidly deteriorating memory and personality changes in a seemingly healthy 51-year-old woman. In a post-mortem examination of her brain, he found tangled fibrils within and in place of neurons, as well as whole brain atrophy (Stelzmann et al., 1995). These tangled fibrils are now recognized as amyloid-beta (Aβ) plaques and neurofibrillary tau tangles, the two main pathological hallmarks of AD that are both associated with impairments in normal neural communication (LaFerla et al., 2007; Sanabria-Castro et al., 2017).

There are two recognized types of AD: familial (or early-onset) AD, which generally presents before the age of 65, and sporadic (or late-onset) AD, which is typically diagnosed after the age of 65 and progresses comparatively later in life (Dorszewska et al., 2016). Individuals with familial AD have autosomal dominant inheritance of genetic mutations in three key genes – the APP gene, presenilin 1 (PSEN1) gene, and presenilin 2 (PSEN2) gene – that are important for the cleavage of Aβ. Thus, the presence of these mutations increases the production of the harmful form of Aβ (Piaceri et al., 2013). Sporadic AD, which is more common than familial AD, has also been linked with certain genetic factors, including having the apolipoprotein epsilon 4 (*APOE4*) allele, which is thought to play a role in the conversion of Aβ from non-toxic monomers into neurotoxic oligomers and fibrils (Piaceri et al., 2013). Moreover, the *APOE4* gene has been associated with decreased microglial phagocytosis of Aβ, contributing to

excessive Aβ in the brain (Lin et al., 2018). However, over 60 percent of sporadic AD cases are not linked with having the *APOE4* allele (Piaceri et al., 2013). Most likely, both genetic and environmental factors influence sporadic AD risk (Piaceri et al., 2013; Dunn et al., 2019).

AD pathology

Both types of AD are characterized by the same pathologies (Dorszewska et al., 2016). The first unique pathogenic occurrence is thought to be the formation of Aβ plaques (LaFerla et al., 2007; Sanabria-Castro et al., 2017; Morris et al., 1996). Aβ is created as a result of the processing of the intracellular amyloid precursor protein (APP). APP is cleaved along one of two processing pathways. In the nonamyloidogenic pathway, proteolytic enzymes α-secretase and γ-secretase cleave APP into C-terminal fragment (CTF)-83 and APPsα, which are cleaved into small, non-toxic fragments that are released from the cell. In the amyloidogenic pathway, APP is cleaved by β-secretase (BACE1) into APPsβ and CTF-99, which are subsequently cleaved by γ-secretase into amyloid precursor protein intracellular domain (AICD) and the potentially neurotoxic $A\beta_{40}$ and $A\beta_{42}$ peptides (LaFerla et al., 2007). In AD brains, these $A\beta_{40}$ and $A\beta_{42}$ peptides are produced more quickly than they can be cleared. They are then secreted from the cell and accumulate into progressively larger polymers, resulting in soluble Aβ oligomers. These oligomers eventually aggregate to form the insoluble, fibrillar Aβ plaques that disrupt neural communication and are characteristic of AD (Sanabria-Castro et al., 2017).

The second significant pathogenic occurrence in AD is the hyperphosphorylation of tau, a microtubule-associated protein that helps to support the cytoskeleton in a healthy neuron. In AD, hyperphosphorylated tau tangles form within neurons and interrupt the axoplasmic transport of proteins and nutrients within neurons, leading to neuronal death and brain atrophy (Sanabria-Castro et al., 2017). While tau tangles play an important detrimental role in AD pathology and

are associated with severity of cognitive impairment, they have typically been observed in the later stages of the disease (Tiraboschi et al., 2004). Both of these pathologies result in brain atrophy, as patients with AD lose a significant number of neurons as the disease progresses (Gómez-Isla et al., 1997; West et al., 1994).

The role of amyloid-beta in cognitive and synaptic dysfunction in AD

Of the two main pathological events in AD, the role of Aβ has been examined more extensively in relation to disease pathogenesis compared to hyperphosphorylated tau tangles, as Aβ plaques form earlier in disease progression (Alzheimer's Association, 2023; Morris et al., 1996). While much AD research exploring Aβ pathology in previous decades has focused primarily on insoluble plaques, more recently, research has begun to examine the role and toxicity of soluble, oligomeric Aβ, a precursor to Aβ plaques (Lublin & Gandy, 2010). While it is thought that Aβ oligomers serve as a regulator of learning and memory, studies have demonstrated that large amounts of this soluble, oligomeric form of Aβ can hinder hippocampal long-term potentiation (LTP), an electrophysiological signal of synaptic plasticity correlated with learning and memory (Lublin & Gandy, 2010). Furthermore, evidence demonstrates that Aβ oligomers interfere with synaptic function, decrease neuronal viability, and impair cognition, potentially more severely than the fibrillar Aβ plaques themselves (Cleary et al., 2005; Dahlgren et al., 2002; Haass & Selkoe, 2007; Makin, 2018). Indeed, studies demonstrate that the infusion of human Aβ oligomers in wild-type rodents results in cognitive dysfunction in those animals (Cleary et al., 2005; Kincheski et al., 2017; White et al., 2020). Additionally, research illustrates that soluble Aβ has a greater impact on cognitive dysfunction compared to hyperphosphorylated tau tangles (White et al., 2016).

It remains unknown whether the soluble, oligomeric form of Aβ is equally responsible for the detrimental impact on cognitive and neural function in AD in comparison to Aβ plaques (Makin, 2018). Furthermore, some evidence suggests that number of Aβ plaques is not always indicative of concurrent cognitive and disease state (Lublin & Gandy, 2010; Giannakopoulos et al., 2003). Thus, plaque formation in the early stages of AD could be adaptive or beneficial in decreasing the amyloid burden by sequestering Aβ oligomers into a consolidated area rather than allowing them to spread diffusely throughout the brain (Makin, 2018). However, insoluble Aβ plaques are often surrounded by soluble, oligomeric Aβ, making it difficult to distinguish the effects of Aβ plaques or soluble oligomers alone on cognition and neuronal loss (Haas & Selkoe, 2007; Makin, 2018). As evidence exists to implicate both Aβ oligomers and Aβ plaques in neuronal injury and cognitive dysfunction, it is likely that both have neurotoxic effects (Haas & Selkoe, 2007).

Neurodegenerative impacts of AD pathology

One of the most easily recognized early symptoms of AD is difficulty remembering new information, which occurs due to damage in the hippocampus, an area of the brain critical for learning and memory (Huang and Mucke, 2012). Other early symptoms of AD may include depression, apathy, and agitation, although it is virtually impossible to make a clinical AD diagnosis based on these neuropsychiatric symptoms alone (Li et al., 2014). Unfortunately, evidence shows that AD pathology may begin well before symptoms present, with some clinical changes in biomarkers of AD occurring up to 20 years before the appearance of any noticeable AD symptoms (Bateman et al. 2012; Alzheimer's Association, 2023). Subtle alterations in cognition can occur in preclinical phases of AD, but these changes are not always distinct from normal, age-related cognitive decline (Jessen, 2014).

Sadly, there is no disease-modifying cure for AD, and the therapies that exist only work to slow the progression of the disease. There are currently three cholinesterase inhibitors (Donepezil, Galantamine, and Rivastigmine) and one N-methyl-D-aspartate (NMDA) receptor antagonist (Memantine) used to treat mild and moderate AD (Grossberg et al., 2019). Although these therapeutic agents help improve cognition and global function, reduce behavior changes (Grossberg et al., 2019), and decrease hospitalizations for those with AD (Möllers et al., 2019), they can only be implemented after disease symptoms have been recognized. Clinically, the development of AD is broken down into three defined stages – preclinical AD, mild cognitive impairment (MCI) due to AD, and Alzheimer's dementia. The Alzheimer's dementia stage can be further distinguished as mild, moderate, or severe AD (Alzheimer's Association, 2023). In the initial preclinical AD phase, patients do not show significant, recognizable symptoms, but increased Aβ and other biomarkers of AD pathology are present in the brain. Research has demonstrated that Aβ plaque accumulation, synapse loss, and neuronal atrophy emerge first in the hippocampus and entorhinal cortex, areas which are crucial for learning and memory, and that these alterations can begin to emerge up to 1–2 decades before recognizable cognitive or behavioral symptoms of AD arise (Thompson et al., 2007; Reiman et al., 2012; Bateman et al., 2012). Noticeable changes in cognition and memory begin to appear in the MCI phase, where patients show an 11–25 % decrease in hippocampal volume compared to cognitively healthy, age-matched control individuals (Thompson et al., 2007). Finally, in the dementia phase, AD patients exhibit progressive and dramatic loss of both cognitive and physical functions (Alzheimer's Association, 2023).

Given the presence of pathological changes prior to the onset of well-documented symptoms, methods for earlier diagnosis are critical for clinicians to offer early interventions,

which can be more effective compared with interventions implemented in later disease states. The earlier therapeutic interventions can be administered, the more effective they are at slowing the progression of cognitive decline and behavioral changes, improving the prognosis for maintaining a high quality of life for AD patients as long as possible (Grossberg et al., 2019). Early diagnosis can also allow for patients to make important decisions, such as planning for care when they are no longer able to do so themselves, while still relatively cognitively healthy (Mattson et al., 2010). However, many primary care physicians are unable to identify patients in the early stages of AD, and it is estimated that as few as 20 percent of people in the U.S. who are living with various stages of AD have been formally diagnosed with the disease (Grossberg et al., 2019).

In addition to lacking a therapeutic mechanism to effectively treat or cure AD, much remains unknown regarding the underlying causes of the disease. Over 95% of AD cases are classified as sporadic, with no explainable etiology (Zetterberg & Mattsson, 2014). As important as it is to identify and develop therapeutic agents and methods for early diagnosis, it is just as imperative to elucidate the underlying causes of AD so that the disease can be better understood, and so that potential preventative measures can be taken early in adulthood. Thus, research efforts seek to explore potential lifestyle or environmental factors that could pose an increased risk for developing AD.

Inflammation and AD pathology

One pathophysiological change that can occur before or during the onset of AD is an increase in both central and peripheral proinflammatory agents, such as proinflammatory cytokines, which are small proteins that act as signaling agents at the site of an injury or infection (Tarkowski et al., 2003; Zotova et al., 2010; Park et al., 2020). In the short term, cytokines such

as IL-1β, TNFα, and IL-6 are a crucial facet of the innate immune response, as they are influential in cytotoxic T-cell differentiation and destruction of pathogens (Slavich & Irwin, 2014). However, chronic inflammation is thought to play a large role in the progression and, potentially, the initiation of the AD, as brains of AD patients show upregulated markers of inflammation compared to healthy individuals (Akiyama et al., 2014).

Increases in proinflammatory agents lead to enhanced amyloidogenic cleavage of APP by BACE1 into the potentially harmful Aβ fragments (Block, 2008). As these extracellular Aβ fibrils accumulate, microglial cells are activated to clear the excess of neurotoxic proteins and debris from dead neurons. In turn, activated microglial cells increase their production and release of proinflammatory cytokines to recruit other immune cells to the site. This microglial activation is an essential part of normal immune function, and proinflammatory cytokines alleviate some of the Aβ burden in the short term (Mandrekar & Landreth, 2010). However, evidence illustrates that repeated or prolonged activation of the immune response and extended production and release of proinflammatory cytokines can be damaging to neuronal function and can lead to an overall increase in Aβ deposition in the cerebral cortex (Lee et al., 2008; Kahn et al., 2012; Weintraub et al., 2014). Furthermore, inflammation itself is associated with increased cognitive dysfunction (Mandrekar & Landreth, 2010). This self-perpetuating cycle is detrimental to neuronal function and is thought to contribute to the cognitive impairment observed in AD patients (Block, 2008; Mandrekar-Colucci & Landreth, 2010).

Importantly, the innate immune response in the periphery also influences neuroinflammation (Dunn, 2002; Kempuraj et al., 2017). Cytokines in the periphery can induce cytokine production in the brain via signaling through vagal afferents, transportation through the blood-brain barrier by selective transporters, acting directly on brain tissue in circumventricular

organs, and by stimulating synthesis of other molecules that can pass through a weakened blood-brain barrier and cause an increase in central cytokine production (Dunn, 2002; Kempuraj et al., 2017; Quan & Banks, 2007). Thus, systemic inflammation can exacerbate neurodegeneration and can lead to neuronal death and cognitive decline (Kempuraj et al., 2017). As inflammation itself shares this link with AD pathology, causes of chronic inflammation should be closely investigated as potential risk factors for AD.

Sleep loss as a risk factor for AD

It is imperative to explore potential controllable risk factors for developing AD, as the disease etiology remains largely unknown, and it could be preventable in many cases. Several environmental and lifestyle-related risk factors for developing or exacerbating the disease pathology have been identified, including chronic consumption of a Western-style diet, smoking, poor cardiovascular health, lack of exercise, low cognitive stimulation during adulthood, chronic stress, and chronic sleep loss (Xu et al., 2015; Kivipelto et al., 2018; Bisht et al., 2018; Brice et al., 2020). Disrupted sleep/wake cycles are a common feature of AD, indicating a relationship between sleep and AD (Lucey & Bateman, 2014)**.** In recent years, data has mounted to support a bidirectional relationship between sleep loss and AD (Bedrosian & Nelson, 2012; Lucey & Bateman, 2014; Irwin & Vitiello, 2019). For example, evidence suggests sleep loss as an accelerator of cognitive aging, as chronic insomnia and reported sleep disturbances are associated with faster cognitive decline in older age (Cricco et al., 2002; Irwin & Vitiello, 2019). Obstructive sleep apnea (OSA), a disorder in which individuals repeatedly stop breathing during sleep, is also associated with cognitive aging increased biomarkers of AD, and earlier onset of MCI (Andrade et al., 2018). In fact, individuals with OSA had approximately twice the risk of developing AD compared to individuals without OSA, although the use of a continuous positive

airway pressure (CPAP) machine is somewhat protective against these risks (Andrade et al., 2018).

However, while approximately 5.9 million people in the U.S. are diagnosed with OSA, 50–70 million people in the U.S. suffer from a chronic sleep disorder such as insomnia, REM sleep behavior disorder, or restless leg syndrome (Ojile, 2018). These statistics are alarming, as chronic sleep loss is a pervasive problem for much of the U.S. population. Well over one-third of U.S. adults report obtaining less than seven hours of sleep per night (CDC Data and Statistics, 2020), which is below the minimum amount recommended for the average adult by the Sleep Research Society and the American Academy of Sleep Medicine (Watson et al., 2015). Frighteningly, many people get even less sleep, as the number of adults in the U.S. that report getting less than six hours of sleep per night has increased over the past 50 years (Frenda & Fenn, 2016). Furthermore, between 2017 and 2020, 30.5% of adults in the U.S. reported experiencing at least one hour of sleep debt (Di et al., 2022). Unfortunately, evidence suggests that habits that promote healthy sleep, such as keeping a consistent meal routine, mitigating light exposure two hours before bed, and being physically active during the day are not prioritized (National Sleep Foundation, 2022). In addition to these findings, in the past century, reported time spent trying to sleep has decreased by 20 percent (Sharma et al., 2015). These trends are not limited to adults, as over 50 percent of individuals between 15 and 17 report getting less than 7 hours of sleep each night (Engle-Friedman, 2014), although 8–10 hours are recommended for that age group (Paruthi et al., 2016). Particularly among younger individuals, the rise in use of smartphones and social networking sites likely contributes to insufficient sleep, as excessive social media use is associated with poor sleep quality (Alonzo et al., 2021). These patterns are alarming, as both younger and older individuals are increasingly affected by chronic sleep loss.

Sleep loss and AD pathology

While mechanisms through which chronic sleep loss shares a relationship with AD are not completely understood, evidence suggests that amyloid-beta (Aβ) likely plays a role. For instance, individuals with OSA show increases in serum Aβ, perhaps providing a clue to the link between the two diseases (Bu et al., 2015). Importantly, brain levels of Aβ fluctuate in a diurnal pattern – during wakefulness, less Aβ is present, while more Aβ is present during sleep (Cedernaes et al., 2017). Moreover, adults who reported experiencing excessive daytime sleepiness or problems sleeping were more likely to have increases in Aβ in the anterior cingulate and parietal cortices compared to adults with fewer complaints about sleepiness and sleep problems (Carvalho et al., 2018). Corroborating this, disrupting slow wave sleep in cognitively healthy adults for one night was associated with increased spinal CSF levels of Aβ the following morning (Ju et al., 2017). Likewise, in rats, total sleep deprivation for both 2 and 4 days was associated with elevated Aβ in the cerebral cortex (Chen et al., 2017). However, no compensatory upregulation in Aβ degradation enzymes neprilysin or insulin-degrading enzyme was observed, indicating that Aβ was not being broken down as quickly as it was being produced in the sleep-deprived rats (Chen et al., 2017). Importantly, wild-type mice that underwent six weeks of chronic sleep restriction had increased $A\beta_{42}$ in the hippocampus and exhibited cognitive impairment in contextual fear conditioning compared to control mice (Brice et al., 2020).

Glymphatic system function may be partially responsible for this increase in Aβ following sleep loss. Normally, Aβ is cleared through the brain's glymphatic system, which is more active during sleep and less active during wakefulness (Rainy-Smith et al., 2018). In humans, evidence demonstrates that one night of total sleep deprivation led to reduced clearance

of a CSF tracer, with levels of the tracer remaining elevated even after a full night's sleep, indicating that human glymphatic system function doesn't recover immediately following sleep deprivation (Eide et al., 2021).

In addition to being linked with increased Aβ, sleep loss is associated with alterations in tau pathology in the human brain (Lucey et al., 2019). For example, 30–60-year-old adults who underwent 36 hours of sleep deprivation exhibited increases in CSF levels of unphosphorylated tau and alters the sites of tau phosphorylation (particularly at the T217 site), potentially leading to hyperphosphorylation in CSF (Barthelemy et al., 2020). Moreover, in wild-type mice, chronic short sleep experienced early in life is associated with late-life upregulated phosphorylation of tau, along with increased $A\beta_{42}$ and exacerbated neurodegeneration, particularly in the CA1 region of the hippocampus (Owen et al., 2021). This indicates that chronic sleep loss early in life may play a role in AD pathogenesis in older age, even with a long period of recovery. While the mechanisms behind these associations remain elusive, one hypothesized link between chronic sleep loss and the pathogenesis of AD is chronic inflammation.

Sleep loss and inflammation

There is a well-established link between chronic stress and inflammation (Black, 2002; Cohen et al., 2012; Rohleder, 2019). In response to acute stressors, activation of the HPA axis results in increased glucocorticoid release, which are anti-inflammatory and immunosuppressive in the short term (Cohen et al., 2012). However, prolonged HPA axis activation and glucocorticoid release leads to glucocorticoid receptor resistance and failure to regulate inflammatory processes, resulting in increased risk of developing inflammatory diseases such as diabetes mellitus type II, cardiovascular disease, and AD (Cohen et al., 2012; Stark et al., 2001). In addition, evidence demonstrates that psychological stressors alone can increase

proinflammatory cytokines (Black, 2002). Furthermore, excessive psychological stress is associated with an exacerbated immune response and elevated production of proinflammatory cytokines in the hippocampus in response to immune challenges (Black, 2002; Johnson et al., 2002).

Sleep loss itself is a type of physiological stressor, as it leads to decreased parasympathetic activity and increased blood pressure, evening cortisol, and proinflammatory cytokine production (McEwen, 2006). For example, in humans, one week of mild sleep loss is associated with increased plasma IL-6 in men and women, and increased plasma TNFα in men (Vgontzas et al., 2004). Additionally, sleep disturbances were associated with increased C-reactive protein (CRP), an acute phase protein indicative of an active inflammatory state (Irwin, 2019). In male rats, 5 days of sleep restriction was associated with increased IL-1β mRNA in the hippocampus and prefrontal cortex (Zielinski et al., 2014). Chronic sleep loss also affects inflammation, as male rats that underwent chronic sleep restriction for 18 hours per day for 21 days showed elevations in plasma TNFα and IL-1β (Venancio & Suchecki, 2015). Similarly, in male mice, four weeks of chronic sleep restriction is associated with increased hippocampal IL-1β mRNA (Kincheski et al., 2017).

The relationship between sleep and cytokine expression is likely very complex and bidirectional in nature. For example, proinflammatory cytokines themselves are known have sleep-promoting effects (Imeri & Opp, 2009; Opp, 2004). Evidence further suggests that TNFα and IL-1β help initiate non-REM sleep and suppress REM sleep, while other evidence illustrates that receiving a high dose of IL-1β has different effects on non-REM sleep onset and duration, depending on the time of day (Imeri & Opp, 2009). Nonetheless, surmounting evidence indicates

that sleep loss may induce increased Aβ production through its effects on immune function, thereby increasing risk for developing AD.

Poor diet as a risk factor for AD

Another identified risk factor for AD is the chronic consumption of a poor diet (Kanoski & Davidson, 2011; Yusufov et al., 2017). Frighteningly, it is estimated that over 73% of the U.S. population over age 20 is classified as overweight, with an estimated 42.5% of those individuals having obesity (9% with severe obesity) (Fryar et al., 2021). Furthermore, evidence suggests that obesity at midlife increases risk of AD and other dementias by six-fold (Kivipelto et al., 2005). Obesity has been increasing over the last several decades in both men and women between the ages of 20–74 (Fryar et al., 2021), which is not entirely surprising, given the evidence that consumption of highly-processed foods has increased in recent years (Juul et al., 2021). From 2001–2018, dietary quality decreased among U.S. adults over the age of 65, with increased consumption of foods higher in saturated fat and cholesterol, and lower in fiber and essential micronutrients (Long et al., 2022). This is particularly concerning, given that AD risk begins to increase significantly at this age, and most sporadic cases of AD are diagnosed after the age of 65 (Castellani et al., 2010; Dorszewska et al., 2016).

Dietary trends and food processing procedures have evolved in contemporary Western populations, changing important components of the human diet, such as fatty acid and macronutrient composition, micronutrient density, fiber content, and glycemic load (Cordain et al., 2005). Principle components of a modern, typical American-style diet (TAD) include refined carbohydrates, simple sugars, and saturated fats – all of which are associated with increased AD risk and cognitive dysfunction – while it is deficient in fiber and essential micronutrients (Kanoski & Davidson, 2011). Sadly, it was estimated in 2016 that total sugar consumption was

around 92.5 grams per day in U.S. adults, which is well over 300% of the recommended daily sugar intake (Faruque et al., 2019). In contrast, in 2019, only 12% of the U.S. population was estimated to consume the recommended 1.5–2 cup-equivalents of fruit per day, and a mere 10% of the population met the recommended 2–3 cup-equivalents of vegetables each day (Lee et al., 2022). Accordingly, fiber content is low in the TAD, with adults consuming around 10–15 grams less than the recommended 25–30 grams of fiber per day (Cordain et al., 2005). Furthermore, sugars with a high glycemic load, or potential to raise blood glucose, such as high-fructose corn syrup, sucrose, and glucose, supply over 18% of total energy in a TAD, while refined grains with high glycemic load constitute over 20% of total energy (Cordain et al., 2005). The TAD also tends to be high in sodium, with around 10% of salt intake coming from naturally-occurring sources, while the remaining 90% is manufactured salt added to processed or cooked foods (Cordain et al., 2005). Unsurprisingly, it is well-known that regular consumption of these foods are associated with increased risk for adverse health outcomes, such as obesity, diabetes mellitus type II, high blood pressure, and coronary heart disease (Brand-Miller, 2003; Cordain et al., 2005; Hunter et al., 2022).

Unbalanced fatty acid composition is also a component of the TAD. Consuming a balance of monounsaturated and polyunsaturated fats is associated with health benefits (Cordain et al., 2005; Mashek & Wu, 2015; Saini et al., 2021). However, while it is recommended to consume a diet that consists of a 2:1 ratio of omega-6 to omega-3 polyunsaturated fatty acids, the average adult in the U.S. consumes an omega-6 to omega-3 ratio of 16–20:1 (Simopoulos, 2021). Furthermore, adults in the U.S. typically consume excess saturated and trans fat in foods such as processed meat, butter, margarine, high-fat dairy products, and baked goods, which are associated with increased LDL cholesterol (Cordain et al., 2005). From 1999–2016, U.S. adults

significantly increased consumption of saturated fatty acids, consuming around 12% of total energy from saturated fatty acids every day (while it is recommended to consume no more than 10% of daily energy from saturated fatty acids) (Shan et al., 2019). This could be related to excessive consumption of low-quality fast foods high in saturated fats, as over one-third of U.S. adults report that they eat fast food on a given day (Fryar et al., 2018). In contrast, surmounting evidence has suggested that adhering to a Mediterranean-style diet, rich in essential monounsaturated and polyunsaturated fats obtained largely through olive oil, nuts, and fish, is associated with longevity and may be a beneficial preventative strategy, or even an intervention, for AD and related dementias, as this diet is associated with protection from brain atrophy and decreased markers of AD (Ballarini et al., 2021; Mosconi et al., 2014; Ricordi et al., 2015; Solch et al., 2022).

Typical American diet and AD pathology

Evidence demonstrates a link between consumption of a TAD and AD-like effects in humans and rodents (Hoscheidt et al., 2022; Hsu & Kanoski, 2014; Thériault et al., 2016; Więckowska-Gacek et al., 2021). For example, in male APP/PS1$_{swe}$ mice, Western-style diet consumption for 3 months was associated with earlier deposition of Aβ plaques in the hippocampus compared to mice fed the standard chow, as well as elevated levels of APP after only three weeks of Western diet consumption (Więckowska-Gacek et al., 2021). Furthermore, eight months of Western diet consumption induced obesity and increased microglial activation in both male and female APP/PS1$_{swe}$ mice and C57BL/6 mice (Graham et al., 2016). In addition, in the APP/PS1$_{swe}$ mice, the Western diet led to increased Aβ plaques and elevated levels of Aβ peptides in the hippocampus (Graham et al., 2016). This increase in Aβ could be due in part to the upregulated production of neurotoxic Aβ peptides, as high-fat diet consumption is associated

with enhanced cleavage of APP by BACE1 (Maesako et al., 2015; Thirumangalakudi et al., 2008). Furthermore, in humans, having more visceral adipose tissue is independently associated with decreased hippocampal volume (Isaac et al., 2011). Moreover, neuronal loss has been observed in the hippocampus in C57BL/6 mice following Western diet consumption, indicating a relationship between chronic TAD consumption and brain atrophy (Graham et al., 2016).

Unsurprisingly, consumption of a TAD is also associated with impaired cognitive functioning (Francis & Stevenson, 2013; Hsu & Kanoski, 2014). For example, 3 months of Western diet consumption led to cognitive impairment in a cued fear conditioning paradigm in male C57BL/6 mice (Gabriel et al., 2020). Further evidence suggests that the combination of refined sugar and saturated fat can be especially harmful to cognitive function, as mice fed a Western diet high in saturated fat and sugar demonstrated deficits in an odor-related learning and olfactory memory task before mice fed a diet only high in saturated fat (Lietzau et al., 2020). Evidence also suggests that relatively short periods of high saturated fat consumption can disrupt cognitive function. For instance, rats that consumed a diet high in saturated fat and simple carbohydrates for three days exhibited working memory and nonspatial reference learning deficits in a radial arm maze (Kanoski & Davidson, 2010). Likewise, in college students, consuming a breakfast high in saturated fat (a toasted sandwich and a chocolate milkshake) for four days was associated with a decline in performance in hippocampus-dependent learning and memory tasks (Attuquayefio et al., 2017)

Typical American diet and inflammation

As is the case with chronic sleep loss, it is possible that one of the mediators between chronic consumption of a TAD and the pathogenesis of AD is inflammation. Components of the TAD, such as saturated fat and refined carbohydrates, are associated with increased

inflammation. For example, mice that consumed a diet high in refined carbohydrates (74.2% kcal from carbohydrates, with 30% kcal from refined sugars such as sucrose) showed increased proinflammatory cytokines from adipose tissue after only one day of high-carbohydrate diet consumption, and after 12 weeks of high-carbohydrate diet consumption (Oliveira et al., 2013). Moreover, in human serum, refined grain consumption is associated with higher CRP, while whole grain consumption was negatively associated with CRP (Taskinen et al., 2022).

Research in male C57BL/6 mice has shown that consuming a diet high in saturated fatty acids (42% kcal from milk fat) for only 4 weeks leads to increases in TNFα, IL-6, and IL-1β mRNA in the hypothalamus (Valdearcos et al., 2014). Moreover, Valdearcos and colleagues (2014) also found that administering milk fat via gavage for 3 days increased TNFα, IL-6, and IL-1β mRNA in the hypothalamus, consistent with the effects found in the high-fat diet-fed mice, while olive oil gavage administration did not increase these markers. Furthermore, mice that consumed a high-fat, high-sucrose diet beginning at 20 weeks of age for 13 weeks exhibited increased inflammation in the hippocampus and prefrontal cortex compared to mice consuming the control diet (Baranowski et al., 2018).

In humans, a longitudinal study determined that higher saturated fatty acid consumption was associated with increased risk for dementia, while consumption of fish (which is high in omega-3 polyunsaturated fatty acids) was associated with decreased risk of dementia (Kalmijn et al., 1997). Thus, inflammation may provide a link between saturated fat itself and AD. Foods that are high in saturated fat cause an increase in blood glucose levels, which leads to increased activation of NF-kB, a transcription factor that stimulates proinflammatory cytokine production (Huang et al., 2012). Furthermore, saturated fatty acids activate TLR4 signaling in the

hypothalamus, which induces cytokine production and is thought to play a role in the resistance to anorectic peptides in the development of obesity (Milanski et al., 2009).

Obesity itself is linked with impaired immune function, including increased vulnerability to bacterial and viral infection, as well as elevated risk for severe complications resulting from these infections (Almond et al., 2013; Baumgarner et al., 2014; Falagas & Kompoti, 2006). Unsurprisingly, obesity also leads to increased inflammation, as white adipose tissue releases IL-6 and TNFα, causing a decrease in insulin sensitivity and an inflammatory state associated with an increase in reactive oxygen species and resulting oxidative stress (Fernández-Sánchez et al., 2011). Additionally, chronic consumption of a TAD and obesity both increase the risk of developing diabetes mellitus type II, which itself is considered a risk factor for AD (Janson et al., 2004). Given the obesity epidemic in the U.S., the prevalence of processed foods high in saturated fat and sugar, and the projected incline in AD cases, it is crucial that we discern the relationships between diet, immune function, and AD.

Current research

The present research investigated chronic sleep loss and consumption of a typical American-style diet as potential risk factors for AD. Specifically, Experiment 1, detailed in Chapter 2, explored whether chronic sleep restriction would alter inflammation and the immune response to an inflammatory stimulus. Experiment 2, discussed in Chapter 3, investigated whether the addition of a high-fat, typical American-style diet would, in combination with chronic sleep restriction, impair cognitive function and lead to elevated Aβ in the brain. Experiment 3, described in Chapter 4, explored whether the addition of this typical American-style diet would exacerbate the effects of chronic sleep restriction hypothesized in the first experiment. Given the statistics regarding poor sleep, diet, and obesity in the U.S., it is likely that

a significant portion of the population regularly lacks sleep and consumes a poor diet, in addition to experiencing a multitude of other factors which likely cause stress, leading to increased risk for adverse health events. Therefore, it is important to gain an understanding of how these two lifestyle factors may influence AD-like pathology, especially since diet and sleep habits are largely controllable.

CHAPTER 2: THE EFFECTS OF CHRONIC SLEEP RESTRICTION AND 1 LPS INJECTION

1. Abbreviated Introduction

Despite being associated with elevated inflammation, evidence suggests that sleep loss can impair immune function. Sleep loss activates the sympathetic nervous system, which stimulates proinflammatory cytokine production and decreases antiviral responses. In addition, there is a modest increase in HPA axis activation following sleep loss, which also inhibits anti-viral response (Irwin, 2012). Consequently, sleep loss can lead to inadequate immune responses to pathogens and increase susceptibility to infectious diseases, while increasing risk for developing inflammatory diseases (Irwin, 2012). Research has demonstrated that sleeping less than 6–7 hours per night is associated with increased susceptibility to the common rhinovirus (Cohen et al., 2009; Prather et al., 2015; Prather & Leung, 2016). Moreover, short sleep duration is linked with lower antibody responses to vaccination and decreased likelihood of clinical protection due to vaccination, which is of particular relevance given the recent COVID-19 pandemic (Prather et al., 2012; Schmitz et al., 2022).

Previous work has shown that exposure to stress creates a proinflammatory state and leads to enhanced inflammatory response and increased microglial activity in response to one injection of LPS (Diz-Chaves et al., 2013). As sleep loss is physically stressful, and as sleep loss is associated with an inflammatory state, it follows that sleep loss would exacerbate the inflammatory response to LPS. Prior research in mice demonstrated that 12 weeks of circadian disruption via advancing the light cycle 6 hours per week was associated with exacerbated IL-6 release from trunk blood cells in response to one LPS injection (Brager et al., 2013). Moreover, evidence suggests that short-term sleep deprivation may impair the immune response to LPS, as male C57BL/6 mice that underwent 72 hours of sleep deprivation via the Modified Multiple

Platform Method (MMPM) had a decrease in circulating lymphocytes, and a decrease in T and B cells in the spleen, in comparison to non-sleep-deprived control mice (Zager et al., 2012).

Much remains to be elucidated regarding the relationship between chronic sleep loss and immune function. In addition, there may be sex-specific effects at play in this relationship. Specifically, it is not known whether chronic sleep loss in wild-type mice will exacerbate or dampen the inflammatory response to one LPS injection, or if this response differs between male and female mice. The present study explored how six weeks of chronic sleep restriction (CSR) would impact the inflammatory response to one LPS injection, as well as BDNF mRNA, in both male and female C57BL/6 mice.

Hypotheses

Based on the literature suggesting that chronic sleep loss is associated with an inflammatory state, we hypothesized that chronic sleep restriction would exacerbate the inflammatory response to one LPS injection. Therefore, we expected to observe significantly elevated cytokine mRNA and increased cytokine levels in the serum of mice that underwent CSR and received an LPS injection in comparison to other groups. Furthermore, we hypothesized that CSR alone would lead to increased inflammation in both the brain and periphery compared to the HCC group. In addition to these primary aims, we also explored how chronic sleep restriction and one LPS injection might impact BDNF mRNA in the hippocampus. We hypothesized that the CSR mice would have decreased BDNF mRNA compared to HCC mice, and that LPS would also be associated with decreased BDNF mRNA.

2. Methods

Subjects

The current research utilized experimentally naïve male and female C57BL/6J mice, bred in the Texas Christian University vivarium from a breeding stock acquired from the Jackson Laboratory (The Jackson Laboratory, Bar Harbor, ME). All mice will be housed in groups of 3–4 with age-matched pups from other litters in standard polycarbonate mouse cages (30 x 20 x16 cm). All mice received care in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996) and were housed under a 12-hour light/dark cycle (lights on from 0700h to 1900h), with food and water available *ad libitum*. All experiments described herein were approved by the Texas Christian University Institutional Animal Care and Use Committee (IACUC) (protocols #2021-11 and #2021-14). In Experiment 1, mice were between 4.5–5.5 months of age at the beginning of the study protocol, making them 6–7 months of age at the time of tissue collection (See Fig. 1 for study timeline).

Chronic Sleep Restriction

All mice were assigned to one of two sleep conditions: chronic sleep restriction (CSR), or home cage control (HCC). The method of sleep restriction used was the Modified Multiple Platform Method (MMPM), which is a widely-utilized technique that has been validated by rodent EEG to confirm sleep loss (Machado et al., 2004; Machado et al., 2006). At 0800h each morning, mice in the CSR group were placed into large, polycarbonate cages (26.67 cm X 48.26 cm X 15.56 cm) containing 14 small platforms arranged in a staggered pattern, about 4.5 cm apart, in the cage. Each platform was constructed from a PVC pipe plug and was roughly 3.2 cm tall, 3.3 cm in diameter, and was glued to the bottom of the cage with aquarium sealant (See Fig. 1 for MMPM cage). These CSR cages were filled with about 2 cm with lukewarm water,

creating small, island-like platforms. All CSR cages were placed on heating mats (Vivosun, Los Angeles, CA), that kept the water temperature at 25º C (Yin et al., 2017), preventing subject hypothermia. Food and water were available *ad libitum*, as there were four different platforms from which to access food, and four different platforms from which to access water, such that no single mouse could prevent cage mates' access to food or water. CSR mice were able to readily move from platform to platform and were easily able to stand. However, once the mice reached the paradoxical stage of sleep (also called rapid eye movement, or REM, sleep), in which they lose muscle tone, they fell from the platforms into the lukewarm water. This caused them to wake, but not undergo hypothermia. CSR mice remained in these sleep restriction cages for 10 hours each day, from 0800h to 1800h, and were returned to their home cages with normal bedding, food, and water following this sleep restriction period. HCC mice were placed with their cage mates into new cages identical to their home cages, complete with normal food, water, and bedding, at the same time and in the same room as the CSR mice to control for handling stress. All mice were weighed once per week throughout the six-week protocol.

Figure 1. Modified multiple platform method of sleep restriction. Water fills the bottom of the cage, leaving the 14 small platforms exposed. Mice remain on the platforms for 10 hours each day and can access food and water as needed from several different platforms.

Intraperitoneal injections

Prior studies have demonstrated that injecting LPS into the peritoneal cavity elicits an immune response that is similar to that following a bacterial infection. LPS binds to CD14 receptors, which activates Toll-like receptor 4 (TLR4), leading to a cascade of events that causes an increase in the production and release of proinflammatory cytokines (and anti-inflammatory cytokines as a compensatory mechanism) from immune cells (Palsson-McDermott & O'Neill, 2004). Shortly after this immune response begins in the periphery, proinflammatory cytokines are also upregulated in the brain. For instance, prior work in our laboratory demonstrates one 250μg/kg dose of LPS, administered intraperitoneally, leads to an increase in IL-1β in both the cortex and hippocampus four hours following the injection (Kranjac et al., 2011). Mechanisms through which these cytokines may evoke central inflammation include transporters that carry peripheral cytokines into the brain, signaling via vagal afferents, immune cells that can enter the brain and produce cytokines, and circumventricular organs, where peripheral cytokines can act to signal the brain in the absence of a blood-brain-barrier (Dunn, 2002).

The morning after the final day of the sleep restriction protocol, all mice were assigned to one of two injection treatments – LPS or saline. Mice in the LPS group received one 250μg/kg dose of LPS (*Escherichia coli* serotype O26:B6; Sigma, St. Louis, MO), injected into the peritoneal cavity. Mice in the saline group received one 200μL injection of sterile saline (Dulbecco's PBS; Caisson Laboratories, Smithfield, UT), injected in the same location. All injections took place between 0800 h and 1100 h.

Serum and Tissue Collection

Four hours following the LPS and saline injections, mice were euthanized via rapid decapitation. Trunk blood was collected in microcentrifuge tubes and placed on ice for 15

minutes, followed by incubation at room temperature for an additional 30 minutes. Blood sample tubes were then centrifuged (18,620 x g/14,000 rpm) at 4º C for 10 minutes. Serum was collected, snap-frozen on dry ice, and stored at -80º C until further sample processing.

During trunk blood collection, tissue was collected from the dorsal hippocampi from both hemispheres of the brain, as IL-1β can act on this area of the hippocampus to disrupt learning and memory (Barrientos et al., 2002). Bilateral hippocampal samples were placed into an RNA-preservation solution (RNA*later*™ Stabilization Solution, ThermoFisher Scientific, Waltham, MA). Tissue was cut with microscissors approximately four times before samples were stored at -20º C.

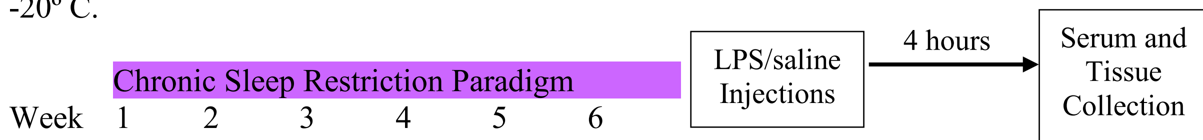

Figure 2. Experimental timeline for Study 1. Adult male and female C57BL/6J mice underwent six weeks of CSR, followed by one injection of LPS or saline. Four hours post-injection, serum and hippocampal tissue were collected.

Peripheral Cytokine Quantification

Serum levels of IL-1β, TNFα, and IL-10 were quantified using MesoScale Discovery Proinflammatory Panel 1 (mouse) multiplexing kits (Meso Scale Diagnostics, Rockville, MD) according to manufacturer instruction in provided 96-well precoated plates. Briefly, wells were washed three times with wash buffer, serum was added to wells in a 1:1 dilution with a proprietary diluent, and the plate was allowed to incubate at room temperature with shaking (700 rpm) for 2 hours. Next, the wells were washed three times, and a detection antibody cocktail was added to each well and allowed to incubate at room temperature with shaking for 2 hours. The wells were washed three more times, the provided read buffer was added to each well, and the electrochemiluminescent signal was read utilizing a QuickPlex SQ 120 instrument (Meso Scale Diagnostics, Rockville, MD). Results were calculated using a 4-parameter fit curve.

Hippocampal mRNA Quantification

Reverse transcriptase polymerase chain reaction (RT-PCR) was conducted to measure the amount of mRNA for various cytokine targets and BDNF in the hippocampus. Messenger RNA (mRNA) was isolated using Maxwell® LEV simplyRNA Purification Kits (Promega, Madison, WI). Samples were measured using a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA) to determine the amount of nuclease-free water to add to each sample to standardize samples to 100ng/reaction. Then, 16µL of each diluted sample was placed into a separate set of microcentrifuge tubes. The reverse transcriptase step was conducted utilizing a 7500 Real-Time PCR Thermal Cycling System (Applied Biosystems, Foster City, CA) by adding 4µL of Script™ Reverse Transcriptase Supermix (Bio-Rad, Hercules, CA) to mRNA diluted with nuclease-free water for a total volume of 20µL. Thermocycling parameters were as follows: 5 minutes at 25º C, 30 minutes at 42º C, and 5 minutes at 85º C. Complementary DNA (cDNA) samples were stored at -20º C until the qPCR step. qPCR was conducted by adding samples, nuclease-free water, iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA), and PrimePCR™ Probe Assay (Bio-Rad, Hercules, CA) probes in triplicate in a 96-well plate. Thermocycling parameters on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA) were as follows: 30 seconds at 95º C, then 5 seconds at 95º C, followed by 30 seconds at 60º C, repeated 39 times. Using CFX Manager software (Bio-Rad, Hercules, CA) cDNA for each target was compared to the amount of β-actin, a housekeeping gene commonly used as a control. Samples with a quantitation cycle (Cq) standard deviation greater than .250 were examined, and the outlying triplicate was removed. If the Cq standard deviation for the remaining duplicates was greater than .250, the sample was eliminated from analysis. Statistical

analyses on relative expression were completed in Prism (GraphPad Software, Inc., San Diego, CA).

Statistical Analyses

All statistical analyses were performed with alpha levels of 0.05, and any significant interactions were explored using pairwise comparisons.

3. Results

Experiment 1A: Effects of Chronic Sleep Restriction on Body Weight

A repeated measures analysis of variance (ANOVA) (Sleep Condition [chronic sleep restriction vs. home cage control] x Week [Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6]) was performed for both male and female mice to assess the impact of chronic sleep restriction on body weight. In male mice, results revealed a significant main effect of week ($F(6,246) = 75.884$, $p < 0.001$), such that all mice gained weight throughout the sleep restriction paradigm, regardless of Sleep Condition. There was also a significant Week x Sleep Condition interaction ($F(6,246) = 3.388$, $p = 0.003$), such that the CSR mice lost weight during the first week of the CSR paradigm, whereas the HCC mice did not (See Fig. 3A). Similarly, in female mice, results illustrated a significant main effect of Week ($F(6,270) = 81.460$, $p < 0.001$), such that all mice gained weight throughout the sleep restriction paradigm. The Week x Sleep Condition interaction was approaching significance ($F(6,270) = 2.049$, $p = 0.060$), such that the CSR mice lost weight during the first week of the CSR paradigm, while the HCC mice did not (See Fig. 3B). There was no main effect of Sleep Condition for male ($F(1,41) = 0.025$, $p = 0.875$) or female ($F(1,45) = 1.907$, $p = 0.174$) mice.

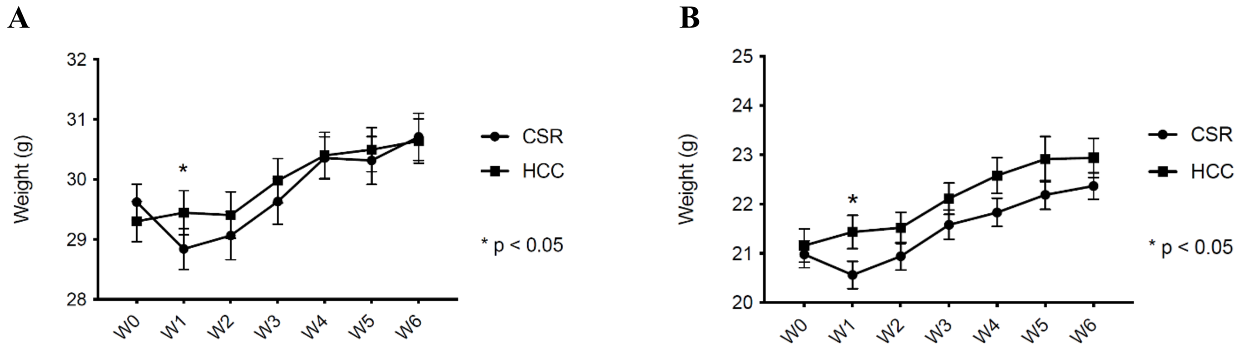

Figure 3. Body weight during CSR in male and female mice. Repeated-measures ANOVAs revealed that both male (A) and female (B) mice gained weight throughout the experiment. Both male and female CSR mice lost weight during the first week of the CSR paradigm. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 21–24.

Experiment 1B: Effects of Chronic Sleep Restriction on BDNF mRNA

Two 2-way ANOVAs (Sleep Condition [chronic sleep restriction vs. home cage control] x Injection Treatment [LPS vs. saline]) were performed to explore the impact of chronic sleep restriction and one injection of LPS on BDNF mRNA in the hippocampus in both male and female mice. In males, results revealed a significant main effect of Sleep Condition ($F(1,37) = 6.956$, $p = 0.012$), such that the CSR mice had less BDNF mRNA compared to HCC mice (See Fig. 4A). This was not true in females, however, as there was no effect of Sleep Condition on BDNF mRNA ($F(1,43) = 0.802$, $p = 0.376$) (See Fig. 4B). There was no effect of Injection Treatment in males ($F(1,37) = 0.069$, $p = 0.795$) or females ($F(1,43) = 0.869$, $p = 0.357$).

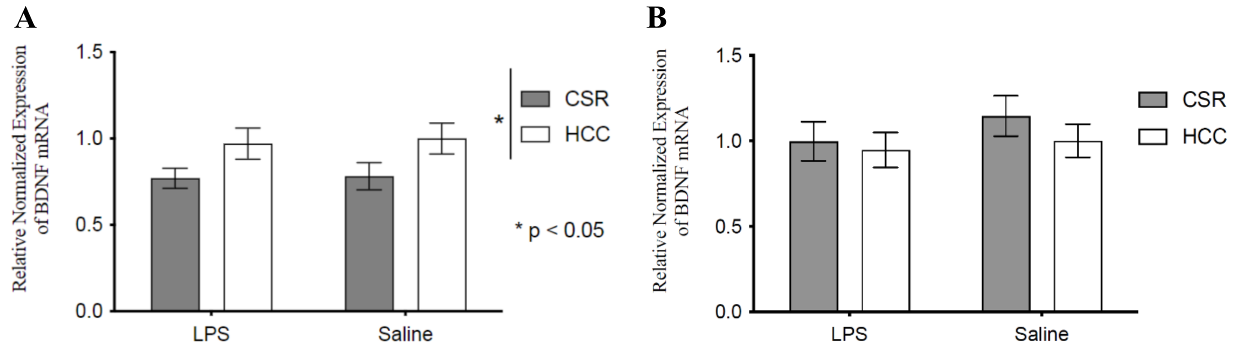

Figure 4. Chronic sleep restriction leads to decreased hippocampal BDNF mRNA in male, but not female, mice. (A) Male mice that underwent CSR had significantly less BDNF mRNA in the hippocampus compared to HCC mice. (B) No differences were observed in female mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 10–11.

Experiment 1C: Effects of Chronic Sleep Restriction on Immune Markers in the Hippocampus

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Injection Treatment [LPS vs. saline]) were completed to explore the impact of one LPS injection following chronic sleep restriction on hippocampal mRNA for cytokines IL-1β, IL-6, IL-10, and TNFα in both male and female mice. Male data will be discussed first, followed by discussion of results for female mice.

Male Hippocampal mRNA Data

For TNFα, results revealed no main effect of CSR ($F(1,28) = 2.012$, $p = 0.1671$) or interaction ($F(1,28) = 1.653$, $p = 0.209$) on hippocampal TNFα mRNA. However, there was a significant main effect of injection ($F(1,28) = 24.800$, $p < 0.001$), such that mice that received an LPS injection had higher TNFα mRNA compared to mice that received a saline injection (See Fig. 5A).

For IL-1β, a 2-way ANOVA revealed a significant interaction ($F(1,37) = 223.60$, $p < 0.001$), such that, among the mice injected with LPS, those that underwent CSR had less IL-1β mRNA compared to HCC mice ($p < 0.001$). Furthermore, there was a significant main effect of

sleep condition ($F(1,37) = 224.60$, $p < 0.001$), such that the CSR mice had less IL-1β mRNA compared to HCC mice. There was also a significant main effect of injection ($F(1,37) = 2388.00$, $p < 0.001$), such that mice that received an LPS injection had higher IL-1β mRNA compared to mice that received a saline injection (See Fig. 5B).

For IL-6, results revealed a significant interaction ($F(1,36) = 10.31$, $p = 0.003$), such that, among the mice injected with LPS, those that underwent CSR had less IL-6 mRNA compared to HCC mice ($p = 0.0001$). In addition, there was a significant main effect of sleep condition ($F(1,36) = 13.71$, $p < 0.001$), such that the CSR mice had less hippocampal IL-6 mRNA compared to HCC mice. There was also a significant main effect of injection ($F(1,36) = 203.80$, $p < 0.001$), such that mice that received an LPS injection had higher IL-6 mRNA compared to mice that received a saline injection (See Fig. 5C).

Finally, for IL-10, a results revealed a significant main effect of sleep condition ($F(1,26) = 31.17$, $p < 0.001$), such that the CSR mice had less hippocampal IL-10 mRNA compared to HCC mice, although there was no significant interaction ($F(1,26) = 2.407$, $p = 0.133$). Inconsistent from the findings in all markers previously discussed, there was no effect of injection for IL-10 mRNA ($F(1,26) = 1.408$, $p = 0.246$) in male mice (See Fig. 5D).

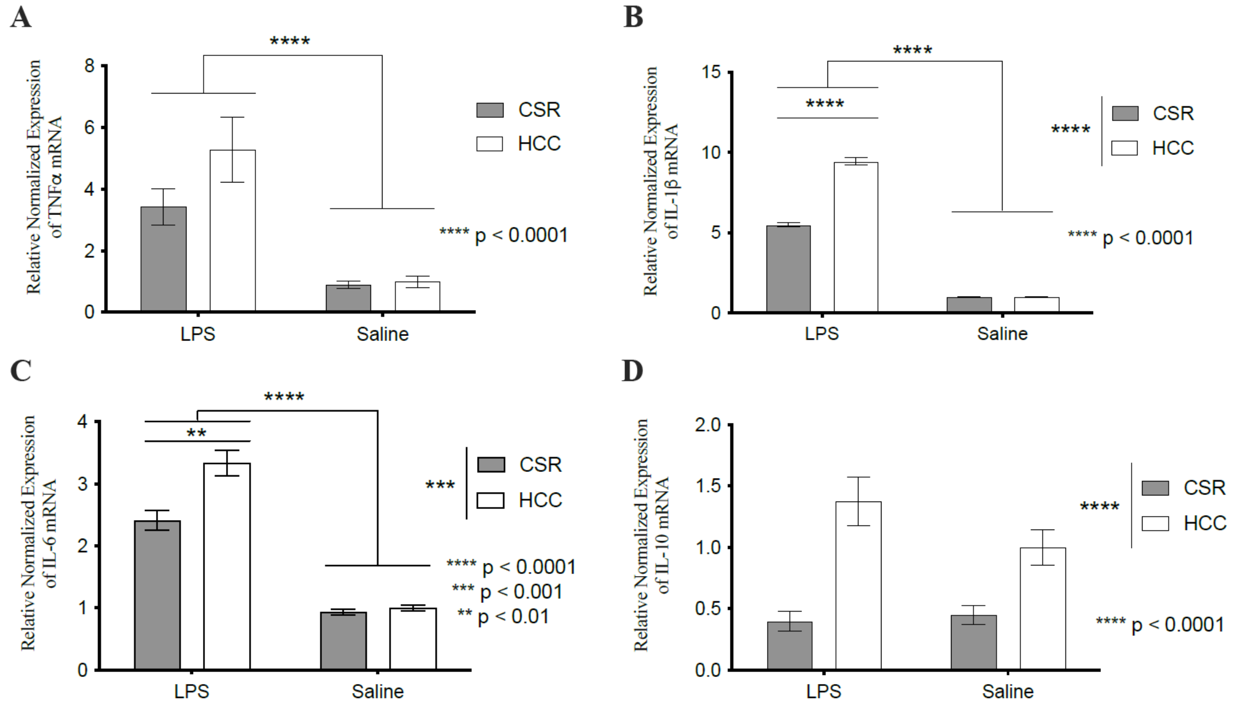

Figure 5. Hippocampal TNFα (A), IL-1β (B), IL-6 (C), and IL-10 (D) mRNA in male mice following CSR and one injection of LPS. 2X2 ANOVAs revealed significant interactions, such that, among mice that received LPS injections, those that underwent CSR had lower IL-1β mRNA (B), and lower IL-6 mRNA (C) compared to HCC mice. CSR mice also had significantly lower IL-1β (B), IL-6 (C), and IL-10 (D) mRNA in the hippocampus compared to HCC mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 5–11.

Female Hippocampal mRNA Data

Identical analyses were performed to for female mice. For TNFα, although there was no significant interaction ($F(1,42) = 2.190$, $p = 0.146$), there was a significant main effect of sleep condition ($F(1,42) = 4.682$, $p = 0.036$), such that CSR mice had less hippocampal TNFα mRNA compared to HCC mice, and a significant main effect of injection ($F(1,42) = 61.45$, $p < 0.001$), such that LPS-injected mice had less TNFα mRNA compared to saline-injected mice (See Fig. 6A).

For IL-1β mRNA, results revealed a significant main effect of injection ($F(1,41) = 62.69$, $p < 0.001$), such that LPS groups had higher IL-1β mRNA compared to saline groups (See Fig.

6B). However, there was no main effect of CSR ($F(1,41) = 1.141$, $p = 0.292$) and no significant interaction $F(1,41) = 2.018$, $p = 0.163$).

For IL-6 mRNA, results revealed a significant interaction $F(1,42) = 4.545$, $p = 0.039$), such that, among the mice that received LPS, those that underwent CSR had less hippocampal IL-6 mRNA compared to HCC mice ($p = 0.030$). Furthermore, there was a significant main effect of sleep condition such that CSR mice had less IL-6 mRNA compared to HCC mice ($F(1,42) = 4.194$, $p = 0.047$), and a significant main effect of injection such that mice injected with LPS had higher IL-10 compared to those injected with saline ($F(1,42) = 52.97$, $p < 0.001$) (See Fig. 6C).

For IL-10 mRNA, there was a significant main effect of injection ($F(1,33) = 20.31$, $p < 0.001$), such that mice that received an LPS injection had higher IL-10 compared to mice that received a saline injection (See Fig. 6D). There was no interaction ($F(1,33) = 0.046$, $p = 0.832$) or main effect of sleep condition ($F(1,33) = 0.485$, $p = 0.491$).

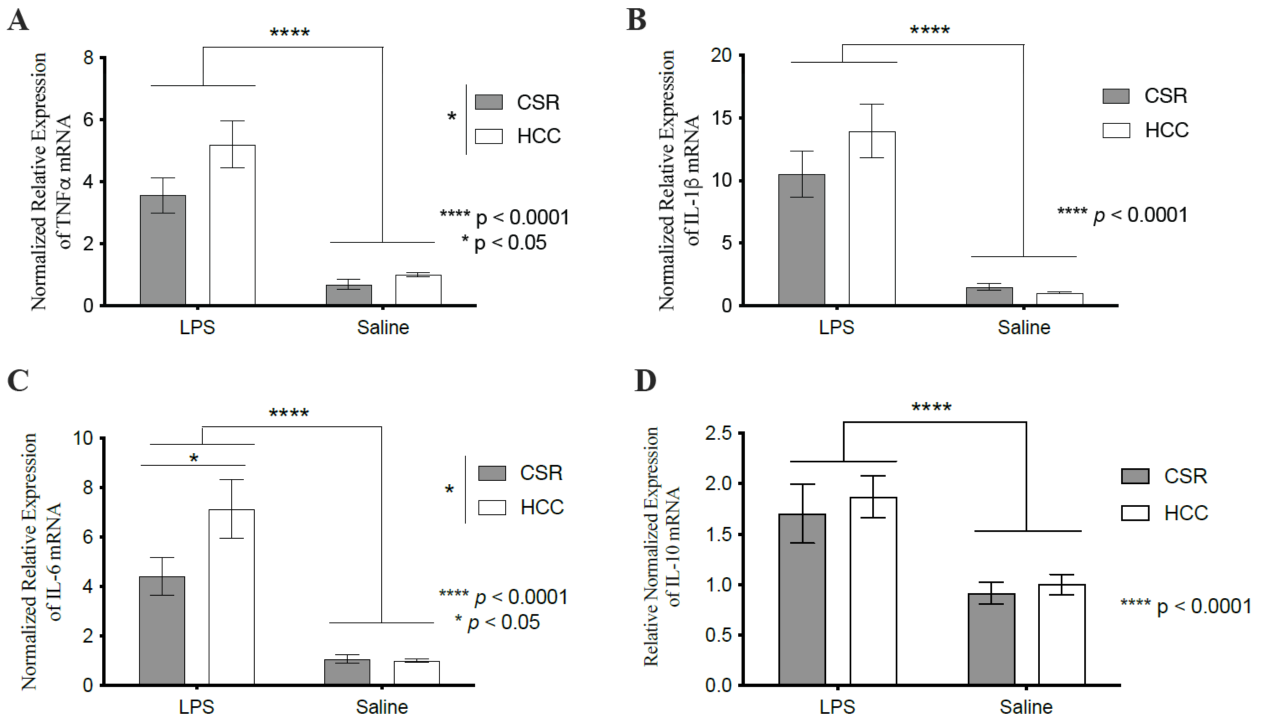

Figure 6. Hippocampal TNFα (A), IL-1β (B), IL-6 (C), and IL-10 (D) mRNA in female mice following CSR and one injection of LPS. Among mice that received LPS injections, those that underwent CSR had lower IL-6 mRNA (C) compared to HCC mice. CSR mice also had significantly lower TNFα mRNA (A) in the hippocampus compared to HCC mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 8–12.

Experiment 1D: Impact of Chronic Sleep Restriction on Serum Immune Markers

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Injection Treatment [LPS vs. saline]) were performed to investigate the effects of one LPS injection following chronic sleep restriction on serum cytokines IL-1β, TNFα, and IL-10, in both male and female mice. Male data will be discussed first, followed by discussion of female data.

Male Serum Data

For TNFα, the interaction was approaching significance ($F(1,37) = 3.765$, $p = 0.060$), such that, among the mice that received LPS, those that underwent CSR had higher TNFα compared to HCC mice ($p = 0.009$). The main effect of sleep condition was also approaching significance ($F(1,37) = 4.031$, $p = 0.052$), such that CSR mice had more serum TNFα compared

to HCC mice. Furthermore, there was a significant main effect of injection ($F(1,37) = 82.455$, $p < 0.001$), such that mice that received an LPS injection had higher serum TNFα compared to mice that received a saline injection (See Fig. 7A). Two samples were removed from analysis due to having CV values over 30%.

For IL-1β, while the interaction was not significant ($F(1,36) = 2.496$, $p = 0.123$), the main effect of sleep condition was approaching significance ($F(1,36) = 2.836$, $p = 0.099$), such that the CSR mice had slightly higher serum IL-1β compared to HCC mice. There was also a significant main effect of injection ($F(1,36) = 72.031$, $p < 0.001$), such that LPS-injected mice had higher IL-1β compared to saline-injected mice (See Fig. 7B). Two samples were excluded from analysis due to falling below the bottom of the standard curve, and one was excluded from analysis due to having a coefficient of variation over 30%.

For IL-10, results revealed a significant main effect of LPS ($F(1,37) = 38.129$, $p < 0.001$), such that LPS-injected mice had higher IL-10 compared to saline-injected mice (See Fig. 7C). The Sleep Condition x Injection interaction was not significant ($F(1,37) = 2.529$, $p = 0.120$). The main effect of Sleep Condition was also not significant ($F(1,37) = 2.776$, $p = 0.104$). Two samples were removed from analysis due to having CV values over 30%.

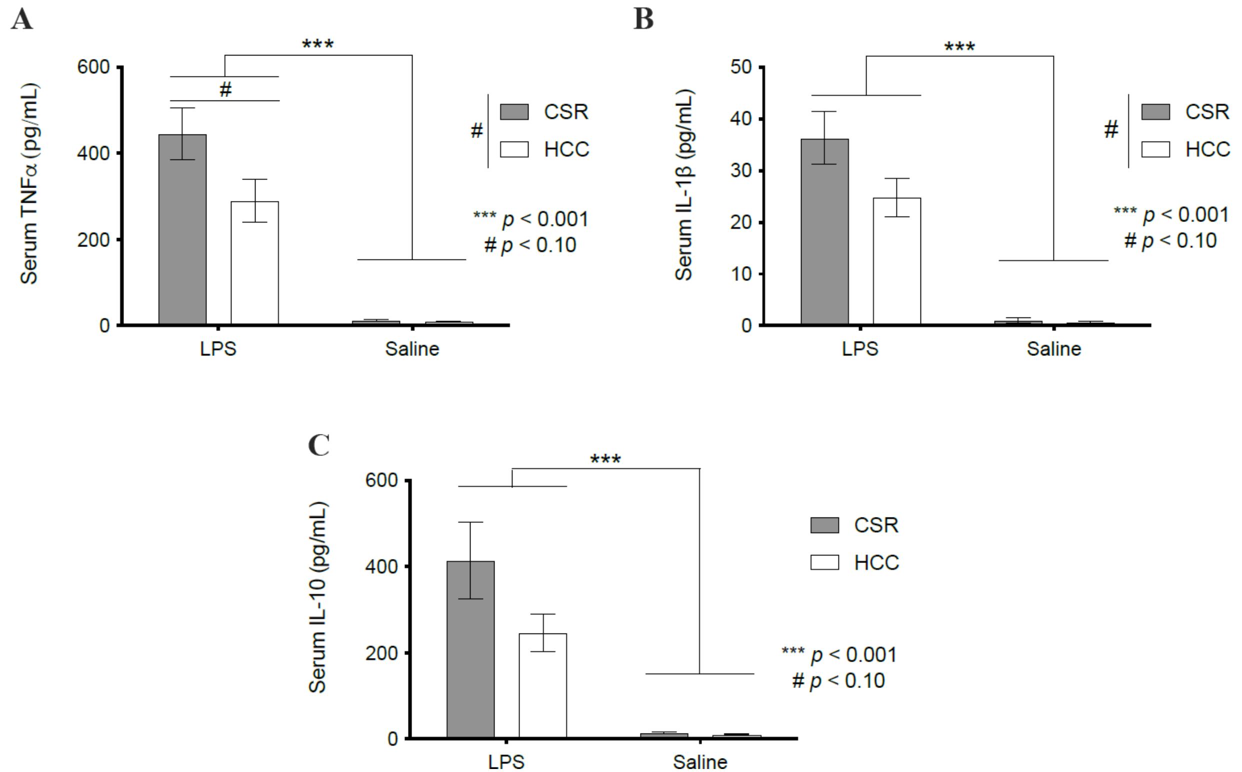

Figure 7. Serum levels of TNFα (A), IL-1β (B), and IL-10 (C) in male mice following CSR and one injection of LPS. CSR mice that received LPS had higher serum TNFα compared to HCC mice that received LPS (A). Mice that received LPS had higher serum IL-1β, TNFα, and IL-10. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 8–11.

Female Serum Data

Identical analyses were performed for female mice. For TNFα, results revealed an interaction that was approaching significance, ($F(1,43) = 3.703$, $p = 0.062$), such that, among mice that received LPS, those that underwent CSR had slightly lower serum TNFα compared to HCC mice ($p = 0.029$). While there was no main effect of sleep condition ($F(1,43) = 2.163$, $p = 0.149$), there was a significant main effect of injection ($F(1,43) = 60.706$, $p < 0.001$), such that mice that received an LPS injection had higher serum TNFα compared to mice that received a saline injection (See Fig. 8A).

For IL-1β, neither the interaction ($F(1,43) = 0.650$, $p = 0.425$) nor the main effect of sleep condition ($F(1,43) = 0.237$, $p = 0.629$) were significant. However, there was a significant main effect of injection ($F(1,43) = 84.204$, $p < 0.001$), such that mice that received an LPS injection had higher IL-1β compared to mice that received a saline injection (See Fig. 8B).

For IL-10, results revealed no significant interaction ($F(1,43) = 0.488$, $p = 0.489$) or main effect of sleep condition ($F(1,43) = 0.090$, $p = 0.765$). However, there was a significant main effect of injection ($F(1,43) = 83.461$, $p < 0.001$), such that mice that received an LPS injection had higher serum IL-10 compared to mice that received a saline injection (See Fig. 8C).

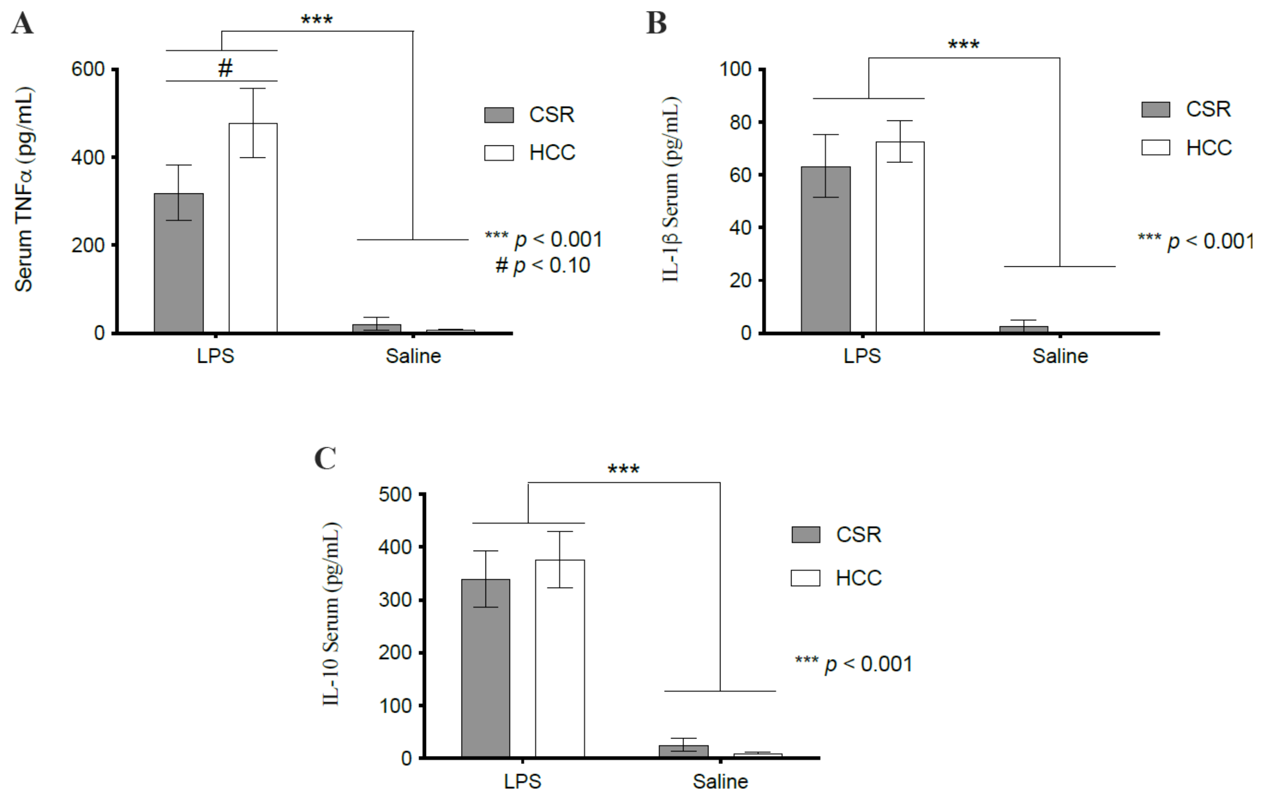

Figure 8. Serum levels of TNFα (A), IL-1β (B), and IL-10 (C) in female mice following CSR and one injection of LPS. CSR mice that received LPS had lower serum TNFα compared to HCC mice that received LPS (A). Mice that received LPS had higher serum IL-1β, TNFα, and IL-10. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 11–12.

Sex Differences in Serum Cytokines

As these patterns of serum cytokine expression appeared to differ between male and female mice, three-way ANOVAs were performed to assess potential sex differences in these markers. For serum TNFα, results revealed a significant Sex X Injection X Sleep Condition interaction ($F(1,80) = 6.373$, $p = 0.014$), such that CSR LPS females had lower TNF compared to CSR LPS males ($p = 0.049$), and that HCC LPS females had higher TNF compared to HCC LPS males ($p = 0.007$). There was also a significant Sex X Sleep Condition interaction ($F(1,80) = 5.531$, $p = 0.021$), such that HCC females had higher TNFα compared to HCC males ($p = 0.048$) (See Fig. 9A).

For IL-1β, results of the 3-way ANOVA revealed a significant main effect of sex such that females had higher IL-1B in serum compared to males ($F(1,77) = 19.100$, $p < 0.001$). There was also a significant Sex X Injection interaction ($F(1,77) = 17.665$, $p < 0.001$) such that females had higher serum IL-1B in response to LPS compared to males that received LPS ($p < 0.001$) (See Fig. 9B). However, the Sex X Sleep Condition X Injection interaction was not significant ($F(1,80) = 1.684$, $p = 0.198$).

Results of the 3-way ANOVA for serum IL-10 revealed a Sex X Sleep Condition X Injection interaction that was approaching significance ($F(1,80) = 2.987$, $p = 0.088$), such that HCC LPS females had higher serum IL-10 compared to HCC LPS males ($p = 0.050$), and LPS males that underwent CSR had higher serum IL-10 compared to HCC LPS males ($p = 0.012$) (See Fig. 9C).

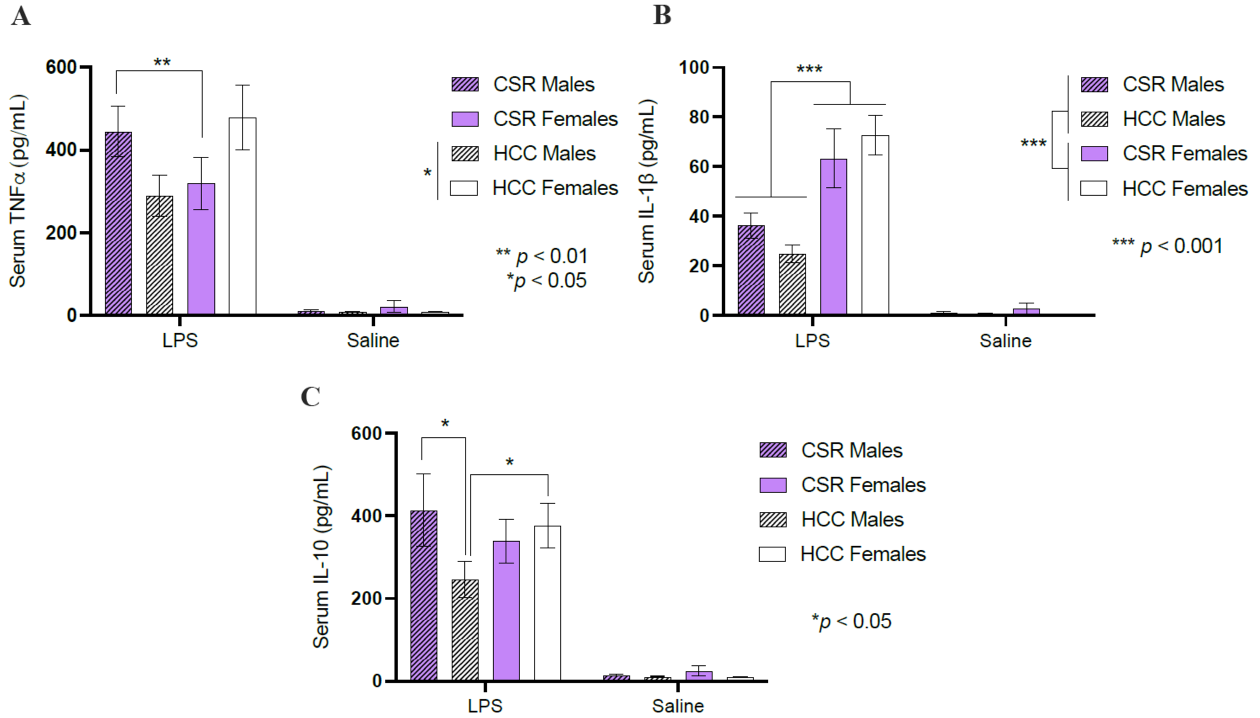

Figure 9. Serum levels of TNFα (A), IL-1β (B), and IL-10 (C) mRNA in male and female mice following CSR and one injection of LPS. (A) CSR males had higher serum TNFα in response to LPS compared to CSR females, and HCC males had lower TNFα in response to LPS compared to HCC females. (B) Females had higher serum IL-1β compared to males and more serum IL-1β in response to LPS compared to males. (C) HCC females had more serum IL-10 in response to LPS compared to HCC males. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 8–12.

4. Discussion

Experiment 1 explored the impact of CSR on cytokine expression in the hippocampus and serum, as well as hippocampal BDNF mRNA. We hypothesized that CSR mice injected with LPS would have higher TNFα, IL-1β, IL-6, and IL-10 mRNA in the hippocampus and increased TNFα, IL-1β, and IL-10 in the serum compared to HCC mice injected with saline. We also hypothesized a main effect of CSR, such that CSR alone would be associated with increased cytokines in the hippocampus and serum compared to the HCC condition. In addition, we hypothesized that the CSR mice would have decreased BDNF mRNA compared to HCC mice, and that LPS would also be associated with decreased BDNF mRNA.

Our hypothesis that CSR would decrease BDNF mRNA in the hippocampus was partially supported, as this effect was observed in male, but not female, mice. This is consistent with the work of Zielinski and colleagues (2014), which demonstrated that 5 days of sleep restriction led to decreased BDNF mRNA in the hippocampus in male rats. Furthermore, total sleep deprivation for 24 hours led to decreased BDNF expression in the PFC in male and female mice (Misrani et al., 2020). On the other hand, some evidence has shown that CSR via the MMPM led to increased BDNF in the hippocampus, although these results were obtained via ELISA (da Silva Rocha-Lopes et al., 2018). In the current study, it is possible that the deleterious effect of CSR on hippocampal BDNF mRNA was only observed in males because of the relationship between estrogen and BDNF. Evidence suggests that estradiol helps regulate BDNF synthesis in the hippocampus (Harte-Hargrove et al., 2013; Scharfman & Maclusky, 2005), which could have been protective in the CSR paradigm used in the current study.

Interestingly, our hypothesis that CSR would exacerbate the inflammatory response to LPS was not fully supported. While there were statistically significant main effects of CSR (or effects approaching significance) for several markers, such as female TNFα mRNA, male IL-6 mRNA, and male IL-1β mRNA, it should be noted that many of these effects were driven by the differences found between the LPS groups, and that CSR alone did not appear to significantly alter cytokine expression. Furthermore, these differences were not in the hypothesized direction, as CSR mice had lower levels of cytokines and cytokine mRNA compared to HCC mice. As CSR alone did not increase inflammatory cytokines, perhaps this particular paradigm did not induce a state of chronic inflammation, but it could have induced an impaired or blunted immune response to LPS. For example, evidence has demonstrated that male C57BL/6 mice that underwent a chronic unpredictable stress paradigm had lower levels of TNFα and IL-1β in the

hippocampus and midbrain compared to non-stressed control mice following an LPS injection, demonstrating an impaired immune response to the LPS administration (Barnum et al., 2012). This is consistent with the current hippocampal cytokine data, as both male and female CSR mice exhibited decreased proinflammatory cytokines in response to LPS compared to HCC LPS mice. While CSR alone did not induce the hypothesized exacerbation in cytokine expression, several of the differences observed between CSR and HCC groups that received LPS were statistically significant. Thus, the CSR paradigm did alter some of the peripheral and hippocampal cytokine responses to an LPS injection.

As hypothesized, there were main effects of LPS in almost all of the cytokines measured in both sexes, such that the LPS injection was associated with an increase in each cytokine. It is widely accepted that LPS leads to increased pro- and anti-inflammatory cytokine expression (Qin et al., 2007; Turrin et al., 2001). This was not true, however, for hippocampal IL-10 mRNA in males in the current study, even though prior research observed increases in IL-10 in hippocampal tissue following an LPS injection in rats. Nonetheless, it appears that there is a complex relationship between sleep loss and immune response to an inflammatory stimulus, and that these relationships may differ based on sex.

Notably, male mice that underwent CSR and received LPS exhibited increased TNFα and IL-1β in the serum compared to HCC LPS mice, a response that appeared opposite to serum cytokines in female mice. Further, the difference between serum TNFα in male and female CSR LPS mice was statistically significant, indicating an effect of sex on serum TNFα cytokines in response to an LPS injection under chronic short sleep conditions.

Although it appears that women are immune-privileged, women are disproportionately affected by AD compared to men, and the mechanisms behind this phenomenon are not well-

understood (Giefing-Kroll et al., 2015; Snyder et al., 2016). There are notable sex differences in inflammation and the immune response, which could provide important insights into this relationship (Klein & Flanagan, 2016). Evidence suggests sex differences in the inflammatory response to endotoxins. For example, in humans, men and women exhibited different responses to intravenous LPS injections, where plasma levels of proinflammatory cytokines TNFα and IL-6 were more elevated in women compared to men, and anti-inflammatory cytokine IL-10 was more elevated in men compared to women (Engler et al., 2016). Likewise, following LPS stimulation, PBMCs from men secreted more IL-10 compared to PBMCs from women (El Temple et al., 2008). Conversely, El Temple and colleagues (2008) also found that, following an LPS injection, PBMCs from men produced 50% more TNFα mRNA, and, when unstimulated, 25% more TNFα mRNA compared to PBMCs from women, highlighting potential sex differences in proinflammatory cytokine production at basal levels and following LPS administration (El Temple et al., 2008). Moreover, sex differences were observed in 14.5-month-old C57BL/6 mice six hours after receiving one injection LPS, such that female mice demonstrated increased sickness behavior and cytokine levels in the striatum and blood plasma in response to LPS compared to males, suggesting that females were more susceptible to the inflammatory effects of LPS (Dockman et al. 2022). These data contrast with the current findings, but it is possible that there is a more complicated relationship between sex, chronic sleep loss, and cytokine response to an inflammatory stimulus. To the best of our knowledge, this is the first study to examine the inflammatory response to an LPS injection following CSR in both male and female wild-type mice.

Both male and female CSR mice lost weight during the first week of the study, which could have been due to the stress of adjusting to the CSR paradigm. Alternatively, it is possible

that this method of CSR may induce weight loss, as da Silva Rocha-Lopes et al. (2018) reported that rats that underwent CSR via the MMPM were lighter compared to control rats. However, previously, the current CSR paradigm utilized in the same lab did not cause differences in body weight between CSR and HCC mice (Brice et al., 2020). Additionally, there were no effects of CSR on weight gain in either male or female mice, despite previous findings that three weeks of sleep deprivation leads to increased weight gain in young female mice (Huang et al., 2022).

CHAPTER 3: THE COMBINED EFFECTS OF TYPICAL AMERICAN-STYLE DIET CONSUMPTION AND CHRONIC SLEEP RESTRICTION

1. Abbreviated Introduction

While rodent studies of environmental factors on AD pathology typically explore how one aspect of lifestyle can affect AD-like markers, it is likely that humans practice more than one harmful lifestyle habit or experience more than one environmental risk factor for AD. In addition to chronic sleep loss, many people struggle to regularly consume a well-balanced, healthy diet, especially in the U.S. (Faruque et al., 2019; Lee et al., 2022). Sleep and diet share a relationship, as evidence suggests a potential bidirectional relationship between lack of sleep and poor diet (St-Onge & Zuraikat, 2019). For example, individuals that get less than 7 hours of sleep per night are more likely to consume significantly more saturated fat and fewer fruits and vegetables compared to individuals who get adequate sleep (St-Onge & Zuraikat, 2019). Thus, sleep quality and quantity may have an impact on dietary choices. For example, five nights of short sleep in adolescents (6.5 hours per night) was associated with increased consumption of carbohydrates, sweetened drinks, and foods with higher glycemic loads, and decreased consumption of fruits and vegetables compared to adolescents in the healthy sleep groups who slept between 8–9 hours each night (Duraccio et al., 2021).

Interestingly, sleep not only affects dietary decisions, but food choices also may have an impact on sleep. In particular, carbohydrates might impact sleep quality (St-Onge et al., 2016). In one study, men who were given a high-carbohydrate snack before bed exhibited decreases in slow-wave sleep architecture compared to those who were given a low-carbohydrate snack (Porter & Horne, 1981). Likewise, another study found that two nights of consuming a high-carbohydrate meal before bed was associated with decreases in slow-wave sleep during the first

sleep cycle (Yajima et al., 2014). Furthermore, in Japanese women, low vegetable and fish consumption and high carbohydrate consumption were both associated with poor sleep quality (Katagiri et al., 2014). Saturated fat may also impact sleep. For example, in male C57BL/6 mice, those that were switched to a high-fat diet demonstrated decreased wake time and increased slow-wave sleep, but had increased sleep fragmentation, indicative of worse sleep quality (Perron et al., 2015). Conversely, Mediterranean-style diet consumption is linked with decreased risk for insomnia and increased subjective and objective sleep duration (St-Onge & Zuraikat, 2019).

There is a gap in the literature regarding the link between a combination of poor diet consumption and chronic sleep loss and AD pathology. In Experiment 2, we aimed to administer a diet that would incorporate as many of the factors of a typical American-style diet as possible to best mimic the human experience, rather than studying one specific component of a typical American diet. The goal of the present research was to explore whether TAD consumption for 12 weeks would exacerbate the effects of CSR in male and female mice.

Hypotheses

We hypothesized that the 12 weeks of TAD consumption would increase body weight, impair cognitive function in CFC, increase hippocampal and PFC Aβ, and increase serum TNFα, IL-1β, and IL-10. We also hypothesized similar effects of CSR, such that CSR mice would demonstrate cognitive impairment in CFC, increased hippocampal and PFC Aβ, and increased serum TNFα, IL-1β, and IL-10. Finally, given the evidence that, independently, chronic sleep loss and TAD consumption both induce chronic inflammation and increase AD pathology, we hypothesized significant interaction effects, such that TAD-fed mice that underwent CSR would

have exacerbated cognitive impairment in CFC, hippocampal and PFC Aβ, and increased serum cytokines compared to all other groups.

2. Methods

Unless otherwise described, all research subjects, behavioral paradigms, tissue collection procedures, and data analyses were performed identically to those discussed in Chapter 2.

Subjects

In Experiment 2, adult male and female C57BL/6J mice were bred and housed as described in Chapter 2. Mice were approximately 4.5–5 months of age at the beginning of the study protocol, making them around 7.5–8 months old at the time of tissue collection (See Fig. 10 for study timeline).

Diet Treatments, Food Consumption, and Body Weight Measurement

In collaboration with Dr. Jada Willis in the Texas Christian University Department of Nutritional Sciences, our lab developed a custom experimental rodent chow that modeled a typical American-style (TAD) diet. This TAD chow was administered in pellet form and was comparable in size to the standard rodent chow fed to all rodents in the TCU vivarium (see Tables 1–3 for details regarding diet compositions). Macronutrient densities in the TAD were similar to that of the average American, with an omega-6 to omega-3 fatty acid ratio of 15:1.

Macronutrients kcal%	Standard Chow	TAD Chow
Protein	21.114%	15%
Fat	13.758%	35%
Carbohydrate	65.128%	50%

Table 1. Diet Macronutrients

Diet component	Standard Chow	TAD Chow
Main protein sources	Fish meal, dried whey, porcine meat and bone meal, L-lysine	Casein, DL-methionine
Main fat sources	Porcine animal fat, soybean oil	Beef fat, bunge, butter, anhydrous, safflower oil
Main carbohydrate sources	Ground corn, wheat middling, soybean meal, ground wheat, cane molasses, ground oat	Corn starch, Maltrodextrin 10
Fiber sources	wheat middling, corn, soy hulls, and soybean meal (12.1% insoluble, 1.53% soluble)	Cellulose, BW200 (insoluble)

Table 2. Macronutrient and fiber sources in the standard chow and TAD.

Fatty acid	Standard Chow	TAD Chow
Saturated fat (%kcal)	3.3	17.0
Monounsaturated fat (%kcal)	3.99	12.3
Polyunsaturated fat (%kcal)	2.89	3.2
Omega-6:Omega-3	7.22:1	15:1

Table 3. Fatty acids in the standard chow and TAD.

Male and female mice were assigned to either the TAD chow (Western Diet #D21112903, Research Diets, Inc., New Brunswick, NJ) or the soy-based standard rodent chow (Prolab RMH 1800-5LL2; LabDiet, St. Louis, MO). Mice in the TAD condition were switched to the TAD chow between 4.5–5 months of age, while the mice in the Standard Diet condition continued to consume the standard rodent chow. Food consumption was measured weekly for the first six weeks (prior to sleep restriction) by standardizing food amounts to 200g every Friday, and the remaining pellets were weighed on the following Monday. Total daily consumption for one mouse in each cage was calculated by subtracting the food weight recorded on Monday from

200g, dividing that number by three (the number of days between weighing sessions), and dividing that number by the number of mice in the cage. Body weight was also recorded weekly.

Throughout the diet consumption period prior to beginning the CSR protocol, food consumption was measured weekly. Briefly, food in all experiment cages was weighed and recorded on Friday mornings. No food was added to the cages until Monday morning, when food was weighed and recorded again. An average was then calculated for food consumed in one day by one mouse in each cage. This was achieved by subtracting the weight obtained on Monday from the starting weight recorded on Friday. Then, that resulting number was divided by the number of mice in the cage, and then again by the number of days between weighing (3 days). Food consumption was not measured during the CSR protocol, as mice created food waste in the CSR chambers that was unable to be accounted for in food consumption analysis.

Body weight was also recorded weekly throughout the duration of the diet and sleep manipulations.

Chronic Sleep Restriction

Following 6 weeks of diet consumption alone, all mice in both standard chow and TAD diet conditions were split into two groups, as described in Chapter 2: CSR and HCC. The experimental protocol for both of these conditions remained the same as in Chapter 2, with the exception of the food conditions. Mice in the standard diet condition remained on the standard chow diet, while mice in the TAD diet condition continued to be fed the TAD diet in the CSR and HCC cages.

Contextual Fear Conditioning

Following the final day of sleep restriction, mice underwent a contextual fear conditioning (CFC) paradigm, between 0800h and 1100h. During the training session, mice were

placed into automated conditioning chambers with a polka-dot wall, a peppermint olfactory cue (peppermint oil in a 1:10 dilution in distilled water), and an electrified grid floor (Coulbourn Instruments, Whitehall, PE, 17.78 cm X 17.78 cm X 30.48 cm). Mice were acclimated to the conditioning chamber for 120 seconds before receiving a two-second, 0.5mA footshock. Following the shock, mice remained in the chambers for an additional 60 seconds before being returned to their home cages. On the next day, 24 hours after the testing session, mice were placed back into the chambers with the same contextual cues for the testing session, where they remained for 180 seconds, but did not receive a footshock. Freezing behavior was assessed utilizing FreezeFrame™ software (Actimetrics Software, Wilmette, IL) to measure the strength of the learned association between the olfactory and visual context of the chamber and the aversive footshock, as mice that learn this association typically freeze more compared to mice that may have impaired learning and memory. All behavioral testing was conducted between 0800h and 1100h.

Serum and Tissue Collection

Following the conclusion of CFC testing, all mice were euthanized via rapid decapitation. Trunk blood was obtained, and serum was collected, as described in Chapter 2. In addition to serum collection, whole, bilateral hippocampi and prefrontal cortex (PFC) were collected separately, placed in microcentrifuge tubes containing a tissue lysis buffer cocktail (PRO-PREP, Bulldog Bio, Portsmouth, NH) with additional protease inhibitors, (phosphatase inhibitor cocktail A, phosphatase inhibitor cocktail B, and protease inhibitor cocktail, Bimake, Houston, TX), and snap-frozen on dry ice. These hippocampal and PFC samples were then stored at -80º C until protein quantification.

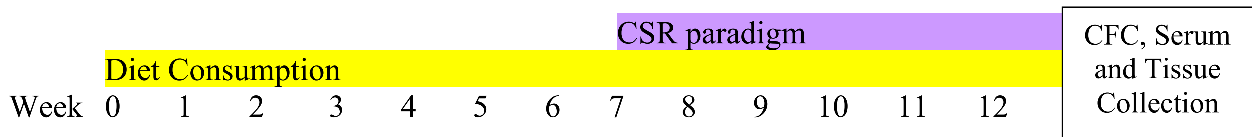

Figure 10. Experimental timeline for Study 2. Adult male and female C57BL/6J mice were fed either a TAD diet or standard chow for six weeks, followed by an additional six weeks of respective diet consumption concurrent with six weeks of CSR. CFC was conducted following the last day of the sleep and diet paradigms, after which serum and brain tissue were collected.

Protein Quantification

Hippocampal and PFC samples were thawed on wet ice and centrifuged at 15,000 rpm (16,820 x g) for 20 minutes at 4º C, and clear lysates were isolated and stored in fresh microcentrifuge tubes. A Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA) was conducted according to manufacturer instructions to measure protein concentrations in hippocampal and PFC lysates. Samples were diluted 1:16 in additional lysis buffer, and 5µL of sample were added to a 96-well microplate. Following the addition of 250µL of Bradford Reagent, the plate was incubated in the dark for 25 minutes before reading. Optical densities were read at 595nm on a BMG LabTech FLUOstar instrument (Omega, Cary, NC).

Soluble Aβ Analysis

After protein quantification, a mouse Aβ1-42 enzyme-linked immunosorbent assay (ELISA) (Invitrogen, ThermoFisher Scientific, Waltham, MA) was conducted to measure amyloid-beta levels in hippocampal and PFC lysates. Briefly, all samples were diluted 1:4 with a sample dilution buffer cocktail containing PRO-PREP mixed with protease and phosphatase inhibitors. 100µL of each sample will be plated in a precoated 96-well micro ELISA plate and allowed to incubate at room temperature for 2 hours. The wells were then washed four times with the provided wash buffer, and Aβ42 Detection Antibody solution was added to each well. The plate was allowed to incubate at room temperature for one hour. Wells were again washed,

and an HRP-tagged conjugate working solution was added. Following a 30-minute incubation period at room temperature, wells were washed, and a stabilized chromagen substrate was added to each well, incubating in the dark at room temperature for 30 minutes before a stop solution was added. The optical densities were then read at 450nm on a BMG LabTech FLUOstar instrument (Omega, Cary, NC). Results were calculated using a 4-parameter fit curve.

Peripheral Cytokine Quantification

Multiplexing assays were performed as described in Chapter 2 to assess serum levels of IL-1β, TNFα, and IL-10.

Statistical Analyses

All statistical analyses were performed with alpha levels of 0.05, and any significant interactions were explored using pairwise comparisons.

3. Results

Experiment 2A: Effects of 12 Weeks of Typical American-style Diet Consumption and 6 Weeks of Chronic Sleep Restriction on Body Weight

Mixed-model ANOVAs, in which the between-subjects variable was Diet (TAD vs. Standard), and the repeated-measures variable was Week (Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6), were performed to assess the impact of TAD consumption on body weight in both male and female mice. In males, results revealed a significant main effect of Week ($F(5,235) = 14.737$, $p < 0.001$), such that mice gained weight throughout the first six weeks of the study. Furthermore, there was a significant Week x Diet interaction ($F(5,235) = 12.639$, $p < 0.001$), such that standard chow mice lost weight during Week 1 ($p < 0.001$) and Week 2 ($p = 0.010$) and gained weight during Week 3 ($p = 0.005$), whereas TAD mice gained weight during Week 1 ($p < 0.001$), lost weight during Week 2 ($p < 0.001$), and gained weight

during Week 3 ($p < 0.001$) and Week 5 ($p < 0.001$). There was also a significant linear trend of Week x Diet ($F(1,47) = 16.894$, $p < 0.001$), such that the TAD mice gained weight more rapidly than did the standard chow mice (See Fig. 11A). There was no main effect of Diet ($F(1,47) = 1.073$, $p = 0.775$).

In females, results revealed a significant main effect of Week ($F(5,225) = 3.879$, $p = 0.002$), such that body weight changed throughout the first six weeks of the study. There was a significant Week x Diet interaction ($F(5,225) = 8.468$, $p < 0.001$), such that TAD mice lost weight during Week 1 ($p < 0.001$), gained weight in Week 2 ($p = 0.006$), lost weight in Week 4 ($p = 0.005$) and gained weight in Week 5 ($p = 0.005$), while body weight of standard chow mice did not differ significantly from week to week (See Fig. 11C). There was no main effect of Diet ($F(1,45) = 0.040$, $p = 0.841$).

A second pair of mixed-model ANOVAs, in which the between-subjects variables were Diet (TAD vs. Standard) and Sleep Condition (CSR vs. HCC), and the repeated-measures variable was Week (Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6), were performed to assess the impact of the combination of TAD consumption and CSR on body weight in male and female mice. In males, results revealed a significant main effect of Week ($F(5,225) = 33.396$, $p < 0.001$), such that mice gained weight throughout the second six weeks of the study. There was also a significant Week x Sleep Condition interaction ($F(5,225) = 8.388$, $p < 0.001$), such that the CSR mice weighed more than HCC mice at Week 7 ($p = 0.032$), although these weights were taken before any mice underwent CSR. Furthermore, the Week x Diet x Sleep Condition interaction was significant ($F(5,225) = 3.509$, $p = 0.004$), such that, among the TAD mice, CSR mice weighed more at Week 7 ($p = 0.001$) compared to HCC mice, although, as stated above, these weights were taken before any mice underwent CSR (See Fig. 11B).

Therefore, these observed differences were not due to effects of chronic sleep restriction on body weight. There were no main effects of Diet or Sleep Condition (Fs(1,45) < 0.034, p > 0.854), and no Week x Diet interaction ($F(5,225) = 1.316$, $p = 0.258$).

In females, results revealed a significant main effect of Week ($F(5,210) = 56.604$, $p <$ 0.001) such that mice were heavier by the last week of the study. There was also a significant Week x Diet interaction ($F(5,210) = 9.276$, $p < 0.001$), such that TAD mice weighed less in Week 7 compared to standard chow mice ($p = 0.016$). The Week x Sleep Condition interaction was also significant ($F(5,210) = 17.685$, $p < 0.001$), such that CSR mice weighed less in Week 8 compared to HCC mice ($p = 0.027$). Finally, there was a significant Week x Sleep Condition x Diet interaction ($F(5,210) = 2.608$, $p = 0.026$), such that, among CSR mice, TAD mice weighed less in Week 7 compared to HCC mice ($p = 0.008$), although these weights were measured prior to the beginning of the CSR portion of the study. Furthermore, among the TAD mice, CSR mice weighed less compared to HCC mice in Week 8 ($p = 0.020$) (See Fig. 11D). There were no effects of Diet or Sleep Condition (Fs(1,42) < 1.985, ps > 0.166), and no Diet x Sleep interaction ($F(1,42) = .946$, $p = 0.336$).

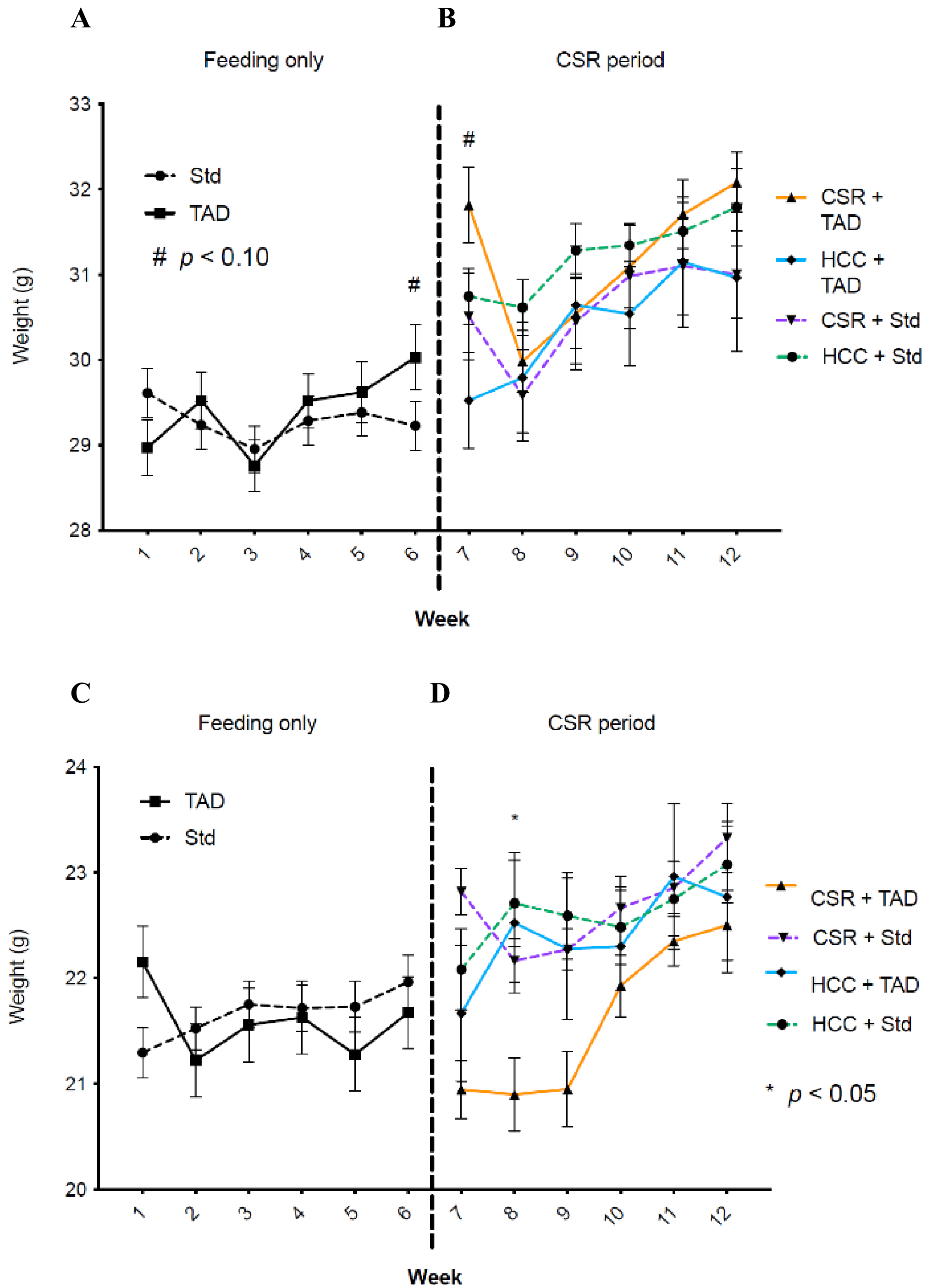

Figure 11. Body weight data during feeding-only and CSR periods for male (A,B) and female (C,D) mice. Male mice that consumed TAD weighed more in Week 6 compared to HCC mice. Female mice that underwent CSR and consumed TAD weighed less in Week 8 compared to HCC females that consumed TAD. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 10–13.

Food Consumption Data

Two mixed-model repeated measures ANOVAs (Diet [TAD vs. Standard] x Week [Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6]) were performed to explore potential differences in food consumption between the two diet conditions during the feeding-only portion of the study in both males and females. In males, results revealed a significant main effect of Week ($F(5,65) = 19.280$, $p < 0.001$), such that food consumption decreased during the six weeks, prior to the beginning of the sleep restriction procedure. There was also a significant main effect of Diet ($F(1,12) = 57.389$, $p < 0.001$), such that TAD mice consumed less food compared to mice receiving the standard chow. The Week X Diet interaction was approaching significance ($F(5,65) = 2.012$, $p = 0.089$), such that TAD males significantly decreased their food consumption from Week 1 to Week 2 ($p = 0.002$), and from Week 5 to Week 6 ($p = 0.036$), while standard chow males decreased consumption from Week 1 to Week 2 ($p = 0.016$), increased consumption from Week 2 to Week 3 ($p < 0.001$), decreased consumption from Week 3 to Week 4 ($p < 0.001$), increased consumption from Week 4 to Week 5 ($p = 0.004$), and decreased consumption from Week 5 to Week 6 ($p < 0.001$). Furthermore, standard chow mice consumed significantly more food compared to TAD mice in each week measured (*p*s < 0.002) (See Fig. 12A).

In females, results revealed a significant main effect of Week ($F(5,65) = 4.270$, $p = 0.002$), such that food consumption increased during the six weeks, prior to the beginning of sleep restriction. Consistent with the male data, here was also a significant main effect of Diet ($F(1,13) = 296.678$, $p < 0.001$), such that TAD mice consumed less compared to the standard chow mice. Results also revealed a significant Week X Diet interaction ($F(5,65) = 2.901$, $p = 0.020$), such that TAD chow females significantly decreased their consumption from Week 1 to

Week 2 (p = 0.020) and significantly increased their consumption from Week 4 to Week 5 (p = 0.010), while standard chow females significantly increased their consumption from Week 2 to Week 3 (p = 0.006) and Week 3 to Week 4 (p = 0.001), and significantly decreased consumption from Week 4 to Week 5 (p = 0.009). Furthermore, standard chow mice consumed significantly more food compared to TAD mice in each week measured (ps < 0.001) (See Fig. 12B).

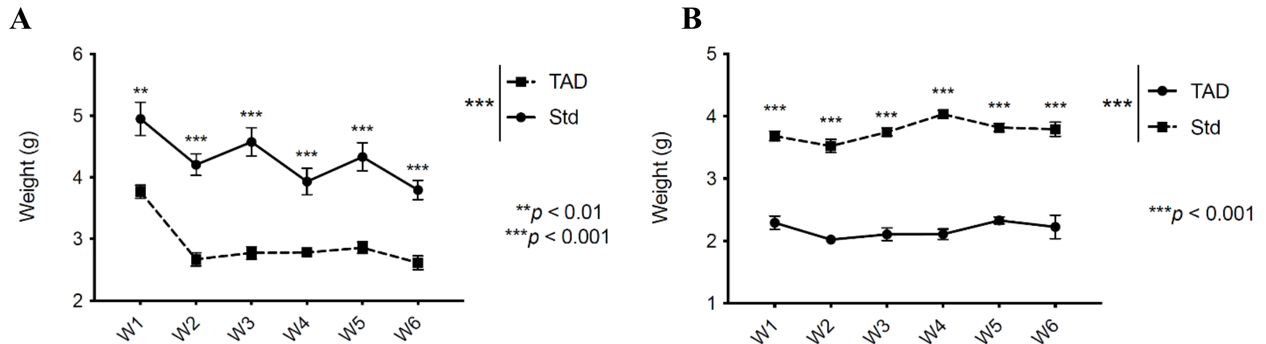

Figure 12. Food consumption data during the first 6 weeks of the study. Males (A) and females (B) that consumed the TAD ate less compared to mice that consumed the standard chow. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 7–8.

Experiment 2B: Effects of 12 Weeks of Typical American-style Diet Consumption and 6 Weeks of Chronic Sleep Restriction on Cognition

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet Condition [TAD vs. Standard]) were performed to assess the impact of 12 total weeks of TAD consumption combined with 6 weeks of CSR on cognitive function in male and female mice. In males, results revealed no effects of Sleep Condition ($F(1,44) = 0.193$, $p = .663$) or Diet ($F(1,44) = 0.113$, $p = .739$), and no Sleep Condition x Diet interaction $F(1,44) = 2.031$, $p = .161$) (See Fig. 13A). One mouse was determined to be an outlier by SPSS's use of the interquartile range rule in the first 120 seconds of the training session (pre-shock) and was excluded from analysis.

In females, results revealed no effects of Sleep Condition ($F(1,42) = 0.244$, $p = .624$) or Diet ($F(1,42) = 0.948$, $p = .336$), and no Sleep Condition x Diet interaction ($F(1,42) = 0.006$, p =

.940). No mice were determined to be outliers during the training session. However, an analysis of covariance (ANCOVA) determined behavior during the first 120 seconds of the training session to be a significant covariate ($F(1,41) = 10.931$, $p = .002$). After accounting for behavior during the first 120 seconds of the training session, there were still no effects of Sleep Condition ($F(1,41) = 0.028$, $p = .869$) or Diet ($F(1,41) = 2.801$, $p = .102$), and no Sleep Condition x Diet interaction ($F(1,41) = 0.034$, $p = .855$) (See Fig. 13B).

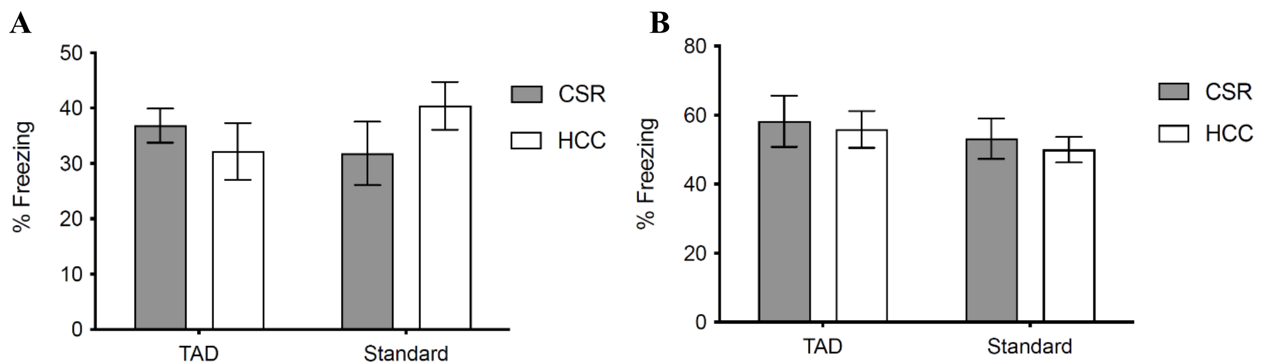

Figure 13. Freezing behaviors in CFC following 12 weeks of TAD consumption and 6 weeks of CSR. No effects of TAD or CSR were observed in male (A) or female (B) mice. Bars represent mean ± SEM. N's = 10–13.

Experiment 2C: Effects of 12 Weeks of Typical American-style Diet Consumption and 6 Weeks of Chronic Sleep Restriction on Aβ

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet Condition [TAD vs. Standard]) were performed to assess the impact of CSR and TAD consumption during adulthood on Aβ in the hippocampus and PFC in male mice. In the hippocampus, results revealed a main effect of Diet that was approaching significance ($F(1,43) = 3.553$, $p = 0.066$), such that the TAD mice had higher hippocampal Aβ (See Fig. 14A). There was no main effect of Sleep($F(1,43) = 0.665$, $p = 0.419$) and no interaction ($F(1,43) = 0.997$, $p = 0.324$). Two samples were removed from analysis for having CV values above 30%.

In the PFC, results revealed significant main effects of diet such that TAD mice had significantly higher hippocampal Aβ in the PFC ($F(1,5) = 7.756$, $p = 0.039$), such that TAD mice had higher Aβ in the PFC compared to standard chow mice (See Fig. 14B). There was no main effect of Sleep Condition ($F(1,5) = 1.700$, $p = 0.249$) and no interaction ($F(1,43) = 3.467$, $p = 0.122$).

Data have not yet been analyzed in female mice, as funding has been allocated to other aspects of the current studies.

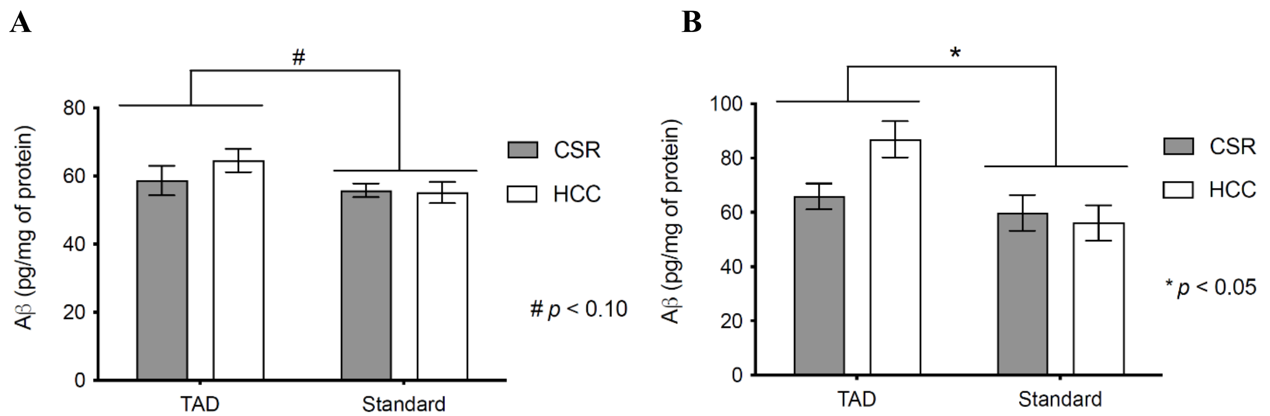

Figure 14. Aβ in male mice following 12 weeks of TAD consumption and 6 weeks of CSR. TAD consumption increases Aβ in the hippocampus (A) and PFC (B) in male mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 10–13 (A) and 2–3 (B).

Experiment 2D: Effects of 12 Weeks of Typical American-style Diet Consumption and 6 Weeks of Chronic Sleep Restriction on Peripheral Cytokines

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet Condition [TAD vs. Standard]) were performed to explore the effects of CSR and TAD consumption during adulthood on serum cytokines IL-1β, TNFα, and IL-10 in both males and females. Male data will be discussed first.

Male Serum Cytokines

In males, results revealed a significant main effect of Sleep Condition on serum IL-1β ($F(1,34) = 7.830$, $p = 0.008$), such that CSR mice had higher serum IL-1β compared to HCC mice. In addition, the main effect of Diet was approaching significance ($F(1,34) = 2.909$, $p = 0.097$), such that TAD mice had slightly higher IL-1β compared to standard chow mice (See Fig. 15A). There was no significant interaction ($F(1,34) = 0.123$, $p = 0.728$). Two samples were excluded from IL-1β analysis due to having coefficients of variation greater than 30% according to the MSD software. However, all of the samples fell below the lower limit of quantification for IL-1β suggested by the manufacturer, so caution should be taken when interpreting these data.

Similarly, for serum TNFα in males, results revealed a significant main effect of Sleep Condition ($F(1,36) = 5.268$, $p = 0.028$), such that CSR mice had higher serum TNFα compared to HCC mice. There was also a significant effect of Diet ($F(1,36) = 6.182$, $p = 0.018$) such that the TAD mice had higher serum TNFα compared to standard chow mice (See Fig. 15B). There was no significant interaction ($F(1,36) = 0.087$, $p = 0.770$).

For male IL-10, results revealed a significant main effect of Diet ($F(1,34) = 15.119$, $p < 0.001$), such that the TAD mice had higher serum IL-10 compared to standard chow mice (See Fig. 15C). There was no effect of Sleep Condition ($F(1,34) = 1.648$, $p = 0.208$) and no interaction ($F(1,34) = 0.669$, $p = 0.419$).

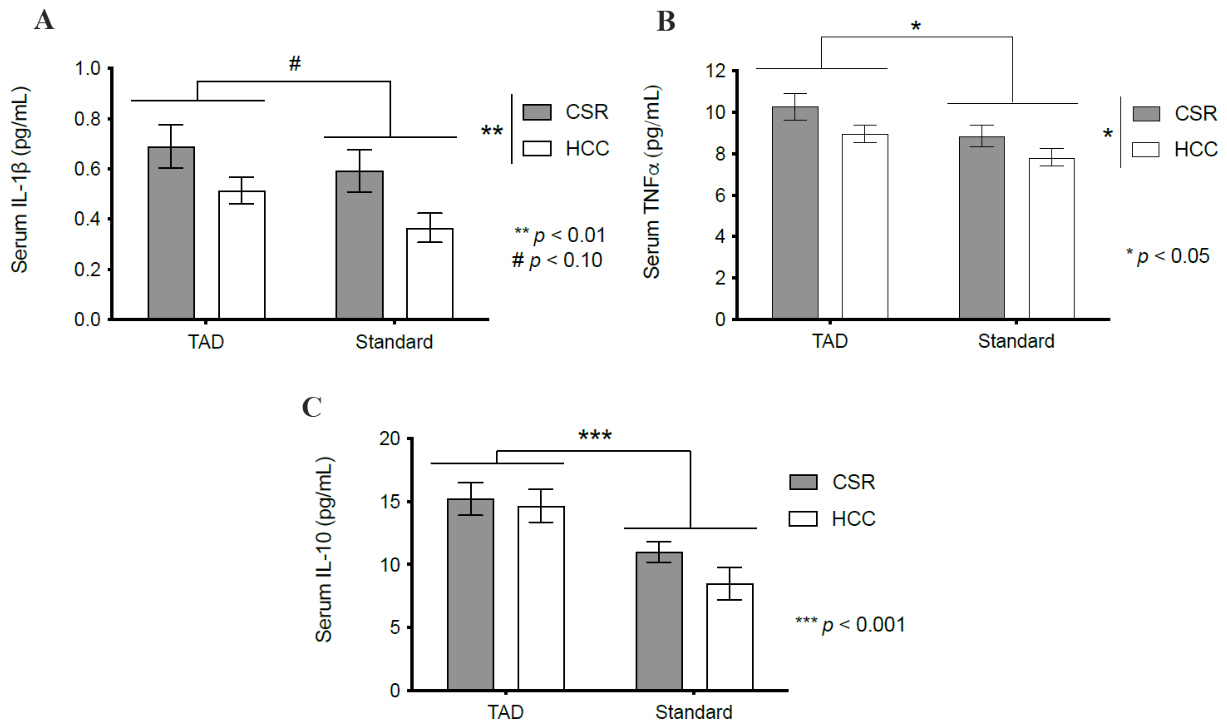

Figure 15. Serum IL-1β (A), TNFα (B), and IL-10 (C) in male mice following 12 weeks of TAD consumption and 6 weeks of CSR. Male TAD mice had higher serum IL-1β, TNFα, and IL-10 compared to standard chow mice, and CSR mice had higher serum IL-1β and TNFα compared to HCC mice. Significant differences ($p < 0.05$) are designated by *. N's = 9–10.

Female Serum Cytokines

In females, results revealed a significant main effect of Diet on serum IL-10 ($F(1,36) = 7.821$, $p = 0.008$), such that TAD mice had higher serum IL-10 compared to standard chow mice (See Fig. 16C). However, there was no significant main effect of Sleep Condition or interaction ($Fs(1,36) < 0.251$, $p > 0.620$). In addition, there were no significant main effects or interactions for serum IL-1β ($Fs(1,33) < 0.678$, $p > 0.416$) (See Fig. 16A), or serum TNFα ($Fs(1,36) < 2.218$, $p > 0.145$) (See Fig. 16B). Two samples were removed from IL-1β analysis due to having coefficients of variation greater than 30% according to the MSD software.

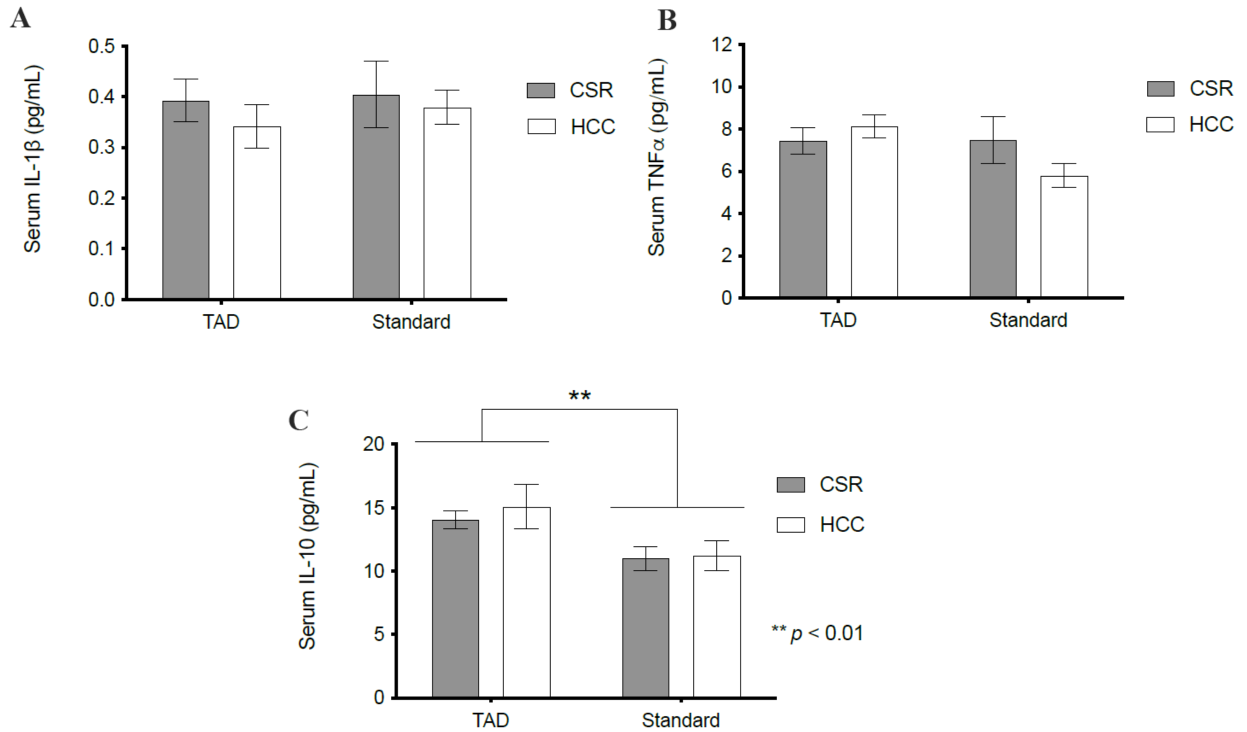

Figure 16. Serum IL-1β (A), TNFα (B), and IL-10 (C) in female mice following 12 weeks of TAD consumption and 6 weeks of CSR. Female TAD mice had higher IL-10 compared to standard chow mice. Significant differences ($p < 0.05$) are designated by *. N's = 9–11.

4. Discussion

Experiment 2 investigated the impact of 12 weeks of TAD consumption combined with 6 weeks of CSR during the last half of the TAD consumption period on body weight, cognition in CFC, hippocampal and PFC Aβ, and serum cytokines in male and female C57BL/6 mice. We hypothesized that the TAD mice would have increased body weight compared to standard chow mice, and that the combination of TAD and CSR would impair cognitive function in CFC, increase hippocampal and PFC Aβ, and increase serum TNFα, IL-1β, and IL-10. We also hypothesized main effects of CSR and TAD independently, such that both variables would impair cognition and increase Aβ and inflammation.

As hypothesized, both CSR and TAD were associated with elevated cytokines in the serum – particularly IL-1β and TNFα – in male mice. However, these differences were not observed in female mice. As discussed previously, it is possible that female mice have an immune advantage and thus were not impacted by the diet and sleep manipulations, whereas the male mice were (Snyder et al., 2016). Furthermore, the only effect on serum cytokines observed in females was the increase in IL-10 following TAD consumption. As IL-10 inhibits IL-1β and TNFα production, perhaps it was exerting its anti-inflammatory role more efficiently in the female mice compared to males (de Waal Malefyt et al., 1991). In addition, Barron and colleagues (2013) investigated sex differences in obesity markers, cognition, and Aβ in the 3xTg-AD mouse following four months of high-fat diet consumption. Evidence demonstrated that high-fat diet was associated with cognitive impairment and increased Aβ in the hippocampus in both male and female mice (Barron et al., 2013). This was consistent with the current results, as male TAD mice showed an increase in Aβ in the hippocampus and PFC. Further, Barron et al. (2013) found that, while both males and females on the high-fat diet had higher body weight compared to control diet mice, male mice fed the high-fat diet demonstrated an increase in blood glucose and insulin compared to females. However, ovariectomized females on the high-fat diet showed an increase in blood glucose, indicating a protective effect of ovarian hormones against insulin resistance (Barron et al., 2013). As the current study did not explore these variables, it is difficult to draw conclusions regarding hormones and the different patterns of serum cytokine responses to CSR and TAD between male and female mice.

Our hypotheses regarding Aβ were partially supported, as elevations in hippocampal and PFC Aβ were observed in male mice that consumed the TAD. However, there were no effects of CSR on Aβ in either brain region, in contrast with previous findings that the same CSR protocol

was associated with increased hippocampal Aβ in comparison to HCC mice (Brice et al., 2020). It is possible that this difference was not observed between the CSR and HCC mice in the standard chow condition because this analysis was underpowered, especially in the PFC analysis. Furthermore, as C57BL/6 mice are non-transgenic and don't overexpress human forms of Aβ, the murine Aβ levels measured in this study were very low, and the values measured were near the bottom of the standard curve, potentially making differences more difficult to detect. Future research should explore this further to determine whether this CSR paradigm reliably leads to increased Aβ in the hippocampus in the context of different study designs. Nonetheless, differences in Aβ due to TAD consumption were observed in the hippocampus, consistent with prior findings (Graham et al., 2016). Interestingly, however, Graham and colleagues (2016) did not observe increased Aβ in the cortex following Western diet consumption, in contrast to the current work, although the current study only explored the PFC specifically.

The hypotheses regarding cognitive impairment in CFC were not supported, as there were no differences in freezing behavior in any of the groups in male or female mice. As freezing behavior was relatively high, it is possible that the two-shock paradigm used in this Experiment made the context too salient to observe differences between groups, meaning that all mice were equally able to learn the association between the aversive footshock and context.

Taken together, the results of Experiment 2 do not indicate that a combination of 12 weeks of TAD consumption and 6 weeks of CSR was severe enough to induce major changes in the variables explored. Thus, Experiment 3 employed a longer feeding protocol to investigate whether lifelong TAD consumption would exacerbate the hypothesized harmful effects of the CSR paradigm.

CHAPTER 4: THE COMBINED EFFECTS OF LIFELONG TYPICAL AMERICAN-STYLE DIET CONSUMPTION AND CHRONIC SLEEP RESTRICTION

1. Abbreviated Introduction

As discussed in Chapter 3, most rodent studies exploring the role of environmental factors on AD pathology typically only investigate one aspect of lifestyle. Furthermore, even among Western-style diet or TAD studies, many explore only one element of a poor diet (Więckowska-Gacek et al., 2021). However, it is likely that humans practice more than one harmful lifestyle habit or experience more than one environmental risk factor for AD, and that, for many individuals, these habits are chronic in nature. As previously discussed, typical American-style diets high in fat cause HPA axis dysregulation and inflammation, which can harm sleep quality and lead to increased Aβ and decreased hippocampal volume. In turn, insufficient or disordered sleep can also induce systemic inflammation, oxidative stress, cognitive disfunction, and downregulation of glymphatic clearance of Aβ from the brain (Pistollato et al., 2016). Furthermore, poor sleep can affect dietary choices, as evidence shows that sleep deprivation leads to enhanced desirability of foods that are high in calories (Greer et al., 2013), leading to increased consumption of foods higher in saturated fats and lower in fiber and essential micronutrients (St-Onge & Zuraikat, 2019). Thus, the current study investigated the combination of lifelong TAD consumption (beginning at 21 days of age) and 6 weeks of CSR in adulthood on inflammatory response to LPS and cognition.

Some evidence suggests that a Western-like diet high in saturated fat exacerbates the inflammatory response to LPS (Więckowska-Gacek et al., 2021). For example, rats fed a high-fat fructose diet for 3 months demonstrated an enhanced increase in inflammation following an LPS injection (Shamseldeen et al., 2021). Interestingly, in contrast, C57BL/6 mice fed a high-fat

(60% kcal from fat) diet had an attenuated inflammatory response to one LPS injection, without demonstrating a reduction in sickness behavior, indicating a blunted immune response to LPS (Baumgarner et al., 2014). Thus, it is not well-understood how consumption of a typical American-style diet alters immune function.

The primary goals of this Experiment were to investigate how the combination of lifelong TAD consumption and 6 weeks of CSR would impact anxiety-like behaviors, cognition, and central and peripheral inflammation. In addition, we also investigated the effects of lifelong TAD consumption, 6 weeks of CSR, and one LPS injection on peripheral and central inflammatory response, colon length, spleen weight, liver weight, and white fat weight.

Hypotheses

Like our Experiment 2 predictions, we hypothesized that lifelong TAD consumption would increase body weight, impair cognitive function in CFC, and increase anxiety and hippocampal and serum expression of cytokines. We also hypothesized similar effects of CSR, such that CSR mice would demonstrate increased anxiety, cognitive impairment in CFC, and hippocampal and serum cytokines. Most importantly, we hypothesized significant interaction effects, such that TAD-fed mice that underwent CSR would have exacerbated anxiety, cognitive impairment in CFC, and increased hippocampal and serum cytokines in response to LPS compared to all other groups. We also hypothesized that TAD would induce inflammation in the colon, indicated by shorter colon lengths in the TAD mice (Hou et al., 2013). Additionally, we hypothesized a decrease in liver and spleen weight due to CSR, as six days of total sleep deprivation led to decreased liver and spleen weight in rats (Balestrieri et al., 1980).

2. Methods

Unless otherwise described, all research subjects, behavioral paradigms, injections, tissue collection procedures, and data analyses were performed identically to those discussed in Chapters 2 and 3.

Subjects

In Experiment 3, male and female C57BL/6J mice were bred and housed as described in Chapter 2. Mice were 21 days of age at the beginning of the study protocol, making them around 6.5–7 months old at the time of tissue collection.

Diet Treatments, Food Consumption, and Body Weight Measurement

As described in Chapter 3, all mice were assigned to either the TAD or the standard chow, and body weight was measured weekly for the duration of the study. Food consumption was also measured until the start of the sleep restriction protocol, as previously described. At the conclusion of the study, total diet consumption was 5.5 months, as many chronic diet studies exploring the effect of diet on AD in rodents feed between 4–7 months (Więckowska-Gacek et al., 2021) (See Fig. 17 for comprehensive study timeline).

Chronic Sleep Restriction

After four months of diet consumption, CSR was performed for six weeks as described in Chapter 2. All animals continued to consume their assigned diets throughout the six weeks of sleep restriction, as described in Chapter 3.

Open Field Testing

On the final day of the CSR protocol, prior to the testing session of CFC, all mice underwent open field testing to assess locomotor activity and anxiety-like behaviors. Mice were placed into open field chambers (27 cm x 27 cm) with a house light as video tracking software

(Med Associates Inc. St. Albans, VT) measured various aspects of locomotor activity. Within the software, the chambers were assigned zones (a center zone and an outer zone), wherein the center zone was defined by dividing the area of the box into 16 squares, with the 4 central squares serving as the center zone (Swiergiel & Dunn, 2007). Anxiety-like behavior was measured as the amount of time spent in the outer zone during the session, as spending time in the center zone suggests less anxiety-like behavior (Seibenhener & Wooten, 2015). Average speed (cm/second), distance traveled (cm), vertical counts, and resting time (seconds) were measured to assess locomotor activity. All mice remained in the chambers for 10 minutes. Following open field testing, mice were placed into their respective experimental cages (i.e., CSR or HCC) for the day. All open field testing was performed between 0700 h and 0900 h.

Fecal Sample Collection

Fecal samples were collected for future analysis. Fecal pellets were obtained from each mouse following open field sessions. After open field testing, fecal samples were stored at -80º C until further processing.

Contextual Fear Conditioning

Following the final day of the CSR protocol and open field testing, CFC was conducted. Protocols used were as described in Chapter 3, but with slightly different parameters. During the training session, mice were left in the chambers for a total of 200 seconds, receiving two, two-second 0.5 mA footshocks – one at 120 seconds, and one at 180 seconds. During the testing session, 24 hours later, mice remained in the chambers for 200 seconds, receiving no footshocks. Again, freezing behavior was measured with FreezeFrame™ software (Actimetrics Software, Wilmette, IL). All CFC was conducted between 0700 h and 1000 h.

Intraperitoneal injections

Following the conclusion of the CFC testing session, all mice were assigned to one of two injection treatments (LPS or Saline) and were injected following procedures described in Chapter 2. All injections took place between 1000 h and 1130 h.

Serum and Tissue Collection

Tissue collection began four hours following the intraperitoneal injections. Trunk blood was collected as previously described. Hippocampal tissue was dissected, and the dorsal third of the hippocampus was collected in microcentrifuge tubes containing RNALater, placed on wet ice, and stored at -20º C overnight to allow RNALater buffer to permeate the tissue. These samples were then stored at -80º C until rtPCR. PFC was collected in separate microcentrifuge tubes containing a tissue lysis buffer cocktail (PRO-PREP, Bulldog Bio, Portsmouth, NH) with additional protease inhibitors, (phosphatase inhibitor cocktail A, phosphatase inhibitor cocktail B, and protease inhibitor cocktail, Bimake, Houston, TX), and were snap-frozen on dry ice and stored at -80º C until processing and analysis. Liver, spleen, and white fat were removed, placed on separate pitri dishes, washed with 1X phosphate-buffered saline (PBS; Dulbecco's PBS; Caisson Laboratories, Smithfield, UT), and weighed. The colon was also dissected from each animal, and colon lengths were measured using a standard ruler.

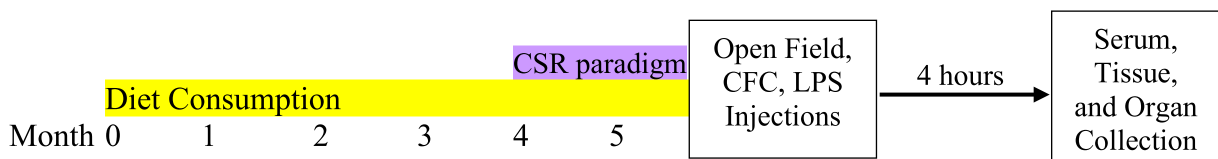

Figure 17. Experimental timeline for Study 3. Male and female C57BL/6J mice were weaned onto either a TAD diet or standard chow. All mice remained on their assigned diets for four months followed by an additional six weeks of respective diet consumption concurrent with six weeks of CSR. Open field was conducted on the final day of the CSR protocol, followed by CFC training the next morning. The CFC testing session was conducted 24 hours later, immediately followed by one LPS or saline injection. Four hours after injections, serum and brain tissue were collected.

Hippocampal mRNA Quantification

RT-PCR was conducted as described in Chapter 2 to assess BDNF, TNFα, IL-1β, IL-6, and IL-10 mRNA in the hippocampal samples.

Peripheral Cytokine Quantification

Multiplexing assays were performed as described in Chapter 2 to assess serum levels of IL-1β, TNFα, IL-6, IFN-γ, IL-10, and IL-4.

Statistical Analyses

All statistical analyses were performed with alpha levels of 0.05, and any significant interactions were explored using pairwise comparisons.

3. Results

Experiment 3A: Effects of Lifelong Consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Body Weight

To assess the impact of TAD consumption on body weight during the first four months of diet consumption only, the average monthly weight was calculated, and mixed-model ANOVAs, in which the between-subjects variable was Diet (TAD vs. Standard), and the repeated-measures variable was Month (Month 1 vs. Month 2 vs. Month 3 vs. Month 4), were performed for both male and female mice. In male mice, results revealed a significant main effect of Month ($F(3,312) = 1040.532$, $p < 0.001$), such that all mice gained weight throughout the study. Results also revealed a significant main effect of Diet ($F(5,312) = 5.898$, $p = 0.017$), such that the TAD mice weighed more compared to the standard chow mice. There was also a significant Month x Diet interaction ($F(3,312) = 4.453$, $p = 0.004$), such that TAD mice weighed more compared to standard mice during Month 3 ($p < 0.001$) and Month 4 ($p = 0.004$) of the study. In addition, there was a significant linear trend of Month x Diet ($F(1,104) = 5.939$, $p = 0.017$), such that TAD

mice gained more weight throughout the first four months of the study compared to the standard chow mice (See Fig. 18A).

To assess the impact of the combination of TAD consumption and CSR on body weight during the six weeks of sleep restriction, mixed-model repeated measures ANOVAs were utilized, in which the between-subjects variables were Diet (TAD vs. Standard) and Sleep Condition (CSR vs. Standard), and the repeated-measures variable was Week (Week 1 vs. Week 2 vs. Week 3 vs. Week 4 vs. Week 5 vs. Week 6). In males, results revealed a significant main effect of Week ($F(5,300) = 28.366$, $p < 0.001$), such that body weight increased throughout the six weeks. The main effect of Diet was approaching significance ($F(1,60) = 3.030$, $p = 0.087$), such that TAD mice weighed more compared to standard chow mice. There was no main effect of Sleep ($F(1,60) = 1.761$, $p = 0.190$), and no Week x Sleep Condition interaction ($F(5,300) = 0.472$, $p = 0.867$), but the Week x Diet interaction was approaching significance ($F(5,300) = 2.174$, $p = 0.057$), such that TAD mice weighed more compared to standard chow mice in Week 4 ($p = 0.038$), Week 5 ($p = 0.050$), and Week 6 ($p = 0.042$). Furthermore, there was a significant linear trend of Week x Diet ($F(1,60) = 4.004$, $p = 0.050$), such that TAD mice continued to gain more weight throughout the last six weeks of the study compared to standard chow mice. The Week x Sleep Condition x Diet interaction was also approaching significance ($F(5,300) = 2.026$, $p = 0.075$), such that, among the CSR mice, TAD mice weighed more compared to standard chow mice in Week 3 ($p = 0.043$), Week 4 ($p = 0.004$), Week 5 ($p = 0.008$), and Week 6 ($p = 0.008$), while there were no differences between TAD and standard chow mice in the HCC group (See Fig. 18B).

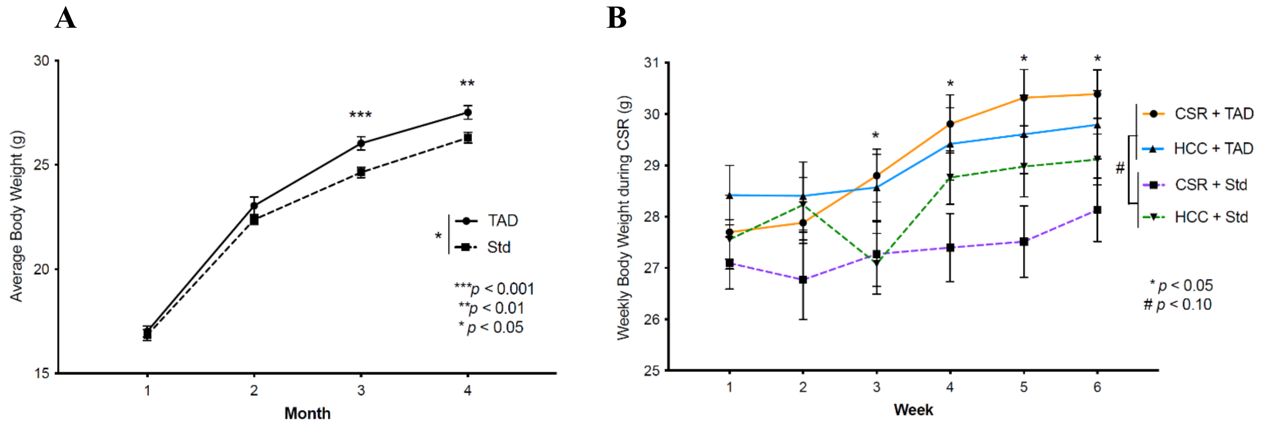

Figure 18. Body weight data during feeding-only and CSR periods in male mice. (A) Male TAD mice gained more weight by Months 3 and 4 compared to standard chow mice. (B) CSR TAD mice weighed more compared to CSR HCC mice in Weeks 3, 4, 5, and 6 of the sleep restriction period of the study. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's 52–54.

For female mice, during the first four months of diet consumption only, results revealed a significant main effect of Month ($F(3,315) = 1486.228$, $p < 0.001$), such that all mice gained weight throughout the consumption-only portion of the study (See Fig. 19A). However, there was no significant main effect of Diet ($F(1,105) = 0.170$, $p = 0.681$), and no Month x Diet interaction ($F(3,315) = 0.833$ $p = 0.476$).

During the six weeks of sleep restriction, results revealed a significant main effect of Week ($F(5,280) = 11.992$, $p < 0.001$), such that mice gained weight throughout the six weeks. There was also a significant main effect of Sleep Condition ($F(1,56) = 4.511$, $p = 0.038$), such that the CSR mice weighed less compared to the HCC mice. The main effect of Diet was not significant ($F(1,56) = 2.547$, $p = 0.116$), but the Sleep x Diet interaction was approaching significance ($F(1,56) = 4.511$, $p = 0.060$), such that, among HCC mice, TAD mice weighed more compared to standard chow mice ($p = 0.016$), and that, among TAD mice, HCC mice weighed more compared to CSR mice ($p = 0.012$). There was also a significant Week x Diet interaction ($F(5,280) = 4.373$, $p < 0.001$), such that TAD mice weighed more compared to standard chow

mice in Week 4 ($p = 0.018$), Week 5 ($p = 0.031$), and Week 6 ($p = 0.023$). Moreover, there was a significant linear trend of Diet x Week ($F(1,56) = 11.113$, $p = 0.002$), such that the TAD mice gained weight more rapidly compared to the standard chow mice. Finally, there was a significant Sleep Condition x Diet x Week interaction ($F(5,280) = 4.589$, $p < 0.001$), such that, among the TAD mice, CSR mice weighed significantly less compared to HCC mice in Week 1 ($p = 0.012$), Week 2 ($p = 0.005$), and Week 3 ($p = 0.006$), and Week 6 ($p = 0.033$), while there were no differences between CSR and HCC mice that consumed the standard chow. In addition, among HCC mice, TAD mice weighed more compared to standard chow mice in each of the six weeks (ps < 0.05), while, among CSR mice, TAD mice weighed less compared to standard chow mice, but only in Week 1 ($p = 0.046$). Furthermore, among HCC mice, standard chow mice gained weight only in Week 4 ($p = 0.020$), and TAD mice gained weight only in Week 5 ($p = 0.011$), whereas, among CSR mice, standard chow mice lost weight in Week 1 ($p =0.045$) and gained weight in Week 3 ($p = 0.006$), while TAD mice gained weight in Week 2 ($p = 0.010$) and Week 3 ($p < 0.001$). Moreover, there was a significant linear trend of Sleep x Diet x Week ($F(1,56) = 10.451$, $p = 0.002$), such that CSR TAD mice gained weight more rapidly compared to other groups (See Fig. 19B).

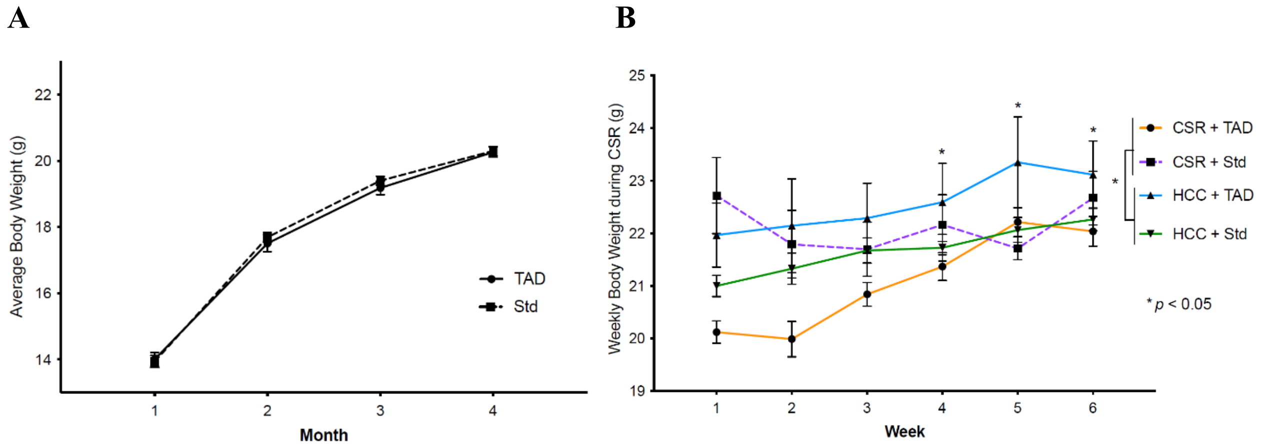

Figure 19. Body weight data during feeding-only and CSR periods in female mice. (A) No differences were observed between TAD and standard chow mice in the first four months of the study. (B) TAD mice weighed more compared to standard chow mice in Weeks 4, 5, and 6, and CSR TAD mice weighed less compared to HCC TAD mice in Weeks 1, 2, 3, and 6 of the sleep restriction portion of the study. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's 53–54 (A), N's 18–28 (B).

Food Consumption Data

To assess differences in food consumption, mixed-model repeated measures ANOVAs, in which the between-subjects variable was Diet (TAD vs. Standard), and the repeated-measures variable was Month (Month 1 vs. Month 2 vs. Month 3 vs. Month 4), were performed for male and female mice. In males, results revealed a significant main effect of Diet ($F(1,27) = 85.501$, $p < 0.001$), such that TAD mice consumed less food compared to standard chow mice (See Fig. 20A). However, there was no effect of Month ($F(3,81) = 0.578$, $p = 0.631$), and no Month x Diet interaction ($F(3,81) = 0.035$, $p = 0.991$).

Similarly, in female mice, results revealed a significant main effect of Diet ($F(1,27) = 58.182$, $p < 0.001$), such that TAD mice consumed less food compared to the standard chow mice (See Fig. 20B). There was no effect of Month ($F(3,81) = 1.090$, $p = 0.358$) and no Month x Diet interaction ($F(3,81) = 1.107$, $p = 0.351$).

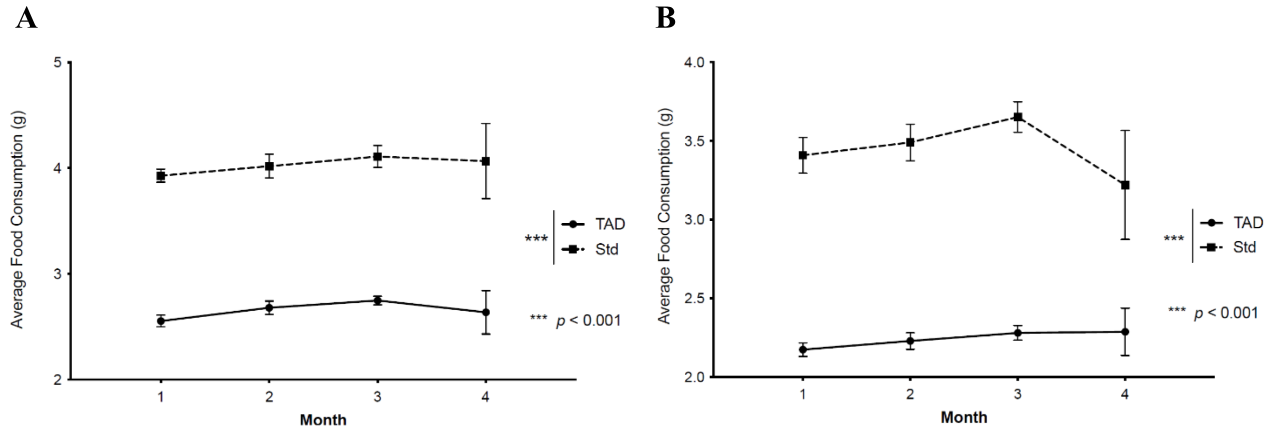

Figure 20. Food consumption data during the first four months of the study. (A) Male and (B) female mice that consumed the TAD ate less food compared to standard chow mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's 14–15.

Experiment 3B: Effects of Lifelong Consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Anxiety and Locomotor Behaviors

To assess the impact of CSR and lifelong TAD consumption on locomotor activity and anxiety-like behavior in the open field paradigm, two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. HCC] were conducted. The behaviors assessed were distance traveled, vertical counts, average speed, resting time, and time in center. Data were analyzed separately within the first five minutes of testing (Bin 1), within the second five minutes of testing (Bin 2), and for the entire ten-minute session (Total).

Distance Traveled

For males, results revealed a significant main effect of Sleep Condition in Bin 1 ($F(1,65) = 39.538$, $p < 0.001$), such that the CSR mice traveled farther in Bin 1 compared to HCC mice. There were no significant effects in Bin 2 ($Fs(1,65) < 2.024$, $ps > .160$). However, results of Total duration analysis revealed a significant main effect of Sleep Condition ($F(1,65) = 19.643$, p

< 0.001), such that CSR mice traveled farther during the 10-minute testing session compared to HCC mice, consistent with the results of Bin 1 (See Fig. 21A).

In females, there were no significant effects of Diet or Sleep Condition in Bin 1 (Fs(1,63) < 2.671, ps > .107), Bin 2 (Fs(1,63) < 2.686, ps > .106), or the total duration (Fs(1,63) < 2.283, ps > .136) (See Fig. 21B).

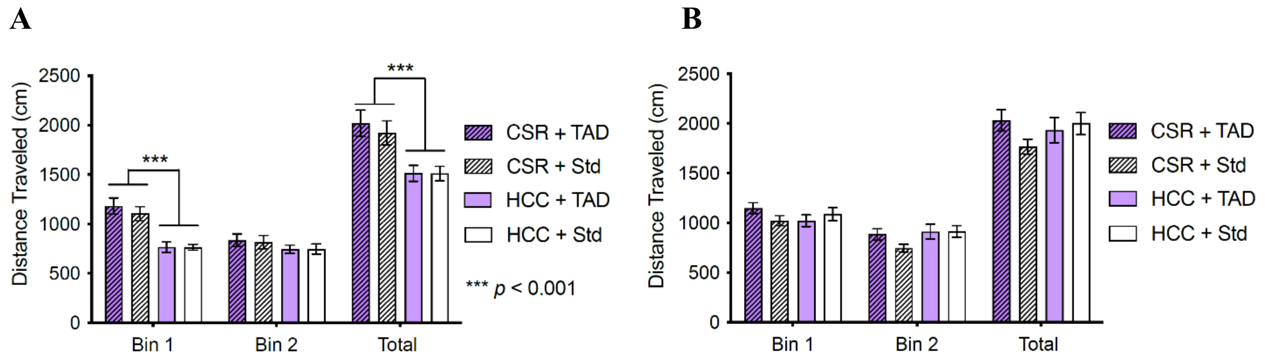

Figure 21. Distance traveled in open field. (A) Male CSR mice traveled farther compared to HCC mice in Bin 1 and in the Total testing session. (B) No differences were observed in female mice in distance traveled. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 14–20.

Vertical Counts

For males, results revealed a significant main effect of Sleep Condition ($F(1,65) = 25.625$, $p < 0.001$), such that CSR mice had more vertical movements compared to HCC mice. There were no significant effects in Bin 2 (Fs(1,65) < 1.019, ps > .317). However, there was a main effect of Sleep Condition in the Total testing session ($F(1,65) = 5.756$, $p = 0.019$), such that CSR mice had more vertical movements compared to HCC mice (See Fig. 22A).

For females, analysis of Bin 1 data revealed a significant main effect of Sleep Condition ($F(1,63) = 4.290$, $p = 0.042$), such that CSR mice had more vertical movements compared to HCC mice. Furthermore, the Sleep Condition x Diet interaction was approaching significance ($F(1,63) = 3.326$, $p = 0.073$), such that, among TAD mice, HCC mice had fewer vertical movements compared to CSR mice ($p = 0.005$). In Bin 2, there was a significant Sleep Condition

x Diet interaction ($F(1,63) = 6.716$, $p = 0.012$), such that, among the TAD mice, HCC mice had fewer vertical movements compared to CSR mice ($p = 0.023$), and that, among HCC mice, TAD mice had fewer vertical movements compared to standard chow mice ($p = 0.034$). In the Total testing session, results revealed a significant Sleep Condition x Diet interaction ($F(1,63) = 5.894$, $p = 0.018$), such that, among the TAD mice, HCC mice had fewer vertical movements compared to CSR mice ($p = 0.006$) (See Fig. 22B).

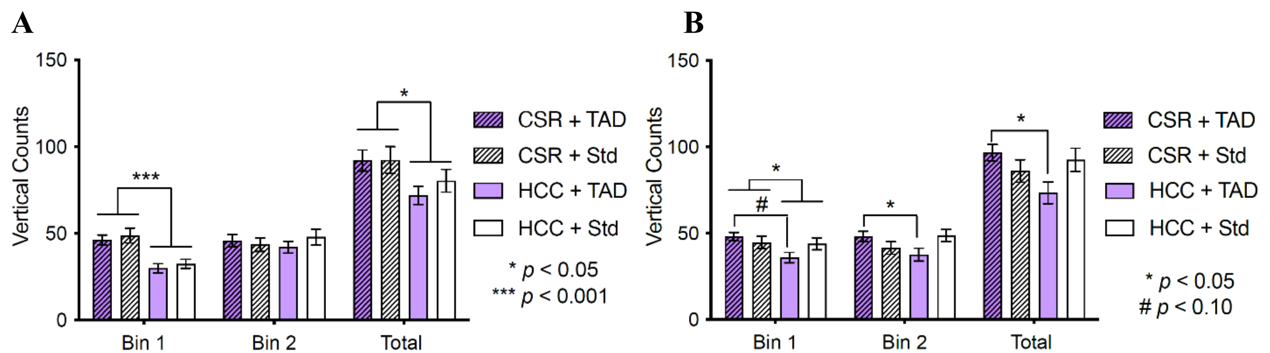

Figure 22. Vertical counts in open field. (A) Male CSR mice had more vertical movements in Bin 1 and in the Total testing session. (B) Female CSR mice had more vertical movements in Bin 1, and CSR TAD mice had more vertical movements compared to HCC TAD mice in Bin 1, Bin 2, and the Total testing session. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 14–20.

Average Speed

In males, analysis of average speed revealed no significant effects of CSR or TAD consumption in Bin 1 ($Fs(1,65) < 0.994$, $ps > 0.322$), Bin 2 ($Fs(1,65) < 1.590$, $ps > 0.212$), or Total ($Fs(1,65) < 0.308$, $ps > 0.581$) (See Fig. 23A).

In females, results revealed no significant effects of CSR or TAD consumption on average speed in Bin 1 ($Fs(1,63) < 0.827$, $ps > 0.367$). However, in Bin 2, there was a significant main effect of Sleep Condition ($F(1,63) = 7.075$, $p = 0.010$), such that CSR mice moved more slowly compared to HCC mice (See Fig. 23B). However, this effect did not persist throughout

the Total testing session, as there were no observed differences in average speed ($Fs(1,63) < 2.556$, $ps > 0.115$).

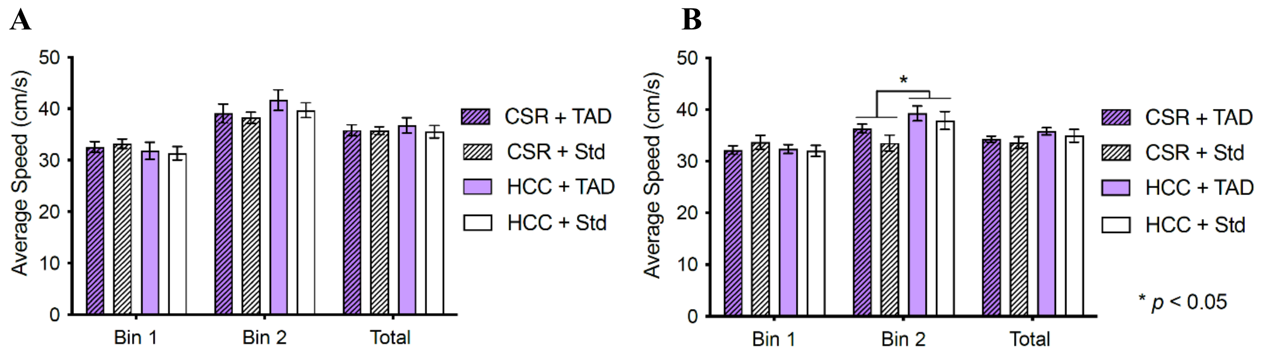

Figure 23. Average speed in open field. (A) No differences in average speed were observed in male mice. (B) Female CSR mice moved more slowly compared to HCC mice in Bin 2. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 14–20.

Resting Time

For males, results revealed a significant main effect of Sleep Condition in Bin 1 ($F(1,65) = 27.536$, $p < 0.001$), such that HCC mice spent more time resting compared to CSR mice. There were no differences in Bin 2 ($Fs(1,65) < 0.301$, $ps > 0.585$). However, in the Total testing session, there was a significant main effect of Sleep Condition ($F(1,65) = 11.235$, $p < 0.001$), such that the HCC mice spent more time resting compared to the CSR mice (See Fig. 24A).

For females, results revealed no significant effects in Bin 1 ($Fs(1,63) < 2.231$, $ps > 0.140$). However, in Bin 2, the main effect of Sleep Condition was approaching significance ($F(1,63) = 3.472$, $p = 0.067$), such that the CSR mice spent more time resting compared to HCC mice (See Fig. 24A). However, no significant effects on resting time were observed in the Total testing session ($Fs(1,63) < 2.222$, $ps > 0.141$).

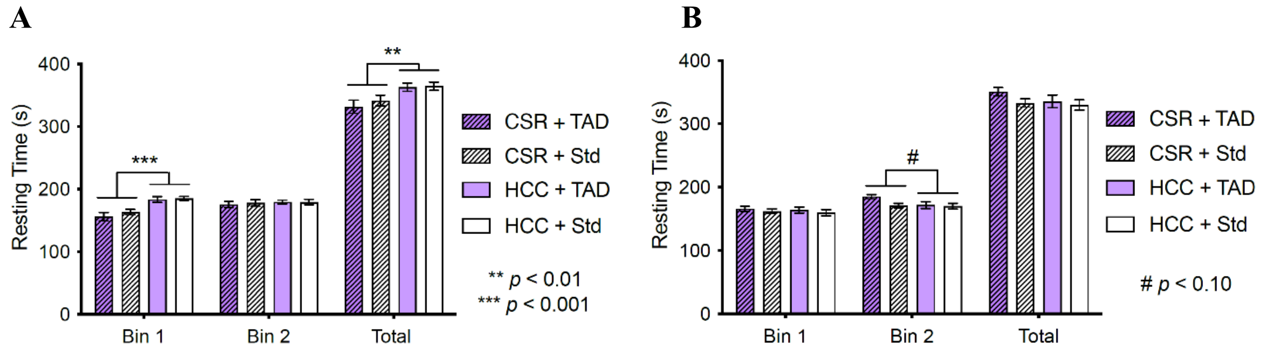

Figure 24. Resting time in open field. (A) Male HCC mice rested more compared to CSR mice in Bin 1 and in the Total testing session. (B) Female CSR mice rested more compared to HCC mice in Bin 2. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 14–20.

Time in Center Zone

For male mice, results revealed no significant effects of CSR or Diet on time spent in the center zone during Bin 1 (Fs(1,65) < 0.190, ps > 0.665), Bin 2 (Fs(1,65) < 1.878, ps > 0.175), or the Total testing session (Fs(1,65) < 1.387, ps > 0.243) (See Fig. 25A).

For female mice, Bin 1 data violated homogeneity of variance, so a natural log transformation was performed prior to analysis. Two-way ANOVA revealed a significant main effect of Sleep Condition on time spent in the center zone ($F(1,63) = 5.161$, $p = 0.027$), such that CSR mice spent less time in the center zone compared to HCC mice. In Bin 2, the main effect of Sleep Condition was approaching significance ($F(1,63) = 2.830$, $p = 0.097$), such that the CSR mice spent less time in the center zone compared to HCC mice. In the Total testing session, there was a significant main effect of Sleep Condition ($F(1,63) = 5.375$, $p = 0.024$), such that the CSR mice spent less time in the center zone compared to HCC mice (See Fig. 25B).

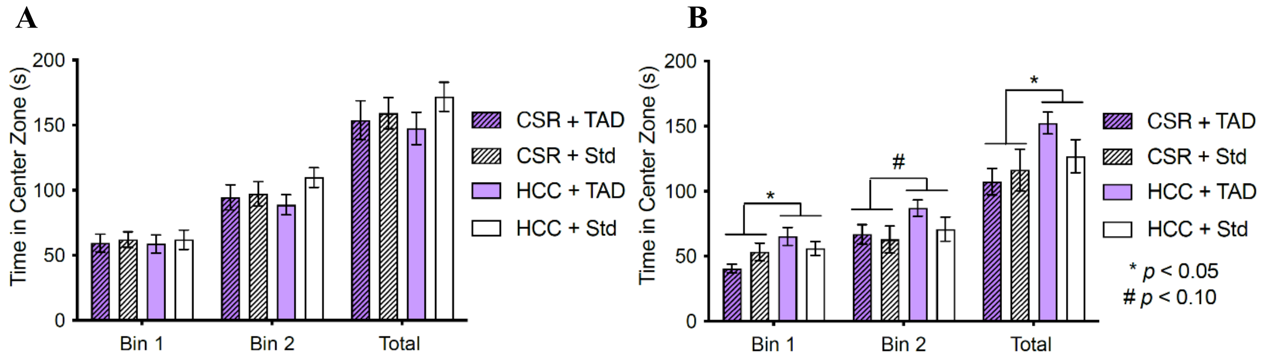

Figure 25. Anxiety-like behavior in open field. (A) No differences were observed in male mice in time spent in the center zone. (B) Female CSR mice spent less time in the center zone compared to HCC mice in Bin 1, Bin 2, and the Total testing session. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 14–20.

Experiment 3C: Effects of Lifelong consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Cognition

Two-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. Standard]) were performed to explore the effects of TAD and CSR on freezing behavior in CFC in both male and female C57BL/6 mice. In males, results revealed a main effect of Diet Condition that was approaching significance ($F(1,48) = 3.310$, $p = 0.075$), such that the TAD mice froze more compared to the Standard chow mice (See Fig. 26A). There was no main effect of Sleep Condition ($F(1,48) = 0.562$, $p = 0.457$) and no significant interaction $F(1,43) = 2.238$, $p = 0.141$). One mouse was excluded due to the presence of what appeared to be a subdural hygroma upon tissue collection (and he was excluded from all other analyses). Two more mice were excluded from CFC analysis due to a tornado emergency which occurred during the training session, and four additional mice were excluded from CFC analysis due to being shocked at incorrect times.

In females, results revealed a significant main effect of Sleep Condition ($F(1,43) = 7.960$, $p = 0.007$), such that the CSR mice froze more compared to the HCC mice. In addition, the main effect of Diet Condition was also approaching significance ($F(1,43) = 3.795$, $p = 0.058$), such

that TAD mice froze less compared to Standard chow mice (See Fig. 26B). There was no Sleep Condition by Diet Condition interaction ($F(1,43) = 0.633$, $p = 0.431$). Two mice were excluded from analysis due to being shocked at incorrect times.

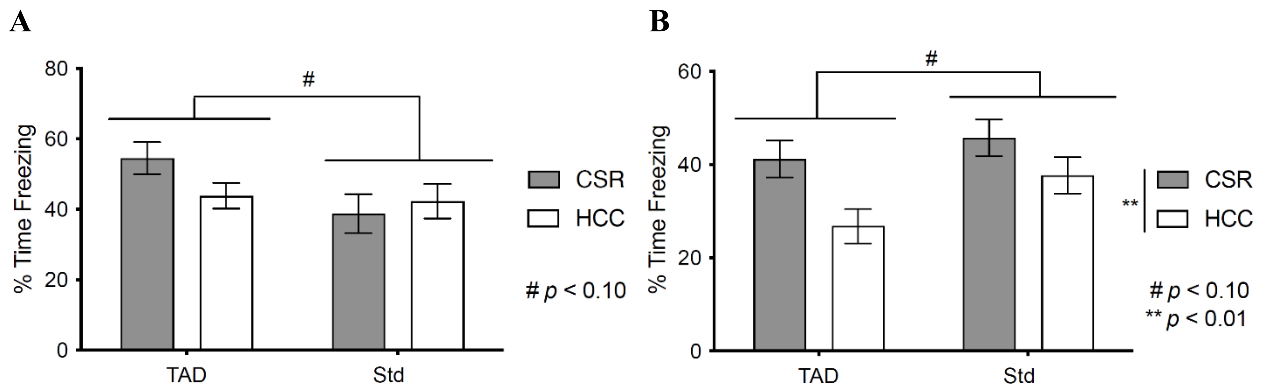

Figure 26. Freezing behavior in CFC. (A) Male mice that consumed the TAD froze more compared to males that consumed the standard chow. (B) Female CSR mice froze more compared to HCC females, and TAD mice froze less compared to females that consumed the standard chow. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 9–15.

Experiment 3D: Effects of Lifelong consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Hippocampal BDNF mRNA

Three-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. Standard] x Injection [LPS vs. Saline]) were conducted to assess the impact of CSR and lifelong TAD consumption on hippocampal BDNF mRNA in male and female mice. In male mice, results revealed a significant main effect of Diet ($F(1,63) = 9.387$, $p = 0.003$), such that TAD mice had lower hippocampal BDNF mRNA compared to standard chow mice. There was also a main effect of Injection ($F(1,63) = 16.590$, $p < 0.001$), such that LPS mice had lower BDNF mRNA in the hippocampus compared to saline mice. In addition, there was a significant Diet x Injection interaction ($F(1,63) = 4.410$, $p = 0.040$), such that, among saline HCC animals, TAD mice had slightly lower BDNF compared to standard chow mice ($p = 0.092$) (See Fig. 27A).

In female mice, results revealed a significant main effect of Diet ($F(1,64) = 14.360$, $p < 0.001$), such that TAD mice had lower BDNF mRNA compared to standard diet mice. There was also a significant Sleep Condition x Diet x Injection interaction ($F(1,64) = 5.436$, $p = 0.023$), such that, among LPS HCC mice, those that consumed the TAD had lower BDNF mRNA in the hippocampus compared to mice that consumed the standard chow ($p = 0.011$) (See Fig. 27A).

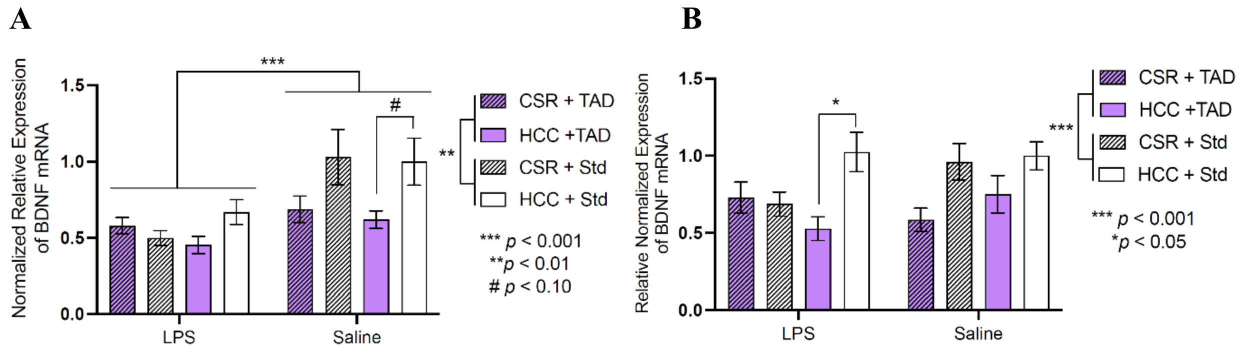

Figure 27. Lifelong TAD consumption decreases BDNF mRNA in the hippocampus. (A) Male TAD mice had lower BDNF mRNA compared to standard chow mice, and LPS mice had lower BDNF mRNA compared to saline mice. (B) Female CSR mice spent less time in the center zone compared to HCC mice in Bin 1, Bin 2, and the Total testing session. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 8–9.

Experiment 3E: Effects of Lifelong Consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Hippocampal Immune Markers

To evaluate the impact of CSR and lifelong TAD consumption on TNFα, IL-1β, IL-6, and IL-10 mRNA in the hippocampus, three-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. Standard] x Injection [LPS vs. Saline]) were performed for male and female mice. Male data will be discussed first, followed by female data.

Male Cytokine mRNA in the Hippocampus

For hippocampal TNFα mRNA, results revealed a main effect of Injection ($F(1,59) = 12.110$, $p = 0.001$), such that LPS mice had higher TNFα mRNA compared to saline mice. The

main effect of Diet was approaching significance ($F(1,59) = 3.142$, $p = 0.082$), such that TAD mice had less TNFα mRNA compared to standard chow mice. There was also a significant Sleep Condition x Diet x Injection interaction ($F(1,59) = 4.896$, $p = 0.031$), such that, among Saline HCC mice, TAD mice had less TNFα mRNA compared to standard chow mice ($p = 0.023$) (See Fig. 28A).

For IL-1β mRNA, results revealed a significant main effect of Injection ($F(1,62) = 46.340$, $p < 0.0001$), such that mice that received LPS had higher IL-1β mRNA compared to mice that received saline (See Fig. 28B). However, no other significant effects were observed ($Fs(1,62) < 1.904$, $ps > 0.173$).

For IL-6 mRNA, results revealed a significant main effect of Injection ($F(1,61) = 15.340$, $p = 0.0002$), such that LPS mice had more IL-6 mRNA in the hippocampus compared to saline mice (See Fig. 28C). No other significant effects were observed $Fs(1,61) < 1.527$, $ps > 0.221$).

For IL-10 mRNA, results revealed a significant main effect of Injection ($F(1,51) = 4.793$, $p = 0.033$), such that mice injected with LPS had lower IL-10 mRNA compared to mice injected with saline. There was also a significant Sleep Condition x Diet interaction ($F(1,51) = 4.016$, $p = 0.050$). When multiple comparisons were conducted utilizing Fisher's LSD, results revealed that, among HCC mice that consumed standard chow, LPS mice had lower IL-10 mRNA compared to saline mice ($p = 0.019$). However, this difference was not significant according to Tukey's multiple comparisons test ($p = 0.255$). Finally, the Sleep Condition x Diet x Injection interaction was approaching significance ($F(1,51) = 3.397$, $p = 0.071$), although Tukey's multiple comparisons test revealed no significant differences between groups (See Fig. 28D).

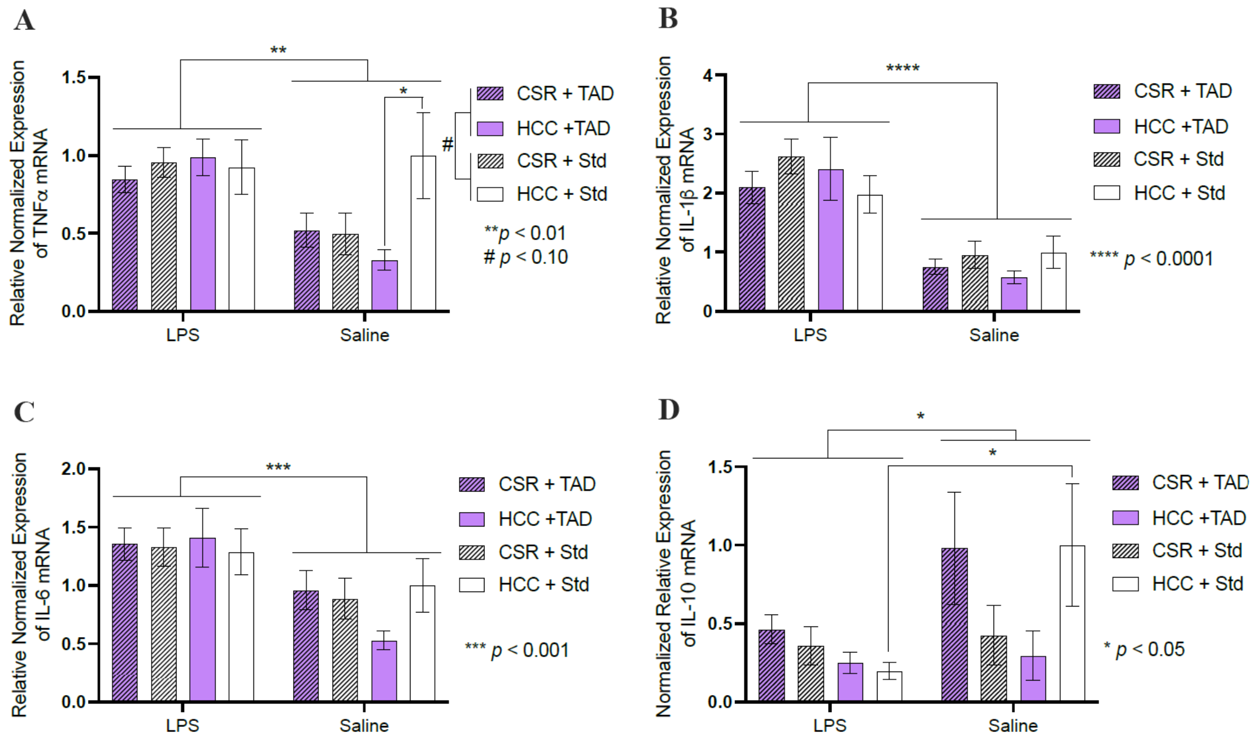

Figure 28. Hippocampal TNFα (A), IL-1β (B), IL-6 (C), and IL-10 (D) mRNA in male mice following lifelong TAD consumption and CSR. Mice injected with LPS had more TNFα, IL-1β, and IL-6 compared to mice injected with saline. TAD mice had slightly reduced TNFα mRNA compared to standard chow mice, particularly among the HCC saline group (A), and HCC standard chow mice injected with saline had higher IL-10 mRNA compared to HCC standard chow mice injected with LPS (D). Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 6–9.

Female Cytokine mRNA in the Hippocampus

For hippocampal TNFα mRNA, results revealed a significant main effect of Injection ($F(1,64) = 5.436$, $p = 0.023$), such that LPS mice had more TNFα mRNA compared to saline mice. Furthermore, results revealed a significant Sleep Condition x Injection interaction ($F(1,59) = 5.134$, $p = 0.027$), such that, among CSR TAD mice, LPS mice had higher TNFα mRNA compared to saline mice ($p = 0.019$), and, among CSR standard chow mice, LPS mice had higher TNFα mRNA compared to standard chow mice ($p < 0.001$), while these differences in injection response were not observed in HCC mice (*ps* > 0.519) (See Fig. 29A).

For hippocampal IL-1β mRNA, results revealed a significant main effect of Injection ($F(1,63) = 48.500$, $p < 0.0001$), such that LPS mice had more IL-1β mRNA compared to saline mice (See Fig. 29B). However, consistent with the male data, no other significant effects were observed (Fs$(1,63) < 1.488$, ps > 0.227).

For hippocampal IL-6 mRNA in female mice, results revealed a significant main effect of Injection ($F(1,62) = 26.850$, $p < 0.0001$), such that LPS mice had more IL-6 mRNA compared to saline mice. Moreover, there was a significant Sleep Condition x Injection interaction ($F(1,62) = 4.082$, $p = 0.048$), such that, among CSR TAD mice, LPS mice had higher IL-6 mRNA compared to saline mice ($p = 0.002$), and, among CSR standard chow mice, LPS mice had higher IL-6 mRNA compared to standard chow mice ($p = 0.035$), while these differences in injection response were not observed in HCC mice (ps > 0.542) (See Fig. 29C).

For IL-10 mRNA, results revealed a significant Sleep Condition x Diet x Injection interaction ($F(1,64) = 9.233$, $p = 0.003$), such that, among HCC TAD mice, those that were injected with LPS had lower IL-10 mRNA compared to those injected with saline ($p = 0.025$), and, among saline mice that consumed TAD, CSR mice had lower IL-10 mRNA compared to HCC mice ($p = 0.0096$) (See Fig. 29D). Furthermore, results revealed a significant Sleep Condition x Diet interaction ($F(1,64) = 4.897$, $p = 0.031$).

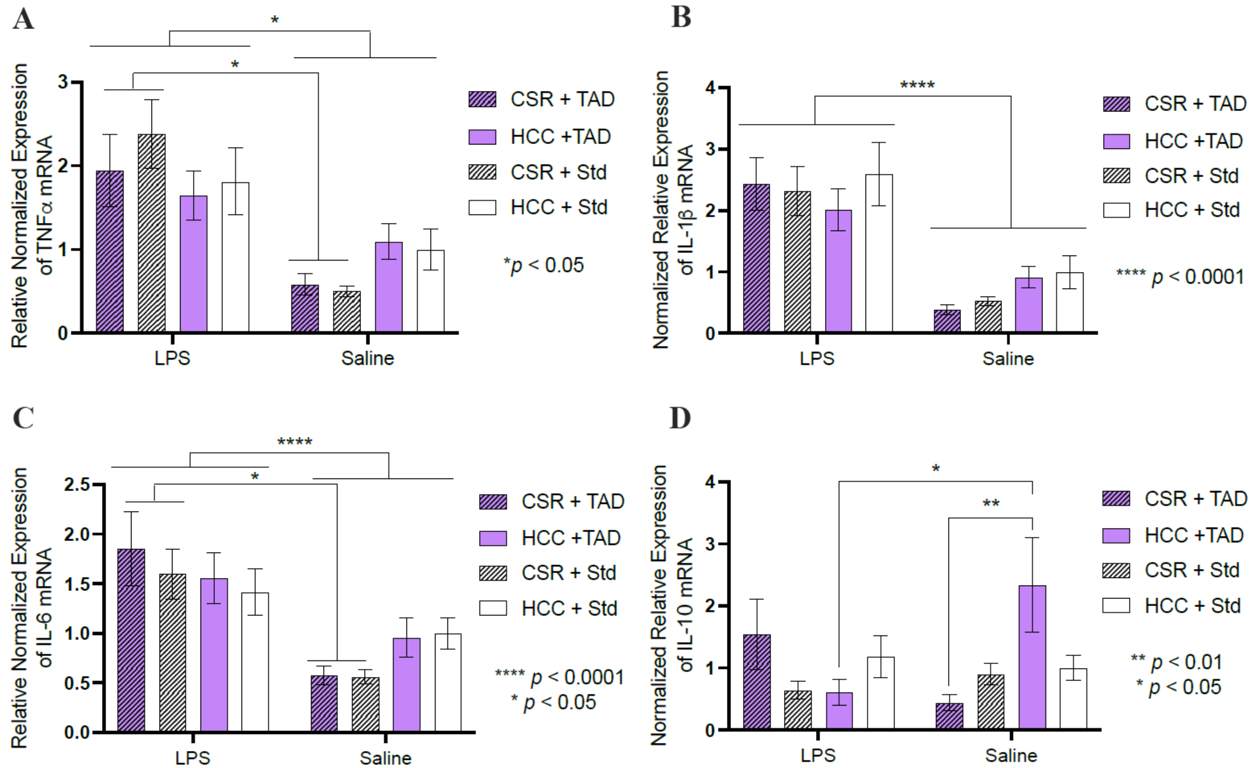

Figure 29. Hippocampal TNFα (A), IL-1β (B), IL-6 (C), and IL-10 (D) mRNA in female mice following lifelong TAD consumption and CSR. Mice injected with LPS had more TNFα, IL-1β, and IL-6 compared to mice injected with saline. Mice that underwent CSR and received LPS had higher TNFα (A) and IL-6 mRNA (C) compared to CSR saline mice. (D) HCC TAD mice injected with LPS had less IL-10 mRNA compared to those injected with saline, and CSR TAD mice injected with saline had less IL-10 mRNA compared to HCC TAD mice injected with saline. Significant differences ($p < 0.05$) are designated by *. N's = 8–9.

Experiment 3F: Effects of Lifelong consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Serum Immune Markers

To assess the impact of CSR and lifelong TAD consumption on serum cytokines, 3-way ANOVAs were conducted (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. Standard] x Injection [LPS vs. Saline]) for male and female data. Male data will be discussed first, followed by female data.

Male Serum Cytokines

For serum TNFα, results revealed a significant main effect of Diet ($F(1,62) = 4.989$, $p = 0.029$), such that the TAD mice had higher serum TNFα compared to the HCC mice. There was also a significant main effect of Injection ($F(1,62) = 94.758$, $p < 0.001$), such that LPS mice had higher TNFα compared to saline mice. Interestingly, there was a significant Diet x Injection interaction ($F(1,62) = 6.222$, $p = 0.015$), such that, among the LPS mice, TAD mice had higher serum TNFα compared to standard chow mice ($p = 0.001$) (See Fig. 30A).

For serum IL-1β, results revealed a significant main effect of Diet ($F(1,60) = 6.885$, $p = 0.011$), such that the TAD mice had higher serum IL-1β compared to the standard chow mice. There was also a significant main effect of Injection ($F(1,60) = 84.455$, $p < 0.001$), such that LPS mice had higher IL-1β compared to saline mice. Furthermore, there was a significant Diet x Injection interaction ($F(1,60) = 7.293$, $p = 0.009$), such that, among the LPS mice, TAD mice had higher IL-1β compared to standard chow mice ($p < 0.001$) (See Fig. 30B). One sample was excluded from analysis for having a CV over 30%.

For serum IL-6, results revealed a significant main effect of Diet ($F(1,62) = 5.299$, $p = 0.025$), such that the TAD mice had higher IL-6 compared to standard chow mice. There was also a significant main effect of Injection ($F(1,62) = 82.477$, $p < 0.001$), such that LPS mice had higher IL-6 compared to saline mice. Importantly, there was a significant Diet x Injection interaction ($F(1,62) = 5.558$, $p = 0.022$), such that, among the LPS mice, TAD mice had higher serum IL-6 compared to standard chow mice ($p = 0.002$) (See Fig. 30C).

Serum analysis of IFN-γ revealed a significant main effect of Injection ($F(1,62) = 29.460$, $p < 0.001$), such that LPS mice had higher IFN-γ compared to saline mice. Interestingly, the Sleep Condition x Diet x Injection interaction was approaching significance ($F(1,62) = 3.002$, p

= 0.088), such that, among TAD mice injected with LPS, those that underwent CSR had lower serum IFN-γ compared to HCC mice ($p = 0.036$). Furthermore, among HCC mice injected with LPS, those that consumed the TAD had higher serum IFN-γ compared to mice that consumed the standard chow ($p = 0.028$) (See Fig. 30D).

For serum IL-10, results revealed a significant main effect of Injection ($F(1,62) = 100.334$, $p < 0.001$), such that LPS mice had higher IL-10 compared to saline mice. Results also revealed that the Diet x Injection interaction was approaching significance ($F(1,62) = 3.064$, $p = 0.085$), such that, among LPS mice, TAD mice had higher IL-10 compared to standard chow mice ($p = 0.021$) (See Fig. 30E).

Serum IL-4 analysis was conducted in males, although many samples were lost due to falling below the limit of detection or having a CV over 30%. Results of the quantifiable data revealed no significant effects (Fs$(1,16) < 0.915$, ps > 0.353) (See Fig. 30F).

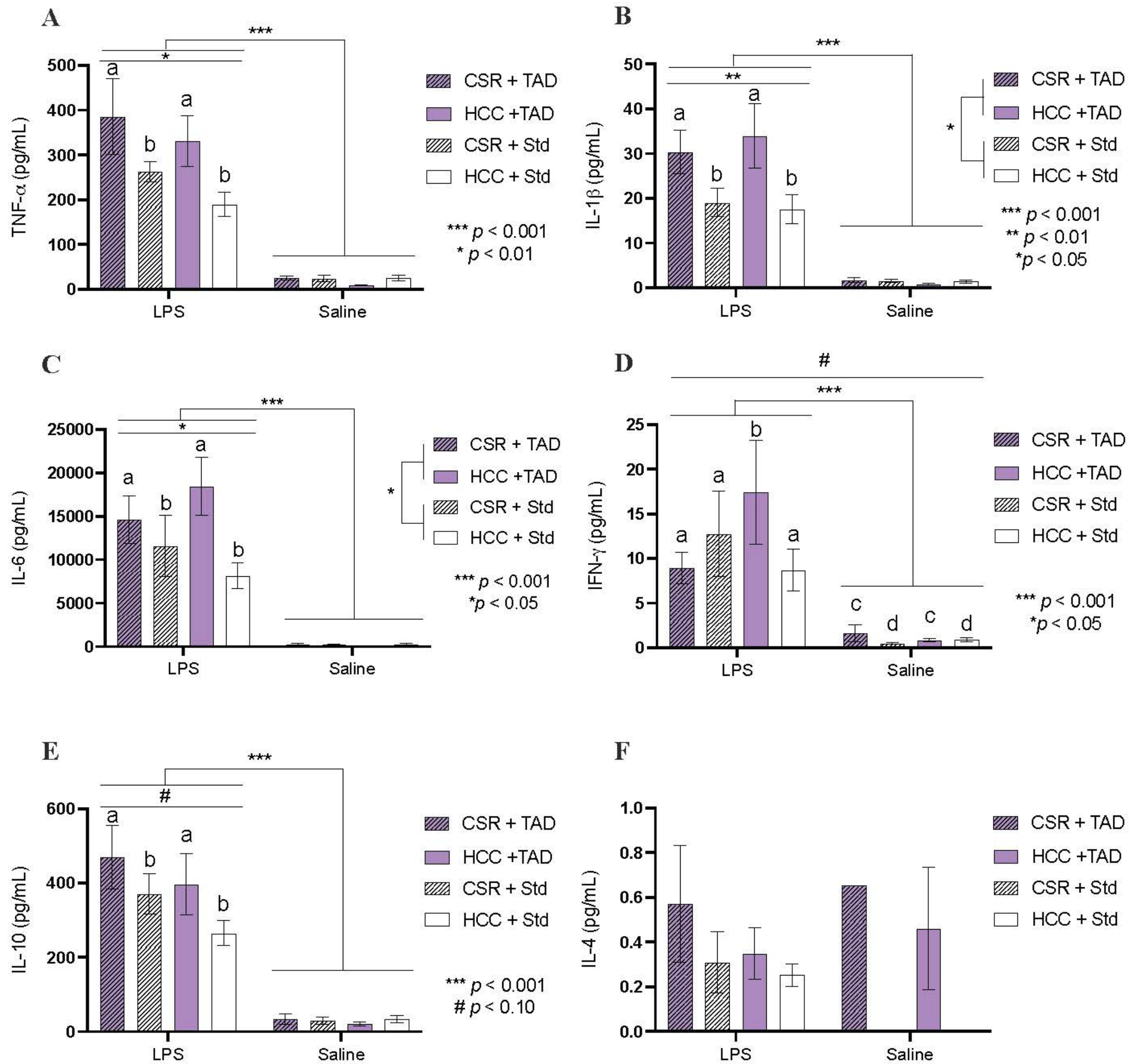

Figure 30. Serum levels of TNFα (A), IL-1β (B), IL-6 (C), IFN-γ (D), IL-10 (E), and IL-4 (F) in male mice following lifelong TAD consumption and CSR. Mice that consumed the TAD had exacerbated TNFα, IL-1β, IL-6, and IL-10 in response to LPS compared to mice that ate the standard chow. TAD mice that underwent CSR had diminished IFN-γ in response to LPS compared to TAD HCC mice. LPS mice had higher pro- and anti-inflammatory cytokines compared to saline mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. Letters denote differences in unpacked interactions. N's = 7–9 (A–E); N's = 0–7 (F).

Female Serum Cytokines

Serum TNFα analysis in female mice revealed a significant main effect of injection ($F(1,59) = 61.524$, $p < 0.001$), such that LPS mice had higher serum TNFα compared to saline

mice. More importantly, there was a significant Diet x Injection interaction ($F(1,59) = 5.052$, p = 0.028), such that, among LPS mice, TAD mice had higher TNFα compared to standard chow mice ($p = 0.012$) (See Fig. 31A).

For IL-1β, results revealed a significant main effect of Injection ($F(1,56) = 56.081$, $p <$ 0.001), such that LPS mice had higher serum IL-1β compared to saline mice. Furthermore, the Diet x Injection interaction was approaching significance ($F(1,56) = 3.349$, $p = 0.073$), such that, among the LPS mice, TAD mice had higher serum IL-1β compared to standard chow mice (p = 0.042) (See Fig. 31B). Two samples were excluded from analysis for having CVs over 30%.

Serum IL-6 analysis revealed a significant main effect of Injection ($F(1,59) = 51.278$, $p <$ 0.001), such that LPS mice had higher IL-6 compared to saline mice (See Fig. 31C). However, there were no other significant main effects or interactions (Fs(1,59) < 1.153, ps > 0.287).

For serum IFN-γ, results revealed a main effect of Diet that was approaching significance ($F(1,59) = 3.209$, $p = 0.078$), such that TAD mice had higher IFN-γ compared to standard chow mice. There was also a significant main effect of Injection ($F(1,59) = 30.928$, $p < 0.001$), such that LPS mice had higher IFN-γ compared to saline mice. Results also revealed a significant Diet x Injection interaction ($F(1,59) = 6.962$, $p = 0.011$), such that, among LPS mice, TAD mice had higher IFN-γ compared to standard chow mice (See Fig. 31D).

Analysis of serum IL-10 in females revealed a main effect of Injection ($F(1,59) = 93.743$, $p < 0.001$), such that LPS mice had higher serum IL-10 compared to saline mice. Results also revealed a Diet x Injection interaction that was approaching significance ($F(1,59) = 3.731$, p = 0.058), such that, among LPS mice, TAD mice had higher IL-10 compared to standard chow mice ($p = 0.017$) (See Fig. 31E).

As was the case with males, female serum IL-4 analysis was conducted, although many samples were lost due to falling below the limit of detection or having a CV over 30%. Results of quantifiable data revealed no significant effects ($Fs(1,26) < 1.777$, $ps > 0.194$) (See Fig. 31F).

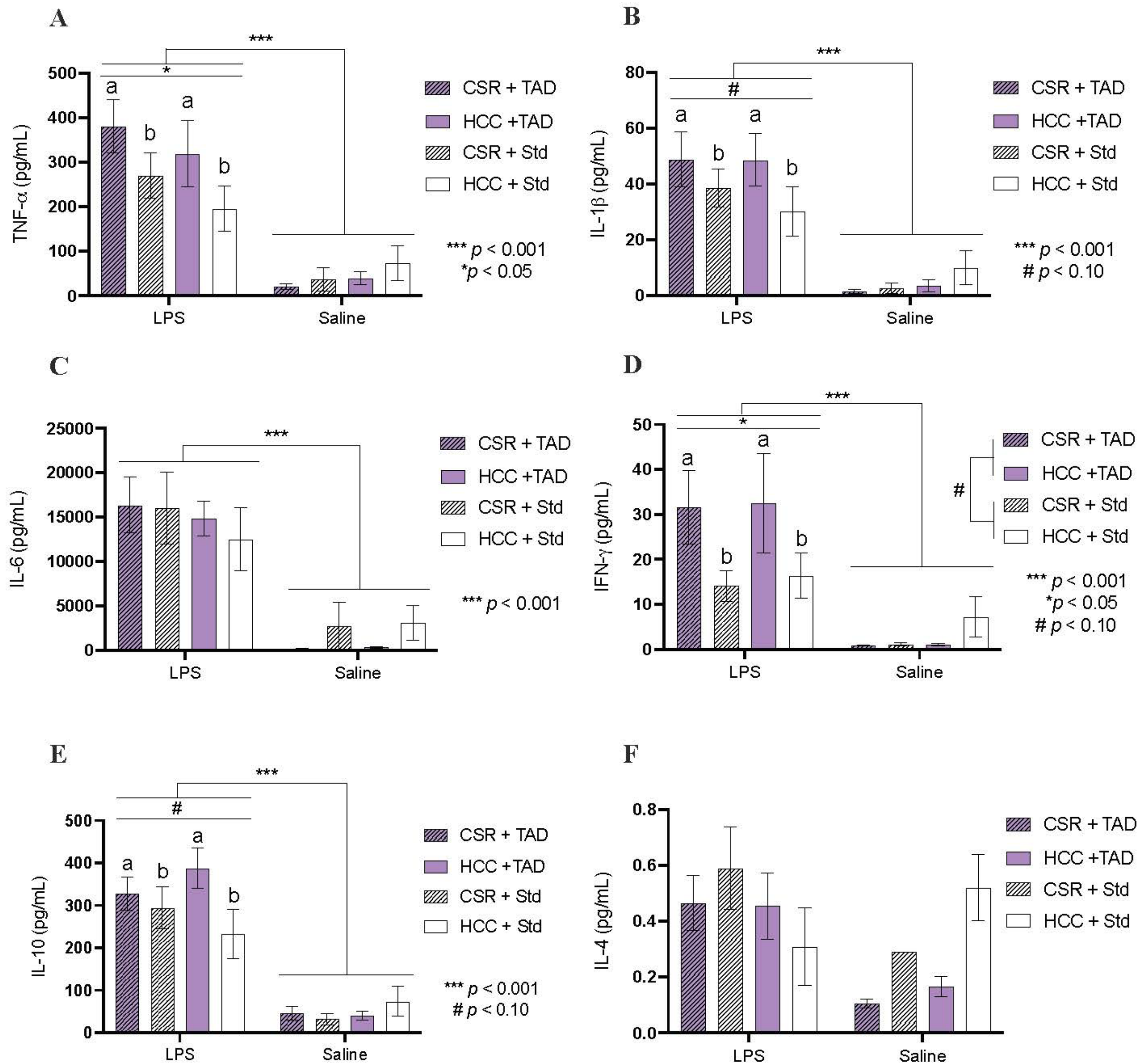

Figure 31. Serum levels of TNFα (A), IL-1β (B), IL-6 (C), IFN-γ (D), IL-10 (E), and IL-4 (F) in female mice following lifelong TAD consumption and CSR. Mice that consumed the TAD had exacerbated TNFα, IL-1β, IFN-γ, and IL-10 in response to LPS compared to mice that ate the standard chow. LPS mice had higher pro- and anti-inflammatory cytokines compared to saline mice. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. Letters denote differences in unpacked interactions. N's = 7–10 (A–E); N's = 1–7 (F).

Experiment 3G: Effects of Lifelong Consumption of Typical American-style Diet and 6 Weeks of Chronic Sleep Restriction on Colon, Spleen, Liver, and White Fat

All analyses on organ data were performed utilizing 3-way ANOVAs (Sleep Condition [CSR vs. HCC] x Diet [TAD vs. Standard] x Injection [LPS vs. Saline]), with male and female datasets analyzed separately.

Colon Length Data

One male mouse was excluded due to being an outlier due to SPSS's use of the interquartile range rule, and three additional mice (two male and one female) were excluded due to tissue damage upon colon extraction. In male mice, results revealed a significant main effect of Diet ($F(1,91) = 41.638$, $p < 0.001$), such that TAD mice had shorter colons compared to standard chow mice. There was also a significant main effect of Injection ($F(1,91) = 14.648$, $p < 0.001$), such that mice receiving LPS injections had shorter colons compared to mice that received saline (See Fig. 32A). In female mice, there was a significant main effect of Sleep Condition ($F(1,95) = 10.944$, $p = 0.001$), such that CSR mice had longer colons compared to HCC mice. There was also a significant main effect of Diet ($F(1,95) = 80.010$, $p < 0.001$), such that TAD mice had shorter colons compared to standard chow mice. In addition, the main effect of Injection was approaching significance ($F(1,95) = 3.672$, $p = 0.058$), such that mice that received LPS injections had shorter colons compared to mice that received saline injections (See Fig. 32B).

A B

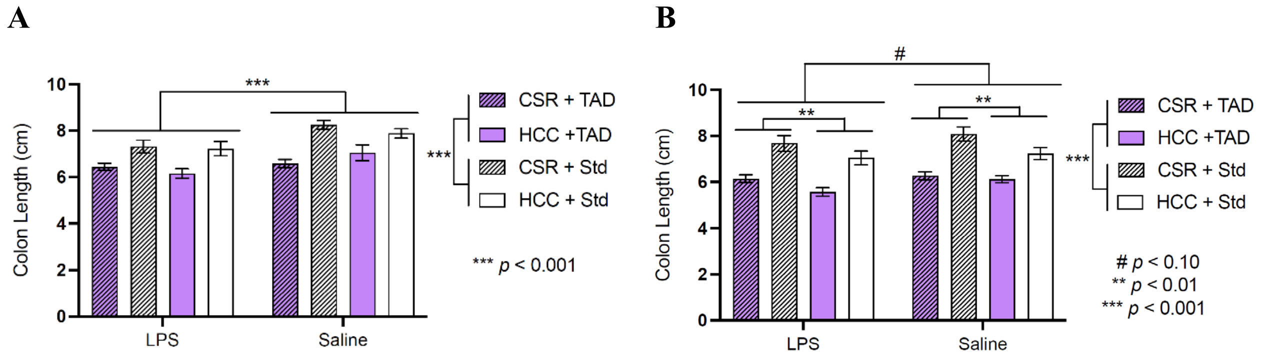

Figure 32. Colon Length data. (A) Male mice that consumed the TAD had shorter colons compared to those that consumed standard chow, and mice that received LPS injections had shorter colons compared to mice that received saline. (B) Female mice that consumed the TAD had shorter colons compared to mice that consumed the standard chow, and CSR females had longer colons compared to HCC females. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *, and differences approaching significance ($p < 0.10$) are designated by #. N's = 12–14.

Spleen Weight Data

In males, results revealed a significant main effect of Diet (F(1,93) = 9.518. p = 0.003), such that TAD mice had heavier spleens compared to standard chow mice (See Fig. 33A). There was no Sleep Condition X Diet X Injection interaction (F(1,93) < 0.001, p = 0.995). In females, results revealed a significant main effect of Sleep Condition (F(1,96) = 6.504, p = 0.012), such that the spleens of CSR mice weighed less compared to those of the HCC mice. Moreover, there was a significant main effect of Diet (F(1,96) = 12.888, p = 0.001), such that TAD mice had heavier spleens compared to standard chow mice. There was also a significant main effect of Injection (F(1,96) = 7.057, p = 0.009), such that mice injected with LPS had heavier spleens compared to mice injected with saline. Additionally, the Sleep Condition X Diet X Injection interaction was approaching significance (F(1,96) = 2.778, p = 0.099), such that, among TAD mice, CSR mice had lower spleen weights compared to HCC mice (See Figure 33B).

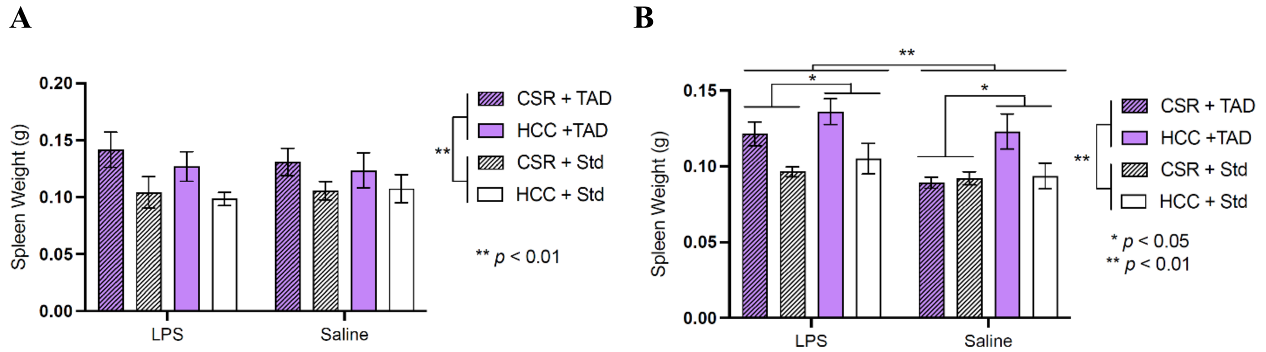

Figure 33. Spleen weight data. (A) Male mice that consumed the TAD had higher spleen weights compared to males that consumed the standard chow. (B) Female CSR mice had lower spleen weights compared to HCC females, female TAD mice had higher spleen weights compared to standard chow females, and LPS females had higher spleen weights compared to saline females. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 12–14.

Liver Weight Data

In males, there was a significant main effect of Diet ($F(1,93) = 5.618$, $p = 0.020$), such that TAD males had lower liver weights compared to standard chow males (See Fig. 34A). However, there was no Sleep Condition X Diet X Injection interaction ($F(1,93) = 0.036$, $p = 0.850$). In females, results revealed a significant Sleep Condition X Diet X Injection interaction ($F(1,96) = 4.342$, $p = 0.040$), such that, among the standard chow mice injected with saline, HCC mice had heavier livers compared to CSR mice ($p = 0.013$). Furthermore, there was a significant main effect of Sleep Condition ($F(1,96) = 4.517$, $p = 0.036$), such that the livers of the CSR mice weighed less compared to those of the HCC mice (See Fig. 34B).

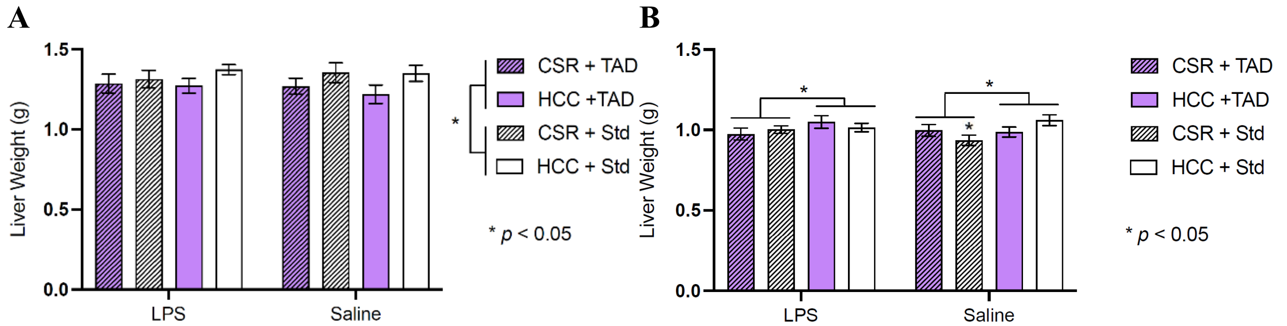

Figure 34. Liver weight data. (A) Male mice that consumed the TAD had significantly lower liver weights compared to males that consumed the TAD. (B) Female mice that underwent CSR exhibited lower liver weights compared to HCC females, and standard chow CSR females injected with saline had lower liver weights compared standard HCC females injected with saline. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 12–14.

White Fat Weight Data

One male mouse was excluded from white fat analysis due to the sample being dropped and compromised. Results revealed a significant main effect of Diet ($F(1,92) = 11.732$, $p < 0.001$), such that TAD mice had more white fat compared to Sleep Condition ($F(1,92) = 13.476$, $p < 0.001$), such that CSR mice had less white fat compared to HCC mice (See Fig. 35A). In female mice, results revealed a significant main effect of Sleep Condition ($F(1,96) = 25.204$, $p < 0.001$), such that CSR mice had less white fat compared to HCC mice (See Fig. 35B). However, there was no main effect of Diet in female mice ($F(1,95) < 0.001$, $p = 0.991$).

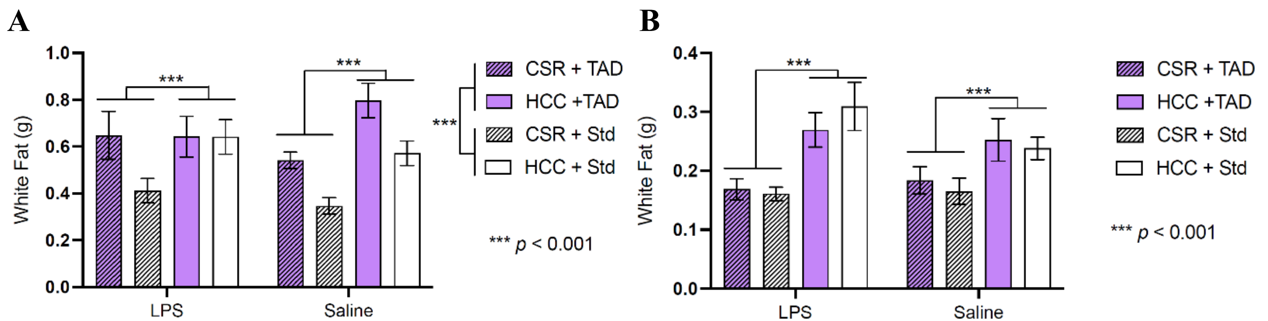

Figure 35. White fat weight data. (A) Male mice that consumed the TAD had more white fat compared to mice that consumed standard chow, and CSR males had less white fat compared to

HCC males. (B) Female mice that underwent CSR had less white fat compared to HCC females. Bars represent mean ± SEM. Significant differences ($p < 0.05$) are designated by *. N's = 12–14.

4. Discussion

The aim of Experiment 3 was to investigate the impact of a combination of lifelong TAD consumption and CSR in adulthood on anxiety-like behaviors, cognition, hippocampal BDNF mRNA, serum and hippocampal inflammation in response to an LPS injection, and organ sizes. Overall, we hypothesized deleterious effects of CSR and TAD independently, as well as significant interaction effects to suggest that the combination of TAD and CSR is especially harmful.

We hypothesized that CSR and TAD mice would have higher levels of inflammation in both the serum and in the hippocampus, and that this would be exacerbated in response to an LPS injection. Some of our hypotheses were supported, as male TAD mice had higher TNFα mRNA compared to standard chow mice, and female CSR mice had higher TNFα and IL-6 mRNA compared to controls. In addition, HCC TAD females that received saline had higher IL-10 mRNA compared to HCC TAD females, indicating a potential blunting of the anti-inflammatory response in females that consumed the TAD. There were no effects of CSR in any of the inflammatory variables in the hippocampus in response to LPS, which is not consistent with the results of Experiment 1.

It is possible that the study was statistically underpowered, as sample sizes for mRNA analysis were 6–9. It should be noted that more samples were collected, with 11–13 mice per group, but funds were limited at the time of sample processing. Future efforts in the lab could focus on exploring these variables in the remaining samples from Experiment 3. Another possible limitation of the rtPCR data analysis is that the BioRad CFX Connect machine does not report data for each individual sample, so there is no way to account for outliers. For example, it

appears that there may be an outlier in the male TNFα data in the HCC standard chow group, which could make those data difficult to interpret.

In serum, our hypothesis that TAD mice would demonstrate an enhanced inflammatory response to LPS was supported, as male TAD mice had exacerbated TNFα, IL-1β, IL-6, and IL-10 in response to LPS compared to mice that ate the standard chow, and female TAD mice had elevated TNFα, IL-1β, IFN-γ, and IL-10. These data are consistent with the work of Shamseldeen and colleagues (2021), who found an enhanced inflammatory response to LPS in rats that consumed a high-fat diet for 3 months. However, inconsistent with the current study, previous work has demonstrated that mice that consumed a high-fat diet for 5.5 months exhibited a blunted immune response in both the hippocampus and periphery four hours following one, intraperitoneal, 250µg/kg dose of LPS (Baumgarner et al., 2014). Additionally, prior research has demonstrated that chronic consumption of a TAD elevated inflammation in male APP_{swe} mice to levels equivalent to that of three injections of LPS, which is not consistent with the results of the current study, as TAD consumption alone did not elevate cytokine levels – main effects that were observed were largely driven by the LPS groups (Więckowska-Gacek et al., 2021). Furthermore, unlike Experiment 1 results, there were no consistent patterns of sex-specific effects on inflammation in Experiment 3. As stated previously, some of this inconclusiveness might be mitigated by increasing the sample size, which can be done for Experiment 3, as more samples were collected than were analyzed.

We hypothesized a significant decrease in BDNF mRNA in the hippocampus as a result of CSR and TAD consumption. The results of Experiment 3 were partially consistent with previous data showing that a combination of Western diet and CSR led to decreased BDNF in the hippocampus in rats (Alzoubi et al., 2013). Both male and female TAD mice had decreased

hippocampal BDNF mRNA compared to standard chow mice. However, the main effect of CSR on BDNF mRNA observed in Experiment 1 was not present in this study, possibly due to the addition of the diet variable.

For CFC, we hypothesized that CSR and TAD mice would demonstrate learning deficits indicated by decreased freezing behavior, and that the combination of these variables would exacerbate those deficits. Our hypotheses were partially supported, as female TAD mice froze less compared to standard chow mice, although the opposite effect was observed in male mice. Prior work in female mice found that three weeks of Western diet consumption induced contextual fear conditioning deficits compared to standard chow mice (Veniaminova et al., 2017). Conversely, high-fat diet consumption was associated with deficits in CFC in male, but not female mice (although female mice fed a control diet did not exhibit freezing behaviors indicative of learning, creating a potential floor effect for this sex difference in CFC performance) (Hwang et al., 2010). Further, in rats, Alzoubi and colleagues (2013) found that the combination of Western diet and 6 weeks of CSR induced cognitive impairment in a radial arm water maze task, indicating that the combination of poor diet and CSR should have exacerbated learning deficits.

We hypothesized that both TAD and CSR would elicit anxiety-like behavior in open field**.** This hypothesis was partially supported in female mice, as CSR was associated less time spent in the center zone of the arena, indicating anxiety-like behavior resulting from the CSR paradigm alone. Prior research has shown that sleep deprivation induces anxiety-like behavior in mice in the open field paradigm, indicated by less time spent in the center of the arena (Sakamoto et al., 2020; Yin et al., 2017). Interestingly, prior work has demonstrated sex differences in anxiety-like behavior in mice following 48 hours of sleep deprivation, with sleep-

deprived females displaying heightened anxiety-like behavior compared to sleep-deprived males, consistent with the current results (Gonzalez-Castañeda et al., 2016).

In the present study, CSR was associated with increased vertical counts, distance traveled, and decreased resting time in male mice, and increased vertical counts in female mice. Although Yin and colleagues (2017) utilized the MMPM of sleep loss, distance traveled was not affected. However, as that study used a total sleep deprivation procedure rather than a CSR paradigm, it might be difficult to compare these findings. Conversely, other work has shown that sleep deprivation and sleep fragmentation via placement on a running wheel over water led to increased exploratory behavior in an open field paradigm (Tartar et al., 2009). In the present research, it is possible that the sleep loss method used could have impacted locomotor activity in open field. The observed increase in activity could have resulted from the CSR mice being constrained on a platform for 10 hours each day. In addition, CSR mice may have more muscle mass, as the MMPM requires maintaining muscle tone, and body composition was not measured in this study. Alternatively, as activity was not monitored during the dark cycle, it is possible that the CSR mice experienced switched circadian rhythms, such that they became more active during the light cycle compared to the dark cycle and were habituated to increased activity during the time at which open field testing was performed. Additionally, inconsistent with prior findings that poor diet leads to increased anxiety-like behavior in rodents (Clark et al., 2022), there were no observed effects of TAD on behavioral measures in open field, with the exception of female HCC TAD mice exhibiting fewer vertical movements compared to CSR TAD females.

Our hypotheses that TAD and CSR would be associated with shorter colons were partially supported. Both male and female TAD mice had shorter colons compared to standard chow mice, consistent with literature demonstrating an association between Western diet

shortened colon length (Benninghoff et al., 2020). However, inconsistent with our hypothesis, CSR mice had longer colons compared to HCC mice, but only in females. Prior work has found no differences in colon length following sleep deprivation (Tang et al., 2009). However, since CSR alone did not appear to induce systemic inflammation, perhaps it is not surprising that our hypothesis that CSR would reduce colon length was not supported.

As hypothesized, mice that consumed the TAD had more white fat compared to mice that consumed the standard chow. However, CSR mice unexpectedly had less white fat compared to HCC mice, inconsistent with previous literature demonstrating that chronic sleep fragmentation increases visceral fat mass (Poroyko et al., 2016). This could be due to the nature of the CSR paradigm used. The MMPM requires animals to maintain muscle tone as they stay on the platforms during the sleep restriction period. This could cause the mice to build muscle mass and decrease fat mass. In this way, the MMPM may create a limitation for studying the effects of CSR on body composition. However, prior work has shown that mice subjected to 15 days of CSR via the MMPM, followed by 6 weeks of high-fat (30% kcal from fat) diet consumption, gained more weight compared to non-sleep-deprived mice fed the high-fat diet (de Oliveira et al., 2015). Moreover, it is possible that the mice in the CSR condition are consuming less food compared to HCC mice, as they may put less effort into obtaining energy and more effort into conserving it. Evidence has demonstrated that 5 days of REM sleep deprivation via the MMPM in rats led to decreased motivation for a food reward in an operant task, despite weight loss during sleep deprivation (Hanlon et al., 2005). However, food consumption was not measured during the sleep restriction portion of the Experiment, as too much food – particularly the standard chow – is wasted during the sleep restriction procedure in the water of the MMPM cages to reliably assess differences in food consumption between CSR and HCC mice.

Nonetheless, based on the initial weight loss observed across diets in the CSR groups in Experiments 1 and 2, it is not surprising that both male and female CSR mice had less white fat across diet groups in Experiment 3.

Liver weights were smaller in female CSR mice compared to female HCC mice, although this difference was not observed in males. This could be due to a decrease in cell size or number as a result of CSR (Balestrieri et al., 1980). Prior work has shown that sleep loss induces liver inflammation, although that was not measured in the current study (Pandey & Kar, 2011). In TAD males, liver weights were smaller compared to standard chow males, inconsistent with previous data showing that 7 weeks of Western diet consumption is associated with increased liver weight (Schierwagen et al., 2015). Consistent with prior findings, the current study demonstrated an association between CSR and decreased spleen weights in CSR female mice, although, again, this difference was not observed in males (Balestrieri et al., 1980). Furthermore, both male and female TAD mice also exhibited lower spleen weights compared to standard chow mice. Prior work demonstrated a decrease in spleen and liver weight in mice consuming a diet-induced obesity chow (44.8% kcal from fat, 36.2% kcal from carbohydrates), but not in a total Western diet chow (34.5% kcal from fat and 50.1% kcal from carbohydrates), highlighting the impact that differences in diet composition may have on organ weights in rodent studies (Monsanto et al., 2016). Taken together, the organ data in Experiment 3 suggest that there were physiological changes to vital organs as a result of lifelong TAD consumption and CSR.

As noted in the methods section, PFC was collected in a lysis buffer cocktail and stored at -80ºC. Our lab aims to obtain funding in the near future to purchase Aβ ELISAs and materials needed to quantify levels of Aβ in those PFC samples to assess the impact of lifelong TAD consumption and CSR on PFC Aβ.

Chapter 5: GENERAL CONCLUSIONS

AD cases are projected to rise significantly in the years ahead, with over 6.5 million people suffering from the disease in the U.S. alone, and no effective treatment is available. The cost of care for patients with AD is well over $300 million dollars per year, and will continue to increase annually (Alzheimer's Association, 2023). As the etiology of AD remains largely unknown, and with so many individuals suffering from AD, it is imperative that we gain an understanding of the causal factors in AD pathogenesis. Of the risk factors identified, many are modifiable, or controllable, aspects of one's lifestyle, such as sleep and diet. In the present research, chronic sleep loss was investigated, individually and in combination with two different timelines of chronic consumption of a typical American-style diet, in order to elucidate the mechanisms through which AD risk is enhanced.

There is a considerable gap in the literature exploring multiple AD risk factors at one time in rodent studies. It is important to consider that humans are likely subjected to more than one risk factor of AD (i.e., lack of exercise, poor diet, chronic stress, and chronic sleep loss). Furthermore, far too few rodent studies of AD utilize both male and female animals, potentially missing important sex differences in the relationships between these lifestyle components and AD risk. The current studies attempted to address these gaps in the literature by combining poor diet and chronic sleep loss, and by utilizing both male and female mice. Exploring sex-specific differences in inflammatory response and AD-like markers can provide new insights into the contributions of lifestyle factors such as poor diet and chronic sleep loss to AD risk. Future research efforts should include both male and female animals to assess where sex differences may exist, as these important data could help elucidate the complexities underlying the sex differences in AD.

Experiment 1 data demonstrated the CSR significantly decreases BDNF mRNA in the hippocampus in male mice compared to controls. Furthermore, male and female mice exhibited a blunted inflammatory response to an LPS injection in the hippocampus following CSR, demonstrating a potential harmful effect of CSR on immune function. Additionally, sex differences in cytokine response to an LPS injection following CSR were observed, such that male CSR mice showed exacerbated serum TNFα levels in response to LPS, while female CSR mice demonstrated blunted serum TNFα in response to LPS. Similar patterns were observed for IL-1β and IL-10, although the effect of sex was not statistically significant when analyzed together.

Results of Experiment 2 demonstrated that 12 weeks of TAD consumption was sufficient to increase Aβ in the hippocampus and PFC in both male and female C57BL/6 mice that were not genetically prone to AD-like pathology. Furthermore, in male mice, those that consumed the TAD had increased serum levels of TNFα, IL-1β, and IL-10 compared to standard chow mice. Although female TAD mice only exhibited increased serum IL-10 compared to standard chow mice, it is important to note that these observed increases in serum cytokines were due to the TAD consumption alone, as no LPS was administered in Experiment 2.

Experiment 3 results showed that lifelong TAD consumption in female mice was associated with increased anxiety-like behavior in the open field paradigm compared to standard chow consumption. Furthermore, TAD consumption led to cognitive impairment in CFC and decreased hippocampal BDNF mRNA in both male and female mice. Moreover, among females, CSR mice injected with LPS had higher TNFα and IL-6 mRNA compared to CSR mice injected with saline, inconsistent with Experiment 1 results. However, among saline-injected females, CSR TAD mice had lower IL-10 mRNA compared to HCC TAD mice, potentially indicating

blunted IL-10 expression in mice that consumed TAD and underwent CSR. Furthermore, in females, TAD was associated with diminished IL-10 response to LPS among HCC females compared to IL-10 levels observed in saline-injected females that consumed TAD.

More effects of TAD were observed in the serum data compared to the mRNA data, as TAD was associated with exacerbated pro- and anti-inflammatory cytokine responses to LPS in the periphery compared to standard chow in both male and female mice. Additionally, TAD mice that underwent CSR showed a diminished response to LPS compared to TAD HCC mice, indicating a potential diminishing effect of CSR, as observed in Experiment 1. Given the Experiment 1 data demonstrating blunted central inflammation in both males and females, diminished peripheral inflammation in females, and exacerbated peripheral inflammation in males in response to LPS after CSR, it may have been difficult to distinguish differences in LPS-induced inflammation due to CSR or TAD consumption in Experiment 3. Overall, the two variables appeared to exert different effects on the inflammatory response to LPS, and these effects may also differ between males and females.

Finally, analysis of peripheral organs in Experiment 3 suggested that TAD consumption led to shortened colons and higher spleen weight in both male and female mice, while only male mice demonstrated lower liver weight and more white fat as a result of TAD consumption. Moreover, CSR was associated with decreased white fat in both males and females, and decreased liver weight in females. Additionally, LPS was associated with shortened colons in males and higher spleen weight in females.

One limitation of the current research is the age of the animals studied. It is likely that age has sex-specific impacts on immune function (Dockman et al., 2022). For example, previous work reported that LPS was associated with a greater increase in proinflammatory cytokines in

adult mice in comparison to pubertal mice, while there was a greater increase in anti-inflammatory cytokines in pubertal mice compared to adult mice (Cai et al., 2016). As the mice utilized in the current experiments were all young-to-middle-aged, important age-related sex differences in response to LPS under poor diet and sleep conditions could have been missed. Future research should explore age as a factor in these relationships, testing immune responsivity in young, middle-aged, and old mice.

Another limitation of Experiments 2 and 3 is that the two diets are not macronutrient matched. This was reflected in the food consumption data for Experiments 2 and 3 in both males and females, as TAD mice consumed less food compared to standard chow mice. As the standard chow is relatively low in fat, it is likely that the standard chow mice had to consume more compared to the TAD mice to have matched energy. However, the majority of studies exploring the impact of a Western-style diet or TAD utilize a standard rodent chow similar in macronutrient densities to that of the present study, and these standard rodent chow formulations are typically lower in fat and higher in carbohydrates (Tuck et al., 2020). An additional limitation of these studies was that the estrous cycle was not monitored in female mice. This could potentially have had an impact on the inflammatory markers, as evidence has shown increased inflammation in response to ozone exposure in female mice during the follicular phase compared to the luteal phase (Fuentes et al., 2019).

Taken together, the results of Experiments 1–3 provide evidence for sex-specific patterns in cytokine responses under sleep-restricted conditions, and for the consideration of both typical American-style diet consumption and chronic sleep loss as deleterious to human health and as potential risk factors for AD. These findings demonstrate the necessity for future research efforts exploring how chronic sleep loss and chronic poor diet consumption might be harmful to health

in aging, and how they might initiate or exacerbate AD. As the prevalence of AD continues to increase, and as poor diet and sleep habits are becoming increasingly common, it is vital to answer the questions about how these lifestyle factors might initiate or exacerbate this devastating neurodegenerative disease.

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Personal Background

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ABSTRACT

EXPLORING CHRONIC SLEEP LOSS AND TYPICAL AMERICAN-STYLE DIET AS RISK FACTORS FOR ALZHEIMER'S DISEASE

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Alzheimer's disease is the most common cause of dementia, currently afflicting over 44 million people worldwide. This number is projected to increase significantly in the coming years, yet much about the disease's etiology remains a mystery, and no cure or preventative measures exist. Two potential risk factors for Alzheimer's disease are chronic sleep loss and long-term consumption of an unhealthy, American-style diet. Harmful effects of both of these lifestyle factors on brain health, immune function, and cognition are often studied separately, but rarely in conjunction with one another. Collectively, the present studies investigated the impact of chronic sleep restriction and typical American-style diet consumption on cognitive impairment, anxiety-like behavior, central and peripheral inflammation and response to one intraperitoneal LPS injection, and amyloid-beta in wild-type mice. Alone, chronic sleep restriction was associated with sex-dependent changes in the inflammatory response to an intraperitoneal LPS injection. Typical American-style diet consumption was associated with increases in basal inflammation, as well as exacerbating the inflammatory response to an LPS injection. Further, consumption of the typical American-style diet was associated with cognitive impairment in females and increased amyloid-beta in the hippocampus and prefrontal cortex in males. Additionally, chronic sleep loss was associated with cognitive impairment in males. Given the increasing prevalence of Alzheimer's disease, understanding how modifiable components of lifestyle can increase the risk of developing this devastating disease is essential.