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Acute Sleep Fragmentation Induces Tissue-Specific Changes in Cytokine Gene Expression and Increases Serum Corticosterone Concentration

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ACUTE SLEEP FRAGMENTATION INDUCES TISSUE-SPECIFIC CHANGES IN CYTOKINE GENE EXPRESSION AND INCREASES SERUM CORTICOSTERONE CONCENTRATION

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I dedicate this thesis to my parents, Pam and Tom Dumaine, who continue to encourage and inspire me to complete everything to the best of my ability. Also, I dedicate this work to my grandfather, Charles Edward Rust Sr., who taught me the importance of hard work and showed me how to face adversity courageously and with a smile, even until the end. Lastly, I dedicate this work to Dr. Darren Rubino, Dr. Luke Starnes, Dr. Walter Bruyninckx, Dr. Daryl Karns, Dr. Steve Steiner, and Dr. Craig Philipp from the Departments of Biology and Chemistry at Hanover College, as they introduced me to research and helped me uncover my true passion.

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Sleep fragmentation induces acute inflammation and increases glucocorticosteroids in vertebrates. Obesity and sleep fragmentation are often concurrent pro-inflammatory conditions in patients with obstructive sleep apnea. Despite the association between the two, their simultaneous effects on immune and endocrine profiles have not been explored. In the first experiment, we investigated changes in pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (TGF-β1) cytokine gene expression in the periphery (liver, spleen, fat, and heart) and brain (hypothalamus, prefrontal cortex, and hippocampus) in mice exposed to various intervals of sleep fragmentation. Serum corticosterone concentration was also assessed. Sleep was disrupted in male C57BL/6J mice using an automated sleep fragmentation chamber (Lafayette Industries), which involved movement of a sweeping bar at specified intervals. Mice were exposed to bar sweeps every 20 sec (high sleep fragmentation; HSF), 120 sec (low sleep fragmentation; LSF), or the bar remained stationary (control). Trunk blood and tissue samples were collected after 24 h of SF. It was found that HSF is a potent inducer of inflammation in the periphery (IL-1β: adipose, heart, and hypothalamus), but leads to upregulation of anti-inflammatory cytokines in the brain (TGF-β1: hypothalamus and hippocampus), despite elevated serum corticosterone. Due to the association between obesity and SF, this experiment was replicated in male C57BL/6J mice (lean) and ob/ob KO mice (obese)

using the previously described methods. We predicted the acute inflammatory response resulting from HSF would be different for the lean and the obese mice, with the greatest cytokine gene expression levels in the OB HSF group, due to a summative effect of the pro-inflammatory conditions. Obesity was the factor that most affected cytokine gene expression profiles. Additionally, the pro- vs anti-inflammatory gene expression profile varied with tissue type. While obesity resulted in neuroinflammation (hypothalamus, prefrontal cortex, hippocampus), it led to decreases in pro-inflammatory cytokine gene expression in the periphery (spleen, fat, heart). Serum corticosterone concentration was significantly elevated due to SF, but was not affected by obesity. As a result, the obese mice likely had neuroendocrine adaptations to combat the pre-existing pro-inflammatory condition of obesity, which impacted the acute inflammatory response to sleep loss.

INTRODUCTION

Sleep is a conserved physiological process that consumes a large portion of mammalian life spans, although its precise function has produced considerable debate. A recent study has suggested that time spent sleeping promotes the clearance of metabolic wastes from the brain through the cleansing of cerebral spinal fluid (Xie et al., 2013). A reduction in the amount of time spent sleeping curtails this cleansing period, which could result in well-documented impairments in learning and memory, as well as an elevated risk in developing chronic disease associated with inflammation, such as cardiovascular disease, insulin resistance, and obesity (Graves et al., 2003; Markwald et al., 2013; Zielinski et al., 2013). Emerging evidence indicates that sleep quantity and quality facilitate the maintenance of neurocognitive function and health, which ultimately contribute to lower morbidity and mortality rates (Simpson and Dinges, 2007). In recent years, fewer people are getting the suggested 7 to 9 hours of sleep a night, likely due to increases in shift work labor, sleep loss, or sleep disordered breathing associated with the worldwide rise in obesity. Since sleep influences memory and cognitive ability, hormone secretion, glucose metabolism, immune function, core body temperature, and renal function, adverse health outcomes have been observed after chronic sleep disturbance (Nair et al., 2011; Simpson and Dinges, 2007; Zielinski et al., 2013). Ample experimental evidence has demonstrated that disrupted sleep contributes to hypertension, diabetes, obesity, depression, and stress, all of which are linked to pro-inflammatory conditions (Naidoo, 2012).

Under normal conditions, the immune system varies throughout the day in conjunction with the sleep-wake cycle. Immune cells in the blood exhibit maximum levels in the early evening and reach minimum circulating levels in the morning (Simpson and Dinges, 2007). Correspondingly, pro-inflammatory cytokine levels coincide with this cycle and approach maximum levels at night, most often times during slow wave sleep (Besedovsky et al., 2012; Simpson and Dinges, 2007). The increase in pro-inflammatory cytokines at the beginning of rest is hypothesized to be a result of the accumulation of endogenous 'danger signals' during waking; however, this is only a proposed mechanism (Besedovsky et al., 2012). Because of the fluctuation in cytokine gene expression, amount and quality of sleep have important effects on immunological integrity, such that disruption of the normal sleep wake cycle results in altered immune function in humans and other mammals. In a normal immune response, an acute inflammatory response induces activation of an immune cascade in response to perceived damage or infection to generate an antigen specific response. Detection of pathogen or danger associated molecular patterns (PAMPs or DAMPs) occurs by means of binding of ligands to the leucine rich repeat domains of toll-like receptor proteins (TLRs) expressed on macrophages or other immune cells (Murphy, 2012). Binding to the TLR induces dimerization of the receptors and a signaling cascade that ultimately brings about the production of pro-inflammatory cytokines, such as Interleukin-1β (IL-1β), Tumor Necrosis Factor alpha (TNF-α), and Interleukin-6 (IL-6), via the Nuclear Factor Kappa B (NF_KB) pathway (Murphy, 2012).

There is growing evidence demonstrating that sleep is influenced by hormones and peptides, known as sleep regulatory substances (SRSs). SRSs are chemicals of

varying molecular sizes that act as neurotransmitters, neuromodulators, or neurohormones to participate in the generation and maintenance of the sleep-wake cycle (Garcia- Garcia et al., 2014). Current research holds that a number of molecules may play a role in sleep regulation, including: IL-1β, TNF-α, growth factor releasing hormone, prolactin, and nitric oxide (Garcia- Garcia et al., 2014). Experimentally, it has been demonstrated that the SRS peptides accumulate within the brain and cerebrospinal fluid (CSF) during prolonged wakefulness, such that CSF or brain extracts taken from a sleep deprived animal promote sleep when injected into a non-sleepdeprived animal (Clinton et al., 2011; Garcia – Garcia et al., 2009; Obal and Krueger, 2003). In addition to having pro-inflammatory roles as previously described, IL-1β and TNF-α also function as sleep regulatory substances (SRS) released by activated microglia that help regulate non-rapid eye movement sleep (Baracchi and Opp, 2008; Clinton et al., 2011). In one proposed mechanism of SRS function, IL-1β and TNF-α are released from glia in response to extracellular ATP. The cytokines then bind to receptors on neurons, resulting in neuromodulator and neurotransmitter receptor up and downregulation, ultimately bringing about alterations in neuronal excitability and function to promote state changes in the local neuronal network (Jewett and Krueger, 2012). Supporting this mechanism is previous experimental evidence that has shown elevated IL-1β expression in the mouse brain following sleep deprivation; however, it must be noted that the expression of TNF-α has varied depending upon the study (Chennaoui et al., 2011; Weil et al., 2009; Zielinski et al., 2014). Additional experiments have expanded upon this idea and have shown the injection of IL-1β and TNF-α in animal models increases the amount of time spent in NREM sleep (Baracchi and Opp, 2008). Growing evidence supports that

recurrent sleep interruptions induce excessive daytime sleepiness via activation of inflammatory mechanisms in a TNF-α dependent pathway (Ramesh et al., 2012). Furthermore, because of the role of IL-1β and TNF-α as SRSs, these molecules can promote NREM sleep and induce symptoms of sleep loss, such as sleepiness, fatigue, and poor cognition (Jewett and Krueger, 2012).

Following a period of sleep curtailment, upregulation of the immune response results, but is not associated with pathogenic challenge (Brager et al., 2013). Sleep loss does not need to be drastic to induce effects on the immune system, as one study demonstrated that with only 4-8 hours of sleep deprivation, there was a significant decrease in natural killer (NK) cell activity (Simpson and Dinges, 2007). Other studies have shown increases in the pro-inflammatory cytokines after just one night of insufficient sleep (van Leeuwen et al., 2009). The non-specific host immune response is characterized by an increase in the number of leukocytes, monocytes, granulocytes, increased cytotoxicity of the NK cells, and decreases in immunoregulatory cytokines, such as IL-4 and IL-10 (Hirotsu et al., 2012; Mullington et al., 2010; van Leeuwen et al., 2009). In addition to an increase in the number of immune cells, there is also activation and translocation of NF_KB in specific brain regions associated with sleep regulation (Kim et al., 2011). This then leads to the production of pro-inflammatory cytokines. There have been numerous cytokines and chemokines hypothesized to affect sleep, including; IL-1α, IL-1 β, IL-2, IL-4, IL-6, IL-8, I-10, IL-13, IL-15, IL-18, TNF-α, TNF-β, IFN-α, IFN-β, IFN-β and macrophage inhibitory protein 1b (Kim et al., 2011). However, of these, experimental models have repeatedly shown increases in expression of the pro-inflammatory cytokines IL-1β and TNF-α in the brain following sleep deprivation

(Ashley et al., 2013; Kaushal et al., 2012b; Majde and Krueger, 2005). In the periphery, increases are observed in IL-1β, IL-6, and TNF-α gene expression (Ashley et al., 2013; Brager et al., 2013). More recently, IL-17 has been identified as another pro-inflammatory cytokine upregulated by sleep restriction (van Leeuwen et al., 2009). Furthermore, it has also been demonstrated in human vaccination experiments that sleep plays a critical role in the formation and maintenance of immunological memory, such that sleep loss results in diminished antibody production (Besedovsky et al., 2012). Although immunoregulatory cytokines such as IL-4 and IL-10 have been studied extensively in relationship to sleep deprivation, TGF-β1 expression, an anti-inflammatory cytokine involved in the regulation of cell growth, proliferation, differentiation, and apoptosis, remains largely unstudied in this context (Murphy, 2012).

Because many pro-inflammatory cytokines are present following sleep alterations, it is understood that insufficient sleep leads to initiation of an acute inflammatory response. However, the mechanism underlying the induction of an inflammatory response has yet to be elucidated. One possible hypothesis includes the reaction to an increase in endogenous danger signals, such as reactive oxygen species, nucleotides, and heat shock proteins, released as a result of various cellular stresses from physical activity, metabolism, and cell injury during the active wake cycle (Besedovsky et al., 2012). Additional experimental results highlight TLR4 as being involved in the inflammatory response to sleep loss (Wisor et al., 2011). TLR4 initiates a signaling cascade in response to detection of lipopolysaccharide (LPS) or endogenous ligands, then results in an increased release of pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β) and other pro-inflammatory agents, such as nitric oxide, cyclooxygenase, prostaglandin E2, and

adenosine triphosphate (Wisor et al., 2011). Because all of these molecules are known to play roles in regulating the timing of sleep/wake cycles, it helps support the proposed relationship between inflammation modulated by TLR4 and sleep loss. In addition, there is upregulated expression of TLR-2 and TLR-4 in patients with obstructive sleep apnea (Akinnusi et al., 2013). However, subsequent investigations to further elucidate this mechnism are lacking. In lieu of this deficiency, additional studies have utilized inflammation mediated by TLR4 in response to sleep loss as a model to show increased pathogenesis as a result of inflammation following sleep loss. It has been demonstrated that within the context of cancer, fragmented sleep accelerates tumor growth and progression through TLR4 signaling (Hakim et al., 2014). Furthermore, TLR4 expression on macrophages is upregulated in obstructive sleep apnea patients before administration of breathing treatment (Akinnusi et al., 2013). Additional experimentation demonstrated that TLR4 signaling changes with age, as there is a decrease in TLR4 mediated inflammation to sleep loss in adults in comparison to young people (Carroll et al., 2015). Because inflammation mediated by TLR4 signaling following sleep interruptions in each of these conditions exacerbates disease pathology, these experiments support the hypothesis that inflammation following sleep curtailment involves TLR4 signaling.

Not only does insufficient sleep lead to an increase in the number of immune cells and an upregulation in cytokine gene expression, but it also stimulates changes in other protein and hormone levels. Because sleep deprivation is associated with the development of chronic disease, such as cardiovascular disease and diabetes, recent studies have investigated the association between sleep loss and C-reactive protein (CRP) levels (van Leeuwen et al., 2009). CRP is an acute phase protein, an inflammatory signal

released by the liver that is commonly used as a marker indicating cardiovascular risk (Murphy, 2012). It has been observed that CRP levels increase with both partial and total sleep deprivation (Simpson and Dinges, 2007). Consequently, this suggests that sleep alteration may result in elevated risks for developing cardiovascular disease.

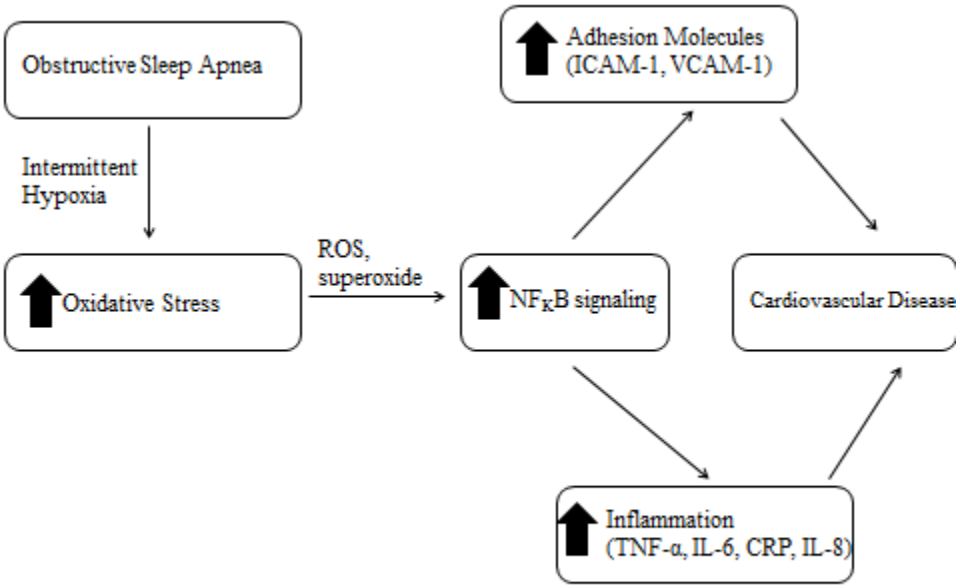

Figure 1. Schematic diagram showing the proposed links between sleep fragmentation of obstructive sleep apnea and the onset of cardiovascular disease (Modified from Pak et al., 2014).

Additionally, it has been demonstrated in mice that chronic sleep fragmentation induces endothelial dysfunction through elastic fiber disruptions in thoracic and arch regions of the aorta (Carreras et al., 2014). Compounding the risk for the onset of cardiovascular disease is the fact that chronic sleep fragmentation, attributable to bstructive sleep apnea, leads to an increase in the expression of cell adhesion molecules, such as ICAM-1 and VCAM-1, which then help contribute to the endothelial dysfunction

(Pak et al., 2014). Cardiovascular disease may arise following chronic periods of sleep interruptions due to the increase in CRP levels, the disruption of vessel wall integrity, and the increase in cell adhesion molecules, all of which help favor the progression towards atherosclerosis.

More recently, it has been found that insufficient sleep affects food intake, as sleep loss results in alterations in satiety hormone concentrations that can ultimately result in weight gain. Results indicate that concentrations of ghrelin, a hunger signaling hormone, and leptin and peptide YY, satiety signaling hormones, are changed following insufficient sleep (Garcia-Garcia et al., 2014). Following sleep alterations, caloric intake increases in conjunction with rises in circulating ghrelin and corresponding decreases in leptin and peptide YY concentrations (Garcia-Garcia et al., 2014; Markwald et al., 2013; Mavanji et al., 2012). The increase in food intake following sleep deprivation is thought to be in response to elevated energetic demands due to prolonged wakefulness; however, because caloric expenditure does not change, and because glucose clearance and insulin sensitivity in adipocytes decrease with time following sleep restriction, the surplus calories become stored as fat (Markwald et al., 2013; Wang et al., 2014; Zhang et al., 2014). Whereas increased food intake leads to weight gain in chronic sleep fragmentation studies, previous experimentation also demonstrated that rodents experience a decrease in weight until day 7 of a chronic sleep fragmentation experiment, indicating the experimental time period may have some effect on this observation (Baud et al., 2013). Other literature indicated that even though altered glucose homeostasis is observed following two weeks of chronic sleep fragmentation, body weight changes are absent;

thereby suggesting the period of chronic sleep interruptions must exceed two weeeks before weight changes are apparent (Carreras et al., 2014).

Obesity is described as a pro-inflammatory condition; however the underlying cause of this inflammation has not yet been determined. Previous literature has attributed the increase in pro-inflammatory cytokines to cytokine recruitment following macrophage infiltration into adipose tissue (Chawla et al., 2011). Macrophages are phagocytic cells of the immune system that ingest cellular debris and pathogens. The engulfment of particles by macrophages can result in the stimulation of lymphocytes and other immune cells to respond to the presence of a pathogen (Murphy, 2012). It is now known that there are two distinct subsets of macrophages, each of which has its own function in the inflammatory response. The M1 macrophages, the classically activated macrophages, are stimulated by the presence of Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) as detected by Toll Like Receptors (TLRs) or by the presence of T_H1 type cytokines, such as IFN-γ (Chawla et al., 2011). M1 macrophages promote inflammatory responses through the secretion of pro-inflammatory cytokines, such as IL-6 and TNF-α (Murphy, 2012). Conversely, the M2 macrophages are alternatively activated macrophages that are activated by T_H2 cytokines, such as IL-4 and IL-13 (Chawla et al., 2011). These macrophages are associated with tissue repair and the down regulation of inflammatory responses (Murphy, 2012).

There is greater polarization towards M1 macrophage infiltration in the adipose tissue of obese individuals, whereas M2 macrophages are found in the adipose tissue of lean individuals (Chawla et al., 2011). Similarly, it has been shown in an experimental model of chronic sleep fragmentation that there is greater polarization towards the M1

macrophage subtype in visceral white adipose tissue following sleep fragmentation, leading to a skewed M1/M2 subtype ratio (Carreras et al., 2015). This difference suggests that the increased infiltration of M1 macrophages into adipose tissue is associated with the increase in pro-inflammatory cytokine expression. Further investigations into the maintenance of metabolism have elucidated potential mechanisms by which obesity and inflammation result in insulin resistance. Recent literature has suggested that superoxide and reactive oxygen species generated by the nicotinamide adenine dinucleotide phosphate oxidase-2 (NOX-2) enzyme in macrophages are the causative agents of tissue damage and inflammation, ultimately leading to systemic and adipose tissue insulin resistance (Zhang et al., 2014). Because the activity of the NOX-2 enzyme increases following sleep fragmentation, the damage due to oxidative stress results in glucose intolerance and reduced insulin sensitivity with time of exposure to sleep fragmentation (Zhang et al., 2014).

The combination of the oxidative stress induced by NOX-2 activity and polarization towards the M1 macrophage subtype leads to a persisting, non-specific inflammatory response characterized by an increase in pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNF-α). If the inflammatory condition persists, then tissue damage will result from the cytotoxic products of the inflammatory state. The cell-mediated products recruited by the cytokines of chronic inflammatory responses have been implicated in the development of diseases such as hypertension, diabetes, and obesity, all of which have been observed as adverse consequences of sleep curtailment (Murphy, 2012).

Furthermore, curtailment of the sleeping period results in an increase in stress hormones (Neto et al., 2010). In addition to activating the sleep homeostatic process,

sleep deprivation models also activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which then acts on the adrenal cortical cells to bring about an increase in glucocorticoids in the circulation (Mongrain et al., 2010). The increase in stress hormone levels (corticosterone for rodents and cortisol in humans) has the potential to influence the resulting hormone levels, as corticosteroids have an anti-inflammatory effect at chronic levels (Miyata et al., 2015; Mongrain et al., 2010).

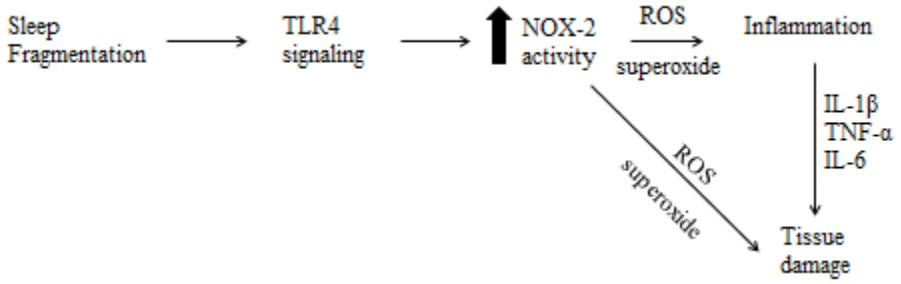

Figure 2. Schematic illustration of the proposed mechanism for tissue damage by means of the increased activity of NOX-2 following sleep fragmentation.

The majority of studies investigating the immunomodulatory role of sleep have utilized a total sleep deprivation method in mice and humans, in which the test subject is prevented from sleeping for an extended period of time (Ashley et al., 2013; Baracchi and Opp, 2008; Brager et al., 2013: Hirotsu et al., 2012). However, a compounding factor when using this model is stress induced by the sleep deprivation process itself. When compared to total sleep deprivation, the sleep fragmentation model induces lower serum corticosterone concentrations within the model organism, ultimately better simulating the result of a single night of interrupted sleep experienced by patients with sleep apnea and

other sleep disorders (Kaushal et al., 2012b). Over time, the acute effects of sleep fragmentation become chronic effects, thereby contributing to the chronic exposure to inflammatory compounds observed in patients with sleep apnea. As a result, there is a need to better characterize the initial response to sleep interruptions and the link it has with the progression to pathology over time, such that measures can be taken to prevent this progression from occurring. Although there is evidence linking sleep deprivation to an elevated immune state, there has been little investigation into whether intermittent sleep can diminish the acute inflammatory response initiated by sleep loss. Previous research has shown that sleep homeostatic responses associated with acute total sleep deprivation cannot be generalized to the response associated with acute sleep fragmentation, due to the fact that sleep loss in sleep fragmentation is markedly smaller (Ramesh et al., 2009). For this reason, the sleep fragmentation model has been chosen for these studies.

Recently, there has been doubt cast upon the accuracy of using murine models for human inflammatory diseases. It has been shown that genomic responses in mouse models poorly mimic human inflammatory diseases, particularly in trauma and burn conditions (Seok et al., 2013). Despite this concern, for sleep deprivation studies, it has been repeatedly shown that the murine model is the closest approximation of the human inflammatory response resulting from sleep deprivation (Baracchi and Opp, 2008; Brager et al., 2013; Majde and Krueger, 2005). Thus, for the purpose of this study, the murine model persists as the most reliable model for the human immune response, apart from using human subjects.

The aim of this study was to examine the effects of acute sleep fragmentation on pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (TGF-β1) cytokine gene expression in brain and peripheral tissue of a murine model, as well as serum corticosterone concentration, to determine whether the degree of sleep fragmentation alters the development of an inflammatory response to sleep loss. IL-1β and TNF-α were chosen as the pro-inflammatory cytokines of interest for this experiment due to the known roles as major pro-inflammatory cytokines and also as sleep regulatory substances affecting the sleep wake cycle (Jewett and Krueger, 2012). TGF-β1 was chosen because of its role as an anti-inflammatory cytokine that is widely expressed in many tissues. The majority of previous sleep studies have investigated IL-10 gene expression; however, TGF-β1 was chosen such that novel information may be uncovered. Recent literature supports selecting these cytokines, as it has been demonstrated that both TNF-α and TGF-β1 play a role in the regulation of clock gene expression, implicating them as effectors of circadian gene expression (Lopez et al., 2014).We hypothesized that mice exposed to a "high" sleep fragmentation model (HSF), with sleep disruptions occurring every 20 seconds, would exhibit an increase in pro-inflammatory cytokine gene expression (IL-1β and TNF-α), an increase in serum corticosterone concentrations, and a decrease in anti-inflammatory cytokine (TGF-β1) gene expression in both the brain and periphery. Mice experiencing a lower frequency of sleep fragmentation (LSF), with sleep interruptions occurring every 2 minutes, were also hypothesized to display upregulated pro-inflammatory cytokine gene expression, downregulated anti-inflammatory gene expression, and increased serum corticosterone concentrations; however, these levels were predicted to be intermediate between those of control and HSF groups.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (n = 35) were housed in a colony room (12 h light/ 12 h dark, lights on 07:00 h; 21°C ± 1°C) at Western Kentucky University. After weaning at 21 d of age, mice resided in polypropylene cages with same sex littermates and were provided corncob bedding, as well as food, and water *ad libitum*. This study was conducted under approval of the Institutional Animal Care Committee at Western Kentucky University and procedures followed the National Institutes of Health Guide for the Use and Care of Laboratory Animals and international ethical standards.

Sleep fragmentation

Adult(>8 weeks of age) male mice were selected for experimentation and were briefly exposed to isoflurane vapors and weighed to the nearest 0.01 g. Mice ranged in age from 23 weeks – 33 weeks. Four mice were then placed in an automated sleep fragmentation chamber (Lafayette Industries, Lafayette, IN USA; Model 80391) with a thin layer of corn cob bedding at 09:00 h. Food and water were provided *ad libitum*. To distinguish among subjects, tails were marked with a different colored pen (Sharpie). At 8:00 the next day, the swipe bar was activated. The chamber (9.625”L x 8.25”W x 7.75”H) resembled a typical polypropylene cage, with the addition of an automated swipe bar that moves horizontally across the cage at set intervals. Thus, this interval reflects a state of sleep fragmentation rather than sleep deprivation and will be referred to as “high” sleep fragmentation (HSF) when set at 20 seconds and “low” sleep fragmentation (LSF) when set at 120 seconds (Ramesh et al., 2009; Ringgold et al., 2013). For the control group, the

bar remained stationary. Thirty-six mice were randomly assigned to either control (n = 12), LSF (n = 11), or HSF (n = 12) groups. One mouse was removed from the LSF group because it climbed into the food hopper unexpectedly and was found asleep there the following morning.

After HSF, LSF, or control conditions for 24 h, animals were deeply anesthetized using isoflurane vapors (< 2 min), weighed, and then rapidly decapitated within 3 min of initial handling. Trunk blood was collected, kept on ice for < 20 min, and then spun at 3000 x g for 30 minutes at 4°C. The serum was drawn off and stored at -80°C for later ELISA analysis. Liver, spleen, inguinal fat, and heart samples were collected and placed into Eppendorf tubes containing RNAlater solution (Life Technologies). The tissue samples were stored at 4°C until RNA extraction was performed. The hypothalamus, prefrontal cortex, and hippocampus were then dissected from the brain and stored in RNAlater at -20°C until RNA extraction. Target tissues were chosen based upon their known roles in immune function, sleep, and involvement in the pathologies associated with chronic sleep fragmentation.

RNA Extraction

RNA was extracted from brain, liver, spleen, and fat using an RNeasy Mini Kit (Qiagen). RNA was extracted from the heart using an RNeasy Fibrous Tissue Mini Kit. RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Reverse Transcription

Total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, #4368813). The total RNA concentration of the tissue was diluted (brain: 9.2 and 10.1 ng/µL; liver, spleen, and fat: 10.2 ng/µL; heart: 22.6 ng/µL). The reaction was carried out according to the manufacturer's instructions. The amplification conditions for the thermocycler were 25°C for 10 min, 37°C for 120 min, 85°C for 5 min (for 1 cycle), and then 4°C .

RT-PCR

Real Time- PCR was performed using an ABI 7300 RT-PCR System. To determine relative cytokine gene expression, Taqman Gene Expression RT-PCR Master Mix and the following primer/probes (Applied Biosystems) were used: IL-1β (Mm00434228_m1), TNF-α (Mm00443260_g1), and TGF-β (Mm01178820_m1), or 18s endogenous primer-limited (Mm03928990_g1). Cytokine probes were labeled with the fluorescent marker 5-FAM at the 5' end and the quencher MGB at the 3' end. The 18s endogenous control used a VIC-labeled probe. The amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 seconds, and 60°C for 1 min. The reaction was carried out according to the manufacturer's instructions, and allowed for the determination of the relative expression of IL-1β, TNF-α, and TGF-β by comparison with the standard curve generated using serial dilutions of pooled cDNA (1:1, 1:10, 1:100, 1:1000, 1:10000) and normalization to the control 18s ribosomal RNA levels.

ELISA

Serum corticosterone concentration was determined using an ELISA kit (ab108821; Abcam) with 96% recovery for corticosterone (0.3 ng/mL sensitivity). The reagents and standards were prepared according to the manufacturer's instructions. Cross reactivities for the kit were < 30% deoxycorticosterone, < 2% aldosterone, < 2% progesterone, and < 1% cortexolone. Per the manufacturer's instructions, serum samples were diluted 1:200. The reaction was carried out in duplicate according to the kit instructions and the average absorbance of the plate was determined using a plate reader and subtracting the absorbance at 450 nm from the absorbance at 570 nm (BioTek Synergy H1 Hybrid Reader). Corticosterone concentrations were extrapolated from a standard curve using a four-parameter logistic curve fit (Graphpad Prism) and multiplying by the dilution factor (1:200). The intraassay variation was 5.0% CV.

Statistical Analyses

All data are expressed as mean ± standard error (SE). One-way ANOVAs were used to detect differences among groups, with sleep treatment (control (no SF), LSF, and HSF) as the main factor and relative gene expression of specific cytokine as the dependent variable. Post hoc tests were carried out using a Tukey-Kramer HSD test. In some cases, logarithmic transformation was necessary to satisfy requirement of homogeneity of variances; in other cases, non-parametric tests were required (Kruskal Wallace H tests). For the latter, Mann-Whitney U tests were used to evaluate differences between experimental groups and a Bonferroni correction was employed to correct from the

number of comparisons made. Statistical analyses were carried out using SPSS and Statview programs.

RESULTS

Highly fragmented sleep decreases body mass

Body mass was significantly decreased following 24 hours of experimentation (one-way ANOVA; $F_{2,32} = 10.513$, $p = 0.0003$; Fig.3). Post-hoc tests revealed that body mass loss was significantly different between the control (-1.14 g ± 0.25 g; mean ± SE) and HSF (-2.67 g ± 0.32 g; mean ± SE) groups ($p < 0.05$, Fig.3) and between the HSF and LSF (-1.29 g ± 0.17 g; mean ± SE) groups ($p < 0.05$, Fig.3). However, there was no significant difference in body mass loss between the control and LSF groups ($p > 0.05$, Fig.3).

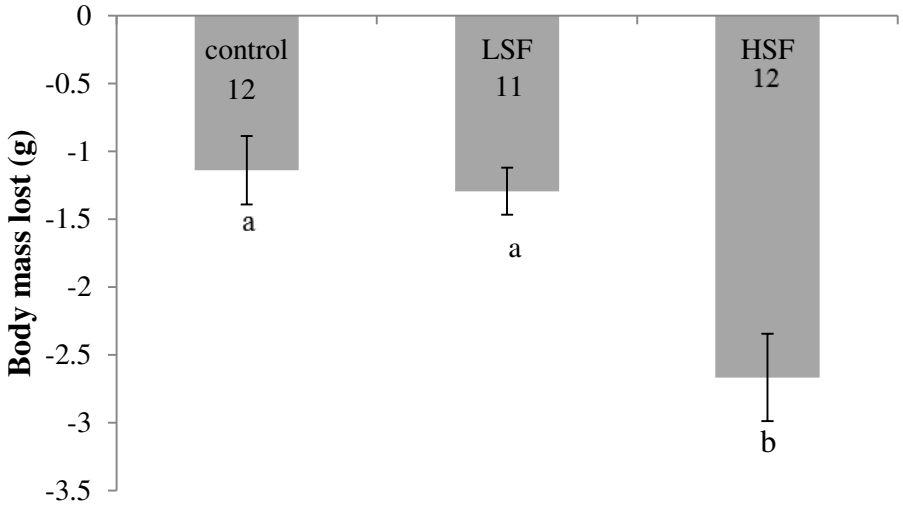

Figure 3. HSF induces changes in body mass after 24 hours. Data are shown as mean body mass lost (g) ± SE for each group. Shared letters indicate no significant difference between groups. The numbers at the base of the column indicate the sample size of each group.

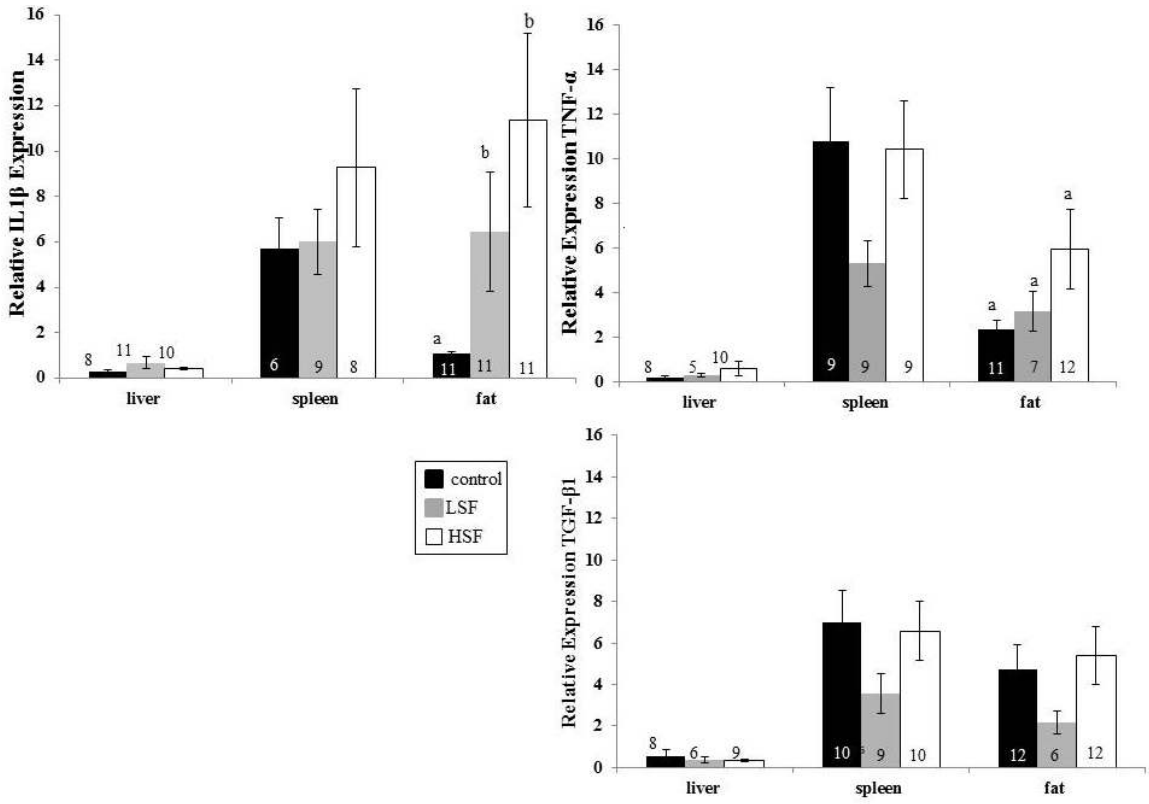

Figure 4. SF increases IL-1β gene expression in inguinal adipose tissue. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissue samples, resulting in a decreased sample size in these groups. Shared letters indicate no significant difference between groups. Since the results are marginally significant for TNF-α gene expression in the inguinal adipose tissue, all bars share the same letter. The numbers at the base of the column indicate the sample size of the group.

Sleep fragmentation increases IL-1β gene expression in adipose tissue

LSF and HSF increased IL-1β gene expression in adipose tissue relative to controls (one-way ANOVA; log-transformed; $F_{2,26} = 9.387$, $p = 0.0009$; $p < 0.05$; Fig.4). Post-hoc tests revealed that IL-1β gene expression was similar between SF groups ($p > 0.05$, Fig. 4). In

contrast, there were no significant differences detected in the liver (Kruskal Wallis; H = 2.364, p = 0.3067, Fig.4) or spleen (Kruskal Wallis; H = 0.770, p = 0.6805, Fig.4). There was a trend for elevation of TNF-α gene expression in adipose tissue of SF mice relative to controls (one-way ANOVA; log-transformed, $F_{2,26}$ = 3.231, p = 0.0546; Fig.4), but there was no significant difference detected in the liver (one-way ANOVA; log-transformed, F = 1.529, p = 0.2410, Fig.4) or spleen (Kruskal Wallis; H = 2.550, p = 0.2794, Fig.4). TGF-β1 gene expression did not differ in the liver (Kruskal Wallis; H = 1.872, p = 0.3921, Fig.4), spleen (one-way ANOVA; $F_{2,31}$ = 1.936, p = 0.1637, Fig.4), or adipose tissue (Kruskal Wallis; H = 3.681, p = 0.1588, Fig.4).

Sleep fragmentation increases IL-1β gene expression in heart tissue

A significant difference in IL-1β gene expression was detected in cardiac muscle tissue following high sleep fragmentation (one-way ANOVA; log-transformed, $F_{2,32}$ = 7.525, p = 0.0021, Fig.5). Post hoc analysis revealed a significant difference in IL-1β gene expression between the HSF group and the control ($p < 0.05$), but there was similar gene expression between the LSF group and the control ($p > 0.05$) and between LSF and HSF ($p > 0.05$). No differences in TNF-α gene expression (one-way ANOVA; log-transformed, Fig.5) or TGF-β1 gene expression (one-way ANOVA, Fig.5) were detected in the heart (all $p > 0.44$).

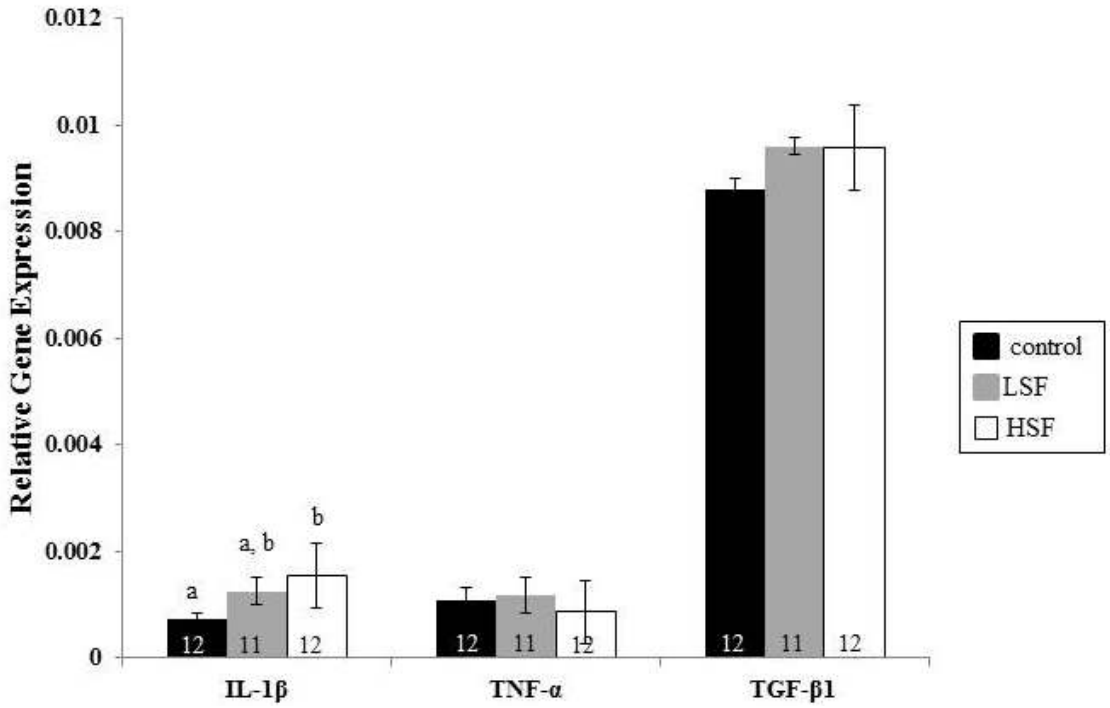

Figure 5. HSF induces an increase in the gene expression of IL-1β in cardiac muscle tissue. Data are shown as mean ± SE for each group. Shared symbols indicate there is no significant difference between groups. The numbers at the base of the columns indicate the sample size.

Sleep fragmentation increases cytokine gene expression in the hypothalamus

IL-1β gene expression varied significantly among groups in the hypothalamus (Kruskal Wallis; $H = 7.212$, $p = 0.0272$, Fig.6), but not in prefrontal cortex (Kruskal Wallis; $H = 1.938$, $p = 0.3794$, Fig.6) or hippocampus (one-way ANOVA; $F_{2,31} = 0.743$, $p = 0.4840$, Fig.6). More specifically, hypothalamic IL-1β gene expression increased in LSF relative to HSF mice (Mann Whitney; $U = 24$; $Z = -2.374$; $p = 0.0176$; adjusted $\alpha = 0.017$, Fig.6). However, there was no difference between LSF and control mice (Mann Whitney; $U = 25$, $Z = -2.308$, $p = 0.0210$, Fig.6) or between the control and HSF mice (Mann Whitney;

$U = 69$, $Z = -0.173$, $p = 0.8625$, Fig.6). There was no significant difference in TNF-α gene expression in the hypothalamus (Kruskal Wallis; $H = 2.455$, $p = 0.2930$, Fig.6), prefrontal cortex (Kruskal Wallis; $H = 2.002$, $p = 0.3675$, Fig.6), or hippocampus (Kruskal Wallis; $H = 1.341$, $p = 0.5115$, Fig.6). Sleep fragmentation induced significant alterations in TGF-β1 gene expression among groups in the hypothalamus (Kruskal Wallis; $H = 15.132$, $p = 0.0005$, Fig.6) and hippocampus (one-way ANOVA; $F_{2,32} = 4.060$, $p = 0.0268$, Fig.6). There was a trend for increased gene expression in the prefrontal cortex (Kruskal Wallis; $H = 5.651$, $p = 0.0593$, Fig.6). Post hoc tests revealed TGF-β1 hypothalamic gene expression was significantly different between LSF and HSF groups (Mann Whitney; $U = 8$, $Z = -3.429$, $p = 0.0006$, Fig.6) and between HSF and control groups (Mann Whitney; $U = 28$, $Z = -2.540$, $p = 0.0111$, Fig.6). However, LSF and control groups exhibited similar levels (Mann Whitney; $U = 27$, $Z = -2.176$, $p = 0.0296$, Fig.6). Post hoc tests for the hippocampus revealed there was a significant difference between HSF and LSF groups ($p < 0.05$). All other comparisons were non-significant.

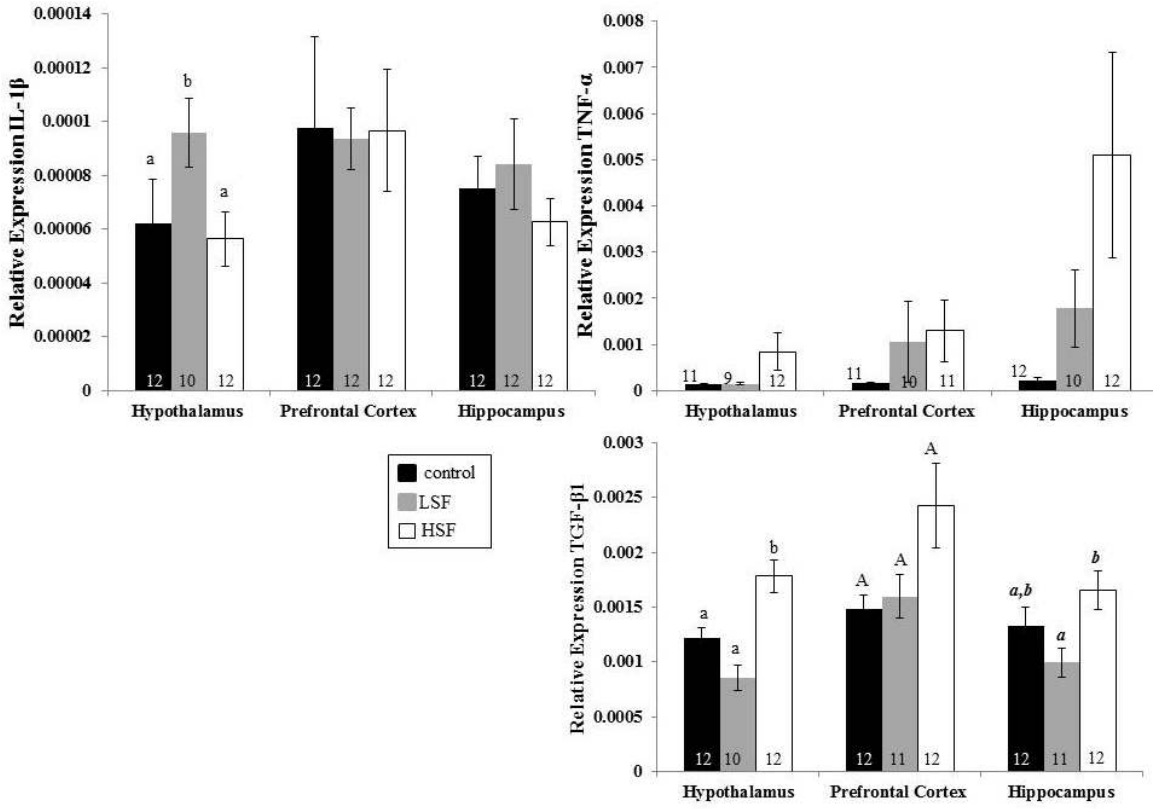

Figure 6. SF induces increased IL-1β and TGF-β1 gene expression in the hypothalamus. SF also amplified TGF-β1 gene expression in the hippocampus. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissues, which resulted in a decreased sample size in these groups. Shared letters indicate no significant difference between groups. Since the results are marginally significant for TGF-β1 gene expression in the prefrontal cortex, all bars share the same letters. Numbers at the base of the column indicate sample size of the group.

Sleep fragmentation increases serum corticosterone

Serum corticosterone concentration varied significantly among treatments (Kruskal Wallis; $H = 9.593$; $p = 0.0083$, Fig.7). Mice exposed to HSF (248.88 ng/mL ± 17.14;

mean ± SE) had significantly increased serum corticosterone (Mann Whitney; $U = 23$; $Z = -2.829$; $p = 0.0047$, adjusted $\alpha = 0.017$, Fig.7) when compared with controls (165.91 ng/mL ± 14.72; mean ± SE). There was also a trend for higher corticosterone levels in mice experiencing HSF compared with mice experiencing LSF (188.31 ng/ mL ± 17.6033; mean ± SE) (Mann Whitney; $U = 32$; $Z = -2.093$; $p = 0.0364$; adjusted $\alpha = 0.017$, Fig.7). Corticosterone concentration did not differ significantly between LSF and control mice (Mann Whitney; $U = 44$, $Z = -1.354$, $p = 0.1757$, Fig.7).

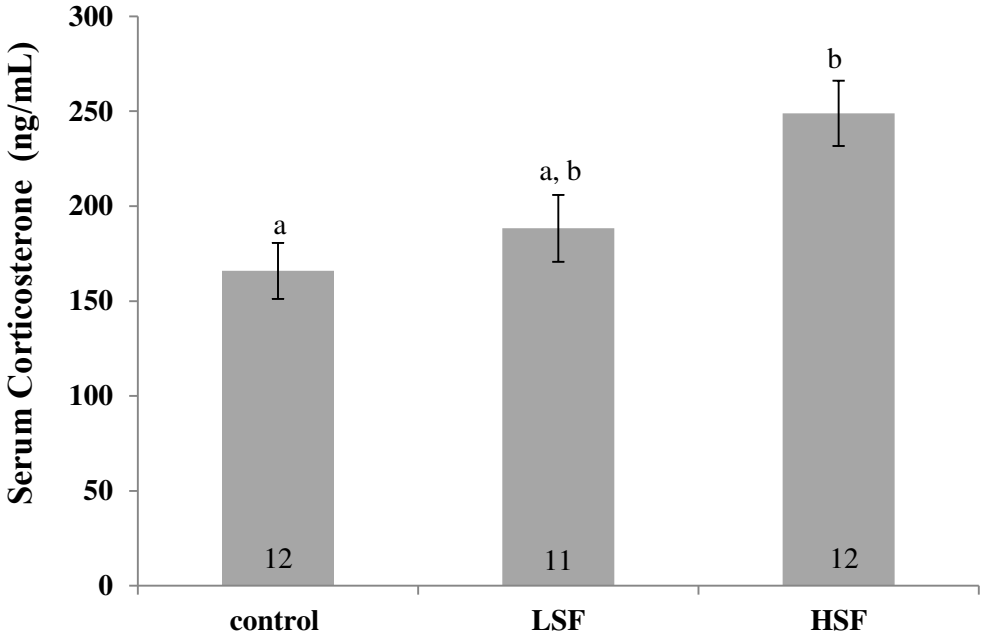

Figure 7. Serum corticosterone concentrations increase following 24 hours of sleep fragmentation. Data are shown as mean (ng/ mL) ± SE for each group. Shared letters indicate there is no significant difference between groups. Numbers at the base of the column indicate sample size.

DISCUSSION

It is widely accepted that sleep is an essential physiological process because acute and chronic sleep loss can lead to deleterious effects upon physiology and behavior (Nair et al., 2011; Simpson and Dinges, 2007; Zielinski et al., 2013). A previous study has suggested that sleep allows for metabolite clearance from the brain and cerebral spinal fluid (Xie et al., 2013). Consequently, curtailment of this cleansing period through sleep loss could potentially induce neuroinflammation towards a build-up of waste products. Current research holds that in addition to acting as modulators of inflammation, IL-1β and TNF-α act as sleep regulatory substances (SRS) that promote non-REM sleep after their release from glia in response to extracellular ATP (Jewett and Krueger, 2012). During periods of prolonged wakefulness, these cytokines accumulate in the brain and cerebral spinal fluid and are thought to be responsible for inducing symptoms of sleep loss, such as sleepiness, fatigue, and poor cognition, and enhanced sensitivity to pain (Clinton et al., 2011; Graves et al., 2003; Jewett and Krueger, 2012; Zielinski et al., 2013). It is well known that sleep deprivation results in an acute inflammatory response, characterized by an increase in pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, and an increase in serum glucocorticoids (Brager et al., 2013; Carreras et al., 2014; Hirotsu et al., 2012; Mullington et al., 2010; Simpson and Dinges, 2007; van Leeuwen et al., 2009). The majority of studies investigating the effects of sleep loss on the immune system have completely deprived subjects of sleep. However, this model may not be applicable for patients suffering from disorders from incomplete sleep loss (multiple arousals per hour) because sleep homeostatic responses are likely different than those responding to complete deprivation (Ramesh et al., 2009). Thus, it is highly likely that

the sleep fragmentation model used in this study more closely approximates these conditions.

In this study, pro- and anti-inflammatory cytokine gene expression were upregulated following 24 hours of acute sleep fragmentation, with the response being heterogeneous in various tissue locations. More specifically, sleep fragmentation led to increased IL-1β gene expression in adipose tissue, heart (HSF only), and hypothalamus (LSF only), while gene expression of TGF-β1, an anti-inflammatory cytokine, was elevated in two out of three regions of the brain measured (hypothalamus and hippocampus) compared with non-sleep restricted controls. SF did not increase TNF-α gene expression in any of the tissues measured. Previous literature describes elevated TNF-α in sleep-restricted mice and rats, but because these studies assessed the effects of chronic sleep fragmentation, the results may not be directly comparable to an acute response (Ramesh et al., 2011; Venancio and Suchecki, 2014; Zhang et al., 2014). As a result, this study provides evidence that cytokine gene expression is altered following sleep fragmentation, and that the degree of sleep fragmentation has an impact upon the development of this inflammation. Furthermore, based upon the cytokines selected for this experiment, the results suggest the induction of a pro-inflammatory environment in the periphery, and the production of a protective anti-inflammatory environment in the brain. The observed increases in cytokine gene expression in this experiment were detected at the mRNA level. Previous experimentation has shown that mRNA and protein expression levels of TNF-α are correlated following experimental sleep loss and vaccine stimulation. However, experiments involving IL-1β have produced conflicting results and TGF-β1 has not been studied within this context (Irwin et al., 2006; Shebl et al., 2010).

Consequently, it is equivocal whether protein levels correlate directly with mRNA expression, but nonetheless, increased mRNA expression still provides insight into the rapid temporal dynamics of cytokine activation at the transcriptional level.

Table 1. The effect of 24 hours of sleep fragmentation on cytokine gene expression.

Tissue	Effect
Liver	-
Spleen	-
Fat	Increase: IL-1β
Heart	Increase: IL-1β
Hypothalamus	Increase: IL-1β and TGF-β1
Prefrontal Cortex	-
Hippocampus	Increase: TGF-β1

Chronic sleep fragmentation has been shown to increase orexigenic behavior, ultimately resulting in weight gain (Carreras et al., 2015; Wang et al., 2014). Since caloric expenditure does not change, and because glucose clearance and insulin sensitivity in adipocytes decrease with time following sleep restriction, the surplus calories become stored as fat (Wang et al., 2014; Zhang et al., 2014). Previous literature has indicated that although altered glucose homeostasis is present after 2 weeks of fragmented sleep, changes in body mass are absent (Carreras et al., 2014). Following a single night of sleep restriction, HSF mice lost significantly more body mass than LSF or control groups. In addition, serum corticosterone was elevated in HSF mice relative to other treatments. This result opposes previous studies indicating serum corticosterone levels that were similar between control and SF groups following 6 h of sleep fragmentation using the same type of sweeping bar apparatus (Ramesh et al., 2009). The discrepancy in corticosterone concentrations is likely due to a physiological stress response occurring in response to sleep loss for a 24 hour period. Alternatively, increases

in serum corticosterone concentration could have been attributed to a shift in natural biological rhythms from forced arousals during the light phase of the LD cycle or to an increase in activity levels in the SF groups as a result of tactile stimulation from the swipe bar. Pro-inflammatory cytokines peak during the rest period, typically during early slow wave sleep (Besedovsky et al., 2012). Because tissue is collected from the animals near the time in which sleep normally begins, it must be acknowledged that natural biological rhthyms could influence the observed results; however it is not likely. The combination of body mass loss and simultaneous increase in serum corticosterone concentrations imply that HSF mice were experiencing a physiological stress response, including activation of the HPA axis, as a result of sleep interruptions occurring so frequently. It is unclear whether HPA activation occurred because of the method of sleep fragmentation used or the direct effect of disrupted sleep *per se*. Consequently, these data suggest that to minimize the effect of glucocorticosteroids on cytokine gene expression profiles following sleep fragmentation, the most effective model may be the LSF model (sleep interruptions every 2 minutes), as body mass loss and serum corticosterone concentrations were not different from those of controls. Since LSF mice exhibited altered adipose and hypothalamic cytokine gene expression relative to controls, this provides evidence that these results are not confounded by increased corticosterone levels, which can have immunoregulatory effects. Use of the LSF model is in agreement with previous literature methodology of chronic SF experiments (Carreras et al., 2014; Carreras et al., 2015; Kaushal et al., 2012b; Ramesh et al., 2009; Zhang et al., 2014). Both the experimental time intervals of sleep fragmentation (HSF and LSF) were selected based upon the results of previous chronic studies (Ramesh et al., 2009; Ringgold et al.,

2013). Due to altered cytokine gene expression and corticosterone concentrations in response to sleep fragmentation, it can be concluded that these time intervals are acceptable for eliciting an immune response using an acute model of sleep fragmentation.

Not only has sleep loss been shown to have metabolic implications in human and mouse models, but it also results in cardiovascular alterations (Carreras et al., 2014; Kheirandish-Gozal et al., 2010; Zhang et al., 2014). Shortened sleep intervals have been shown to increase the production of white blood cells, C - reactive protein, IL-6, and produce a simultaneous decrease in the anti-inflammatory cytokine IL-10, with the observed changes amplifying the risk for the development of future cardiovascular disease (Gozal et al., 2008; Kheirandish-Gozal et al., 2010; Mullington et al., 2009). Furthermore, chronic sleep fragmentation induces endothelial dysfunction and thickening and disruption of elastic fibers in the aortic arch and thoracic aorta (Carreras et al., 2014). Similar to these previous studies, even just 24 hours of sleep fragmentation leads to the inception of inflammation in organs that are susceptible to chronic inflammation in patients with obstructive sleep apnea (OSA), namely, in heart and adipose tissue (Carreras et al., 2014; Carreras et al., 2015; van Leeuwen et al., 2009). IL-1β gene expression was upregulated in cardiac muscle tissue, indicating a pro-inflammatory environment that would be detrimental to surrounding tissue. After a period of chronic sleep loss, exposure to increasing levels of pro-inflammatory cytokines and accumulation of damage, likely due to oxidative stress produced by the NOX-2 enzyme found in macrophages during chronic inflammation, has the potential to lead to the onset of cardiovascular disease, but further investigation into this transition is needed (Carreras et al., 2014; Zhang et al., 2014). Previous literature also indicates the persistence of a pro-

inflammatory environment following chronic sleep fragmentation due to a polarization towards the pro-inflammatory M1 macrophage subtype, ultimately resulting in a skew of the M1/M2 macrophage subtype ratio (Carreras et al., 2015; Zhang et al., 2014). Despite the acute nature of this experiment, a single 24 hour SF period was sufficient time to induce a pro-inflammatory environment in inguinal adipose tissue. Because acute sleep loss can lead to chronic effects over time, further investigation into this transition is needed to better understand the transition to pathology associated with chronic conditions, such as OSA. Additionally, future examination of epigenetic changes, such as changes in SIRT, may lead to a greater understanding of the progression from acute to chronic dysregulation of the immune system.

While a pro-inflammatory response to sleep loss was observed in inguinal adipose and cardiac muscle tissue, an anti-inflammatory response was detected within the brain. This finding supports a previous study that indicates specifically within brain tissue, the anti-inflammatory environment and increased production of IL-10 could protect the brain against ischemic cell death preceding surgical procedures (Weil et al., 2009). Because a similar anti-inflammatory environment was observed in all regions of the brain in this experiment, with the upregulation of TGF-β1 gene expression following acute sleep fragmentation, this provides evidence for neuroprotection. Although there was an increase in IL-1β gene expression in the hypothalamus following sleep fragmentation, it could be explained by an accumulation of SRSs in the brain following a period of prolonged wakefulness and possibly the absence of metabolic clearance of the cerebral spinal fluid that takes place during sleep (Jewett and Krueger, 2012; Xie et al., 2013).

Consequently, further experimentation is needed to elucidate whether IL-1β is acting as an SRS or an inflammatory marker.

In conjunction with the rise in morbid obesity in recent years, obstructive sleep apnea (OSA) and other obesity-related sleep disorders are becoming more common. Sleep interruptions from these disorders influence overall restfulness from sleep and eventually lead to pathology over time due to chronic inflammation (Pulixi et al., 2014). While OSA is a chronic disease, understanding the acute effects of sleep fragmentation is essential, as it is these effects that eventually progress to chronic pathologies, such as cardiovascular and metabolic disease. This study reports a rapid inflammatory response in adipose and cardiac muscle tissue following a single 24 h period of interrupted sleep. Furthermore, pro-inflammatory gene expression increased as sleep became more fragmented. Because adipose and heart tissue are highly susceptible to dysfunction as a result of OSA, the acute elevation of pro-inflammatory cytokine gene expression in these tissue could provide an early indication of disease. Future studies are needed to fully characterize the acute inflammatory response to sleep loss, to understand its progression towards chronic pathology, and to identify epigenetic changes that may alter this acute/chronic transition. From a clinical standpoint, prophylactic measures could be developed to prevent the accumulation of inflammation and subsequent tissue dysfunction that OSA-related disorders create.

CHAPTER 2: ACUTE SLEEP FRAGMENTATION INDUCES DIFFERENT CYTOKINE GENE EXPRESSION PROFILES IN OBESE AND LEAN MICE

Obesity and sleep fragmentation are often times co-occurring pro-inflammatory conditions in patients with obstructive sleep apnea. Despite the known association between the two, the simultaneous effects of the conditions on the immune and endocrine profiles have yet to be explored. Consequently, we investigated changes in pro-inflammatory (IL-1β, TNF-α) and anti-inflammatory (TGF-β1) cytokine gene expression in the periphery (liver, spleen, fat, and heart) and brain (hypothalamus, prefrontal cortex, and hippocampus) of lean and obese mice exposed to sleep fragmentation. Serum corticosterone concentration was also assessed. Sleep was disrupted in male C57BL/6J mice (lean) and ob/ob KO mice (obese) using an automated sleep fragmentation chamber. Trunk blood and tissue samples were collected after 24 h of SF. We predicted that the acute inflammatory response resulting from HSF would be different for the lean and the obese mice, with the highest cytokine gene expression levels being detected in the OB HSF group, due to a synergistic effect of the pro-inflammatory conditions. While, obesity was found to be the factor that differentially affected cytokine gene expression profiles, the effect on cytokine gene expression varied from tissue to tissue. The pro-inflammatory nature of both obesity and sleep loss was not found to be summative overall in this experimental model. Obesity significantly elevated cytokine gene expression in the hypothalamus (IL-1β and TNF-α), prefrontal cortex (IL-1β), hippocampus (IL-1β, TNF-α), liver (IL-1β, TGF- β1), and heart (TGF- β1). Conversely, decreases in pro-inflammatory cytokine gene expression were detected in the spleen (IL-1 β), fat (IL-1β), and heart (IL-1β, TNF-α). Serum corticosterone concentration was

significantly elevated as a result of sleep fragmentation, with HSF mice exhibiting the highest serum corticosterone concentrations. These data indicate that obesity is a potent inducer of neuroinflamation, but leads to an anti-inflammatory response in the periphery, all within the context of elevated stress hormones. As a result, this suggests obese mice may have neuroendocrine adaptations to combat the pre-existing pro-inflammatory condition of obesity and that these adaptations may impact the acute inflammatory response to sleep loss.

INTRODUCTION

In conjunction with the growing obesity epidemic in the United States, the number of individuals suffering from sleep disorders has continued to rise in recent years. It was estimated that in 2007, more than 40% of patients with sleep disordered breathing could be attributed to obesity (McNicholas et al., 2007). More specifically, Obstructive Sleep Apnea (OSA), a sleep disorder characterized by respiratory pauses during sleep, loud intermittent snoring and excessive daytime sleepiness, has risen steadily with the increased incidence of obesity (Ryan et al., 2009). In the general population, the prevalence of OSA is estimated to be between 5 and 10%; however, because often times OSA remains undiagnosed, this statistic is likely underestimated (Witkowski and Kadziela, 2015). The symptoms of OSA can be explained by repeated episodes of upper airway obstruction or collapse during sleep, resulting in increased respiratory efforts, periodic arterial oxygen desaturation, systemic and pulmonary arterial blood pressure increases and sleep disruption (McNicholas et al., 2007). Individuals suffering from severe sleep apnea can have up to thirty hypoxic attacks per hour, equating to sleep interruptions occurring every 2 minutes (Ramesh et al., 2009). While individuals suffering from OSA may or may not be fully aroused during these hypoxic events, the overall restfulness of sleep continues to be adversely affected, as sleep interruptions associated with OSA are known to decrease the amount of time spent in REM sleep (Ryan et al., 2009). Consequently, previous research has found that untreated OSA increases the risk for automobile accidents, worsens the quality of life and mood, decreases cognitive impairment, lessens productivity in the work place, and results in

daytime sleepiness (Akashiba et al., 2002; Finn et al., 1998; Hiestand et al., 2006; Schwartz et al., 2005; Teran-Santos et al., 1999).

Despite these detrimental effects of OSA, of even greater interest to physicians and researchers is the strong association between sleep interruptions and cardiovascular events. It has been found that untreated chronic OSA increases the risk for the development of hypertension, coronary vascular disease, congestive heart failure, cerebrovascular disease, glucose intolerance, impotence, stroke, and myocardial infarction (Hiestand et al., 2006; McNicholas et al., 2007). When left untreated, severe OSA can be linked to an increased risk of mortality due to cardiovascular events. Consequently, the diagnosis and treatment of OSA have become a great health concern in an attempt to prevent the pathology associated with chronic exposure to the condition.

The standard diagnostic technique for sleep apnea and other sleep disorders is overnight polysomnography in a sleep clinic (McNicholas et al., 2007; Ruehland et al., 2009; Witkowski and Kadziela, 2015). Typically patients are recommended for a polysomnography study after a sleeping partner has complained of very loud snoring or gasping sounds during the night, coupled with the individual experiencing excessive daytime sleepiness (Ryan et al., 2009). However, a physician may also suggest a sleep study based upon the presence of additional risk factors indicative of future sleep disordered breathing, such as: a high arched palate, large tonsils, a long uvula, tongue indention, or abnormal posterior positioning of the maxilla or the mandible (Lee et al., 2013). OSA is diagnosed when the apnea-hypopnea index (API) is greater than 15 events per hour during polysomnography (Witkowski and Kadziela, 2015). The apnea-hypopnea

index is a quantifiable measure of the severity of sleep apnea. In order to calculate the API, the number of stopped breathing events (apnea) and events in which the airflow through the abdominothoracic cavity is reduced (hypopnea) are counted and divided by the total number of hours spent sleeping (Ruehland et al., 2009). Taken together, apnea and hypopnea help to explain the increased respiratory efforts and oxygen desaturation typically observed with OSA.

Once OSA has been diagnosed, the standard form of treatment is continuous positive airway pressure (CPAP) during sleep, which essentially acts as a splint to keep the upper airway open, thereby preventing the negative pressure during inspiration from building to facilitate the airway collapsing back on itself (McNicholas et al., 2007; Ryan et al., 2009). Previous research has shown that CPAP dramatically improves the overall well-being of individuals with OSA, as it normalizes their sleep pattern and helps better their day time alertness (Ryan et al., 2009). Additional studies have shown that adenotonsillectomy in non-obese children with OSA can reverse problematic physiological alterations, such as an increase in the pro-inflammatory cytokine IL-6, further highlighting the importance of seeking treatment for OSA to prevent the progression to cardiovascular disease and other complications (Gozal et al., 2008; Kardassis et al., 2013).

Helping to compound the potential dangers associated with untreated OSA is the fact that the incidence of OSA increases with the prevalence of obesity in a population (Hiestand et al., 2006). In a U.S. population study for the National Sleep Foundation, it was found that 59% of obese individuals were at high risk for OSA (Shahar et al., 2001).

Furthermore, it has also been reported that OSA is commonly seen in morbidly obese patients, so much so that BMI has been used in scoring systems in order to predict OSA (O'Keeffe et al., 2004). Because both OSA and obesity have been demonstrated to independently impact inflammation and cardiac function, the summative effect of the two conditions is of particular concern. The association between obesity and OSA can be explained by physiological changes that occur as a direct consequence of increased adiposity. Firstly, the excess weight on the chest when lying in a supine position negatively effects respiratory efforts and may help facilitate the collapse of upper airways during sleep, ultimately increasing the frequency of hypoxic respiratory events (McNicholas et al., 2007). Additionally, other physiological alterations associated with obesity may contribute to negative cardiovascular outcomes as a result of OSA. Obesity is associated with left ventricle hypertrophy, pulmonary hypertension, and diastolic dysfunction, as a result of increased force being exerted on the heart (Otto et al., 2007). However, previous studies have shown that OSA also contributes to left ventricle hypertrophy and other changes associated with obesity, due to an increased degree of diastolic dysfunction observed in obese patients with OSA than those without (Arias et al., 2005; Otto et al., 2007). Consequently, changes in cardiovascular function and physiology attributed to obesity are exacerbated when concurrent with OSA, further underscoring the need to understand the progression towards pathology in both conditions.

Obesity is a low-grade pro-inflammatory condition, with adipose tissue exhibiting both paracrine and endocrine functions by releasing various hormones and cytokines into

the surrounding tissue and blood. Visceral adipose tissue releases a variety of pro and anti-inflammatory markers, namely: adipokines, leptin, adiponectin, resistin, IL-6, TNF-α, monocyte chemoattractant protein-1 (MCP-1) (Kardassis et al., 2013; Ryan et al., 2009). One proposed mechanism for the initiation of an inflammatory response associated with obesity is the disruption of tight junctions between adjacent intestinal epithelial cells; thereby allowing intestinal bacteria to escape into the periphery, and initiating an inflammatory response to the presence of bacterial LPS through TLR4 (Teixeria et al., 2012). However, additional support for this proposed mechanism is still lacking. Obesity has also been associated with a greater polarization towards the M1 macrophage subtype in adipose tissue, ultimately promoting the persistence of a pro-inflammatory environment (Chawla et al., 2011). When compared to the adipose tissue of lean individuals, tissue from obese individuals shows an increase in molecules such as: TNF-α, IL-6, iNOS, TGF-β1, CRP, soluble ICAM and MCP-1, and the pro-coagulant protein plasminogen activator inhibitor type-1 (Weisberg et al., 2003). Because of the persistent pro-inflammatory environment surrounding adipose tissue, obesity has been connected to conditions resulting from damage due to chronic inflammation, such as insulin resistance, diabetes, and cardiovascular disease (Ryan et al., 2009; Zhang et al., 2014). Current hypotheses implicate oxidative stress as the causative factor for the development of insulin resistance, diabetes, and cardiovascular disease, due to cell and tissue damage following the prolonged pro-inflammatory response; however, a complete understanding of this mechanism is lacking (Khalyfa et al., 2014; Pak et al., 2014; Wang et al., 2014).

In the same way that obesity induces a shift towards a pro-inflammatory phenotype, OSA also increases cytokine gene expression due to the sleep fragmentation caused by the condition. Previous literature indicates that sleep fragmentation increases corticosterone concentrations, leads to the upregulation of cell adhesion molecules on monocytes (ICAM-1, VCAM-1), and also increases the expression of inflammatory markers, such as CRP, TNF-α, IL-8, and IL-6 (Ciftci et al., 2004; Kent et al., 2011; Neto et al., 2010; Pak et al., 2014). It must be noted that increased expression of IL-6 and IL-8 is particularly important in the progression towards cardiovascular disease following sleep fragmentation, as this leads to the release of CRP from the liver to upregulate the expression of cell adhesion molecules, and an increase in neutrophil and monocyte adhesion to the vascular endothelium, respectively (Murphy, 2012). The progression towards cardiovascular disease is facilitated by an increase in the expression of cell adhesion molecules and chemokines, allowing macrophages overwhelmed with oxidized lipids to be recruited to the vascular endothelium, where they become stuck, resulting in the development of an atherosclerotic cap in the vessel and subsequent structural changes in the elastic fibers of the vessel (Carreras et al., 2014; Ryan et al., 2009). The increase in pro-inflammatory cytokines due to OSA can be reduced following treatment with CPAP or adenotonsillectomy. Previous experiments have shown that adhesion molecules, such as ICAM-1 and VCAM-1, and cytokines, such as TNF-α, IL-6, and IL-10, return to normal values in human patients with OSA following treatment (Gozal et al., 2008; Ryan et al., 2009). Because both obesity and OSA are pro-inflammatory conditions associated with a simultaneous increase in the expression of cell adhesion molecules, both have the potential to enhance the rate of onset and severity of cardiovascular disease.

Sleep interruptions attributable to OSA have been shown to have adverse metabolic consequences. Curtailment of the sleep period skews the balance between hunger and satiety hormone concentrations and alters normal feeding and activity behaviors, such that weight gain is the impending result. Previous literature indicates that concentrations of ghrelin, a hunger signaling hormone, and leptin and peptide YY, satiety signaling hormones, are altered following insufficient sleep (Garcia-Garcia et al., 2014). These changes are characterized by a decrease in serum concentrations of leptin, simultaneously occurring with an increase in ghrelin concentrations (Garcia-Garcia et al., 2014). As a result, caloric intake increases in conjunction with surges in circulating ghrelin and simultaneous decreases in leptin and peptide YY concentrations (Garcia-Garcia et al., 2014; Markwald et al., 2013; Mavanji et al., 2012). The elevated caloric intake can be explained by changes in the hormone concentrations in the hypothalamus. Under normal conditions, ghrelin binds to neurons expressing NPY and agouti-related peptide to send an orexigenic signal, while leptin binds to neurons expressing POMC and cocaine and amphetamine-related transcripts (CART) to produce an anorexigenic response (Garcia-Garcia et al., 2014). However, due to the changes in circulating hormone concentrations, the orexigenic response is activated over the anorexigenic response.

The increase in food intake following sleep deprivation is thought to be in response to a rise in energetic demands due to prolonged wakefulness; however, because caloric expenditure does not change, the surplus calories become stored as fat (Markwald et al., 2013; Wang et al., 2014; Zhang et al., 2014). Further compounding the propensity

towards weight gain is the fact that sleep loss not only increases appetite, but also shifts an individual's preference towards lipid-dense, high-calorie food. This response provides more opportunity to eat while awake, and results in decreased amounts of physical activity as a result of sleepiness and fatigue (Garcia-Garcia et al., 2014). It must be noted that while the increase in food intake leads to weight gain for in chronic sleep fragmentation studies, previous experiments have also reported that rodents experience a decrease in weight following prolonged wakefulness, indicating the experimental time period may have some effect on this observation (Baud et al., 2013; Hakim et al., 2012; Venancio and Suchecki, 2014). One such experiment indicates that chronic sleep fragmentation must exceed 2 weeks in order for increases in body weight to be present (Carreras et al., 2014).

Further investigations into the metabolic changes associated with sleep fragmentation have postulated a relationship between obesity, inflammation, and the development of insulin resistance and reduced glucose clearance. Chronic sleep fragmentation results in a reduction in insulin sensitivity and glucose clearance in adipose tissue (Carreras et al., 2015; Zhang et al., 2014). Previous literature has implicated two different proposed mechanisms of oxidative stress for the development of insulin insensitivity and reduced glucose tolerance as a consequence of the accumulation of tissue damage (Mesarwi et al., 2015). One such mechanism proposes superoxide and reactive oxygen species generated by the nicotinamide adenine dinucleotide phosphate oxidase-2 (NOX-2) enzyme in macrophages are the causative agents of tissue damage and inflammation, ultimately accounting for adipose tissue insulin resistance previously

described (Zhang et al., 2014). Because the activity of the NOX-2 enzyme increases with the duration of exposure to sleep fragmentation, the damage due to oxidative stress results in glucose intolerance and reduced insulin sensitivity with the passage of time (Zhang et al., 2014). Helping to support this mechanism is the fact that additional experimentation revealed NOX-2 activity was required for increased adiposity and subsequent weight gain in mice following 3 weeks of chronic sleep loss (Khalyfa et al., 2014). As a result, over time, the metabolic changes in adipose tissue associated with sleep fragmentation explain the development of diabetes, hypertension, and other conditions associated with chronic exposure to the condition.

However, another proposed mechanism involves endoplasmic reticulum (ER) stress due to ROS from cholesterol and free fatty acids or calcium depletion from the sarcoplasmic reticulum (Lee and Ozcan, 2014). In this mechanism, ER stress leads to an unfolded protein response. The unfolded protein response results in upregulated expression of protein folding machinery to try to catch up, which then causes suppressed leptin signaling in the hypothalamus, to bring about an increase in caloric intake (Hakim et al., 2015; Lee and Ozcan, 2014). In support of this mechanism, additional experimentation detected an increase in PTP1B activation (phospho-tyrosine protein kinase that negatively regulates insulin signaling) following ER stress in the hypothalamus, resulting in attenuation of leptin receptor signaling (Hakim et al., 2012). The attenuation of leptin receptor signaling provides an additional explanation for the development of obesity and metabolic dysfunction (Hakim et al., 2015). Consequently

this suggests, leptin also plays a major role in the progression towards insulin resistance and cardiovascular mortality associated with OSA (Fu et al, 2015).

Leptin, resistin, and adiponectin are adipokines secreted by adipose tissue that are essential in regulating metabolic functions, such as insulin sensitivity and glucose tolerance (Fu et al., 2015). While leptin is a peptide hormone that is produced by adipose tissue and circulates to the hypothalamus to act as a satiety hormone, resistin and adiponectin regulate insulin sensitivity (O'Donnell et al., 2000). Serum leptin levels are directly related to adipose tissue mass, meaning increased leptin levels are associated with obesity (Al Maskari and Alnaqdy, 2006). Although obesity is associated with an increased concentration of leptin, there is no corresponding loss of appetite, which suggests that leptin resistance is occurring (O'Donnell et al., 2000). In addition to having metabolic functions, leptin is also implicated as a potential player in regulation of the sleep wake cycle, since leptin deficient mice exhibit altered sleep architecture. Leptin deficiency results in more frequent, shorter-duration sleep periods, increased bouts of REM sleep, and decreased recovery to sleep deprivation (Laposky et al., 2006). Furthermore, leptin is a pro-inflammatory signal that supports immune cell activation, proliferation, differentiation and the production of pro-inflammatory cytokines, such as IL-1, IL-12, TNFα and Th1 cytokines like IFN-γ (Besedovsky et al., 2012).

Due to alterations in leptin concentrations following sleep fragmentation in OSA, it has been hypothesized that leptin can be used as a biomarker to detect the presence of sleep disorders (Ozturk et al., 2003; Pan and Kastin, 2014). Despite this predicted relationship, previous attempts have yet to successfully correlate leptin and leptin gene

receptor polymorphisms with OSA (Lv et al., 2015). In lieu of this failed attempt, other previous experiments have shown leptin concentrations are affected by sleep fragmentation; although the conflicting results have been reported as to if the alteration is an increase or a decrease (Fu et al., 2015; Garcia-Garcia et al., 2014; Ozturk et al., 2003). Consequently, leptin concentrations are also affected by both sleep fragmentation and obesity.

Because OSA and obesity are often times comorbid, we wanted to determine if the pre-existing pro-inflammatory environment associated with obesity influences the acute inflammatory response as a result of sleep loss. While pathologies associated with OSA are due to chronic exposure to the condition, we chose to investigate an acute model of sleep fragmentation because acute effects may become chronic effects over time. Since little is understood about the acute effects of sleep fragmentation and the progression towards chronic disease, we chose to begin to characterize this model. In order to do this, the effects of acute sleep fragmentation on pro-inflammatory (IL-1β and TNF-α) and anti-inflammatory (TGF-β1) cytokine gene expression in brain and peripheral tissue of lean and obese mice were investigated. Serum corticosterone concentrations were also quantified. The ob/ob knock-out (KO) mouse lacking the leptin gene was chosen as the model organism of obesity for this experiment. The ob/ob KO mouse exhibits hyperphagia, hyperinsulinemia, hypercorticosteronemia, and sterility as a result of genetic modification, but previous experimentation demonstrated impaired respiratory mechanisms during sleep in this strain, similar to those observed in patients with OSA (Jackson Laboratory website; O'Donnell et al., 2000).

While investigations into the immune and endocrine reaction surrounding the interplay of acute sleep fragmentation and obesity are still lacking, previous literature revealed a difference in the effects of sleep fragmentation on neurobehavior (sensitivity to pain, diet, and anxiety) in lean mice and obese mice (pan-leptin receptor KO) (He et al., 2015). As a result of this experiment, we hypothesized that the response to high sleep fragmentation (HSF) would be different between lean and obese mice. Based upon previous literature, it was hypothesized that this difference could go one of two ways. Firstly, the effects of HSF on inflammation could be attenuated in the obese animals as a result of pre-existing neuroendocrine adaptations intended to minimize the effects of chronic low-grade inflammation associated with obesity (He et al., 2015; Pak et al., 2014). More specifically if this were the case, we would expect that serum corticosterone levels and the gene expression of cytokines (IL-1β, TNF-α, TGF- β1) characterizing the inflammatory response in the brain and periphery of the obese mouse would be lower than that of the lean mouse. We predicted that the highest cytokine gene expression values and serum corticosterone concentrations would be observed in the lean HSF group. However, an alternative hypothesis could be because both obesity and sleep fragmentation are pro-inflammatory conditions, the effects on the inflammatory response would be summative. Consequently, if this were the case, we would expect an increase in cytokine gene expression (IL-1β, TNF-α, TGF- β1) in the brain and periphery and an increase in serum corticosterone concentrations in the obese mice compared to the lean group, with the highest values seen in the obese HSF group.

METHODS AND MATERIALS

Animals

Male C57BL/6J mice (n = 16) and male ob/ob leptin gene knockout mice (n = 18) were housed in a colony room (12 h light/ 12 h dark, lights on 07:00 h; 21°C ± 1°C) at Western Kentucky University. To obtain the ob/ob knockout mice, Jackson Laboratory OB heterozygotes were purchased to form breeding trios. Ob/ob homozygous recessive offspring lacking the leptin gene were identified phenotypically by weight around 4 weeks of age. After weaning at 21 d of age, mice resided in polypropylene cages with same sex littermates and were provided corncob bedding, as well as food, and water *ad libitum*. This study was conducted under approval of the Institutional Animal Care Committee at Western Kentucky University and procedures followed the National Institutes of Health Guide for the Use and Care of Laboratory Animals and international ethical standards.

Sleep fragmentation

Male adult (> 8 weeks of age) ob/ob mice and C57BL6/J mice were selected for experimentation and were briefly exposed to isoflurane vapors and weighed to the nearest 0.01 g. C57BL6/J mice ranged in age from 23 weeks – 31 weeks of age. Ob/ob mice ranged from 8 – 10 weeks of age. Two to four mice were then placed in an automated sleep fragmentation chamber (Lafayette Industries, Lafayette, IN; Model 80391) with a thin layer of corn cob bedding at 09:00 h. Previously it has been demonstrated that because mice are social animals, isolation during sleep deprivation induces differential

effects for socially isolated and paired animals (Kaushal et al., 2012a). As a result, the number of animals in the cage does not affect the resulting inflammatory response, it is only necessary that the animals remain in a social environment and not in isolation. Food and water were provided *ad libitum*. To distinguish among subjects, tails were marked with a different colored pen (Sharpie). At 8:00 the next day, the swipe bar was activated according to the previously described experimental protocol. Based upon the result of previous experiments, the "high" sleep fragmentation (HSF) interval was chosen to represent the sleep fragmentation group and the swipe bar interval was set to 20 seconds (Ramesh et al., 2009). For the control group, the bar remained stationary. Thirty-four mice were randomly assigned to either control (n = 8), HSF (n = 8), obese (OB) control (n = 9), or obese (OB) HSF groups (n = 9). To minimize the number of animals sacrificed, frozen tissue collected from 8 male C57BL6/J during the first experiment was selected for use for the control and HSF groups for this experiment. RNA was extracted from the tissue samples, reverse transcription was performed, and the resulting cDNA was used for RT-PCR analysis. All frozen tissue samples (liver, spleen, fat, heart, hypothalamus, prefrontal cortex, and hippocampus) came from the same randmomly selected individual.

After HSF or control conditions for 24 h, the obese animals were deeply anesthetized using isoflurane vapors (< 2 min), weighed, and then rapidly decapitated within 3 min of initial handling. Trunk blood was collected, kept on ice for < 20 min, and then spun at 3000 x g for 30 minutes at 4°C. The serum was drawn off and stored at -80°C for later ELISA analysis. Liver, spleen, inguinal fat, and heart samples were collected and placed

into Eppendorf tubes containing RNAlater solution (Life Technologies). The tissue samples were stored at 4°C until RNA extraction was performed. The hypothalamus, prefrontal cortex, and hippocampus were then dissected from the brain and stored in RNAlater at -20°C until RNA extraction.

RNA Extraction

RNA was extracted from brain, liver, spleen, and fat using an RNeasy Mini Kit (Qiagen). RNA was extracted from the heart using an RNeasy Fibrous Tissue Mini Kit. RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Reverse Transcription

Total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription kit (Life Technologies, #4368813). The total RNA concentration of the tissue was diluted (brain: 9.2; liver, spleen, and fat: 10.8 ng/µL; heart: 21.9 ng/µL). The reaction was carried out according to the manufacturer's instructions. The amplification conditions for the thermocycler were 25°C for 10 min, 37°C for 120 min, 85°C for 5 min (for 1 cycle), and then 4°C .

RT-PCR

Real Time- PCR was performed using an ABI 7300 RT-PCR System. To determine relative cytokine gene expression, Taqman Gene Expression RT-PCR Master Mix and the following primer/probes (Applied Biosystems) were used: IL-1β (Mm00434228_m1),

TNF-α (Mm00443260_g1), and TGF-β (Mm01178820_m1), or 18s endogenous primer-limited (Mm03928990_g1). Cytokine probes were labeled with the fluorescent marker 5-FAM at the 5' end and the quencher MGB at the 3' end. The 18s endogenous control used a VIC-labeled probe. The amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 seconds, and 60°C for 1 min. The reaction was carried out according to the manufacturer's instructions, and allowed for the determination of the relative expression of IL-1β, TNF-α, and TGF-β by comparison with the standard curve generated using serial dilutions of pooled cDNA (1:1, 1:10, 1:100, 1:1000, 1:10000) and normalization to the control 18s ribosomal RNA levels.

ELISA

Serum corticosterone concentration was determined using an ELISA kit (ab108821; Abcam) with 96% recovery for corticosterone (0.3 ng/mL sensitivity). The reagents and standards were prepared according to the manufacturer's instructions. Cross reactivities for the kit were < 30% deoxycorticosterone, < 2% aldosterone, < 2% progesterone, and < 1% cortexolone. Serum samples were diluted 1:200 per the manufacturer's instructions and plated in duplicate. The reaction was carried out according to the kit instructions and the average absorbance of the plate was determined using a plate reader and subtracting the absorbance at 450 nm from the absorbance at 570 nm per the manufacturer's instructions (BioTek Synergy H1 Hybrid Reader). Corticosterone concentrations were extrapolated from a standard curve using a log transformed-log transfomed curve fit (Excel) and multiplying by the dilution factor (1:200). The intraassay variation was 5.0% CV.

Statistical Analyses

All data are expressed as mean ± standard error (SE). Two-way ANOVAs were used to detect differences among groups, with sleep treatment (control (no SF) vs. HSF), genotype (wildtype vs. ob/ob), and the interaction between two as the main factors, and relative gene expression of each specific cytokine as the dependent variable. Post hoc tests were carried out using a Fisher's LSD test, using a one-way ANOVA to determine the significant interactions. In some cases, logarithmic transformation was necessary to satisfy requirement of homogeneity of variances. Statistical analyses were carried out using SPSS and Statview programs.

RESULTS

Highly fragmented sleep results in body mass loss

Body mass significantly decreased following 24 hours of SF (two-way ANOVA; $F_{1,30}$ = 41.101, $p < 0.0001$; Fig.8). Post-hoc tests revealed that body mass loss was significantly different between the control (-0.85 g ± 0.71 g; mean ± SE) and HSF (-2.69 g ± 1.30 g; mean ± SE) groups ($p < 0.05$, Fig.8) and between the OB control (-0.68 g ± 0.44 g; mean ± SE) and OB HSF groups (-2.49 g ± 0.68 g; mean ± SE) groups ($p < 0.05$, Fig.8). Additionally, there were differences between the control and OB HSF group and between the HSF group and the OB control ($p < 0.05$, Fig.8). However, there were no significant differences in body mass loss due to obesity (two-way ANOVA; $F_{1,30} = 0.414$; p = 0.5347; Fig.8) or the interaction between sleep and obesity (two-way ANOVA; $F_{2,30}$ = 0.003; $p = 0.9563$; Fig.8).

Obesity increases cytokine gene expression in the liver

Obesity increased TNF-α gene expression in liver tissue relative to controls (two-way ANOVA; log-transformed; $F_{1,25} = 6.748$, $p = 0.0155$; Fig.9). Post-hoc tests revealed that TNF-α gene expression was different between the wildtype control and OB control groups ($p = 0.0096$, Fig. 9) and between the control and OB HSF groups ($p = 0.0073$, Fig. 9). In contrast, sleep (two-way ANOVA; log-transformed; $F_{1,25} = 2.076$; $p = 0.1620$; Fig.9) and the interaction between the two factors (two-way ANOVA; log-transformed; $F_{2,25} = 1.330$; $p = 0.2597$; Fig.9) did not alter TNF-α gene expression in the liver. Obesity also increased TGF-β1 gene expression in the liver (two-way ANOVA; log-transformed; $F_{1,28} = 12.352$, $p = 0.0015$; Fig.9). Post-hoc tests showed that TGF-β1 gene expression

was different between both the control and OB control group ($p = 0.0038$; Fig.9) and the control and OB HSF group ($p = 0.0027$; Fig.9). No other factors affected TGF-β1 gene expression in the liver (two-way ANOVA; log-transformed; sleep: $F_{1,28} = 1.257$; $p = 0.2717$; interaction: $F_{2,28} = 0.646$; $p = 0.4282$; Fig.9). There were no significant differences in IL-1β gene expression in the liver (two-way ANOVA; log-transformed; sleep: $F_{1,27} = 0.809$; $p = 0.3765$; obesity: $F_{1,27} = 2.875$; $p = 0.1015$; interaction: $F_{2,27} = 0.993$; $p = 0.3280$; Fig.9).

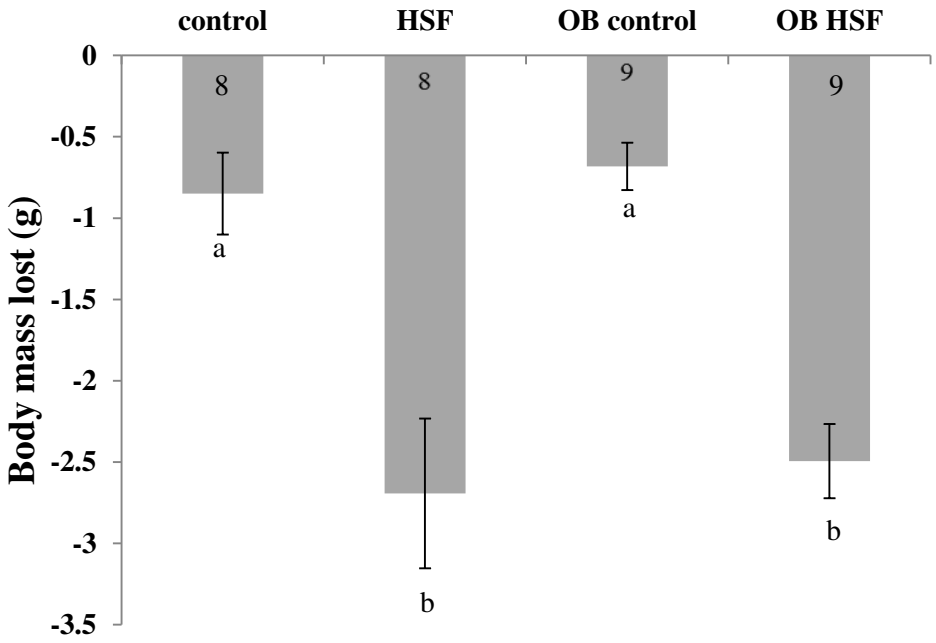

Figure 8. HSF induces changes in body mass in both obese and lean mice after 24 hours. Data are shown as mean body mass lost (g) ± SE for each group. Shared letters indicate no significant difference between groups. The numbers at the base of the column indicate the sample size of each group.

Obesity decreases IL-1β gene expression in the spleen

IL-1β gene expression was significantly decreased in the spleen due to obesity (two-way ANOVA; log-transformed; sleep: $F_{1,26} = 6.391$; $p = 0.0179$; Fig.9). Post-hoc tests revealed that the difference in splenic IL-1β gene expression was between the HSF and OB HSF groups ($p = 0.0153$: Fig.9). Conversely, there was no effect due to sleep or the interaction between the two factors (sleep: $F_{1,26} = 0.154$; $p = 0.6978$; interaction: $F_{2,26} = 0.1.233$; $p = 0.2769$; Fig.9). Similarly, there were no significant differences in TNF-α gene expression detected in the spleen due to any of the factors tested (two-way ANOVA; log-transformed; sleep: $F_{1,28} = 0.022$; $p = 0.8834$; obesity: $F_{1,28} = 0.071$; $p = 0.7920$; interaction: $F_{2,28} = 0.292$; $p = 0.5935$; Fig.9).

Highly fragmented sleep increases TGF-β1 expression in the spleen

Highly fragmented sleep increased TGF-β1gene expression in the spleen (two-way ANOVA; log-transformed; $F_{1,25} = 6.821$, $p = 0.0150$; Fig.9). Post-hoc tests revealed a significant increase in splenic TGF-β1 gene expression between the control and HSF group ($p = 0.0155$; Fig.9), between the control and OB control ($p = 0.0320$; Fig.9), and between the control and OB HSF group ($p = 0.0049$; Fig.9). However, no other factors affected TGF-β1 gene expression in the spleen (two-way ANOVA; log-transformed; obesity: $F_{1,25} = 3.278$, $p = 0.0823$; interaction: $F_{2,25} = 1.857$, $p = 0.1851$; Fig.9).

Obesity and the interaction between sleep and obesity affect pro-inflammatory cytokine gene expression in inguinal adipose tissue

IL-1β gene expression was significantly different in the inguinal adipose tissue due to obesity (two-way ANOVA; log-transformed; $F_{1,26} = 6.391$; $p = 0.0179$; Fig.9) and the interaction between sleep and obesity (two-way ANOVA; log-transformed; $F_{2,26} = 4.627$; $p = 0.0403$; Fig.9). Post-hoc analysis indicated a decrease in IL-1β gene expression due to obesity between the HSF and OB control ($p = 0.0403$; Fig.9). Additionally, the difference due to an interaction between sleep and obesity was also a decrease between the HSF and OB HSF groups ($p = 0.0035$; Fig.9). Furthermore, sleep did not affect IL-1β gene expression in inguinal adipose tissue (two-way ANOVA; log-transformed; sleep: $F_{1,26} = 0.304$; $p = 0.5859$; Fig.9). TNF-α gene expression was significantly altered in the inguinal adipose tissue due to the interaction between sleep and obesity (two-way ANOVA; log-transformed; $F_{2,28} = 8.886$; $p = 0.0059$; Fig.9). Post-hoc analysis revealed that TNF-α gene expression was significantly increased in inguinal adipose tissue between the control and HSF group due to sleep ($P = 0.0015$; Fig.9), while TNF-α gene expression was decreased due to obesity between the HSF and OB HSF groups ($P = 0.0275$; Fig.9). No other factors affected TNF-α gene expression in the inguinal adipose tissue (two-way ANOVA; log-transformed; sleep: $F_{1,28} = 3.963$; $p = 0.0564$; obesity: $F_{1,28} = 0.095$; $p = 0.0.7596$; Fig.9). Similarly, none of the factors tested affected TGF-β1 gene expression in inguinal adipose tissue (two-way ANOVA; sleep: $F_{1,28} = 0.002$, $p = 0.9623$; obesity: $F_{1,28} = 0.037$, $p = 0.8485$; interaction: $F_{2,28} = 0.011$, $p = 0.9175$; Fig.9).

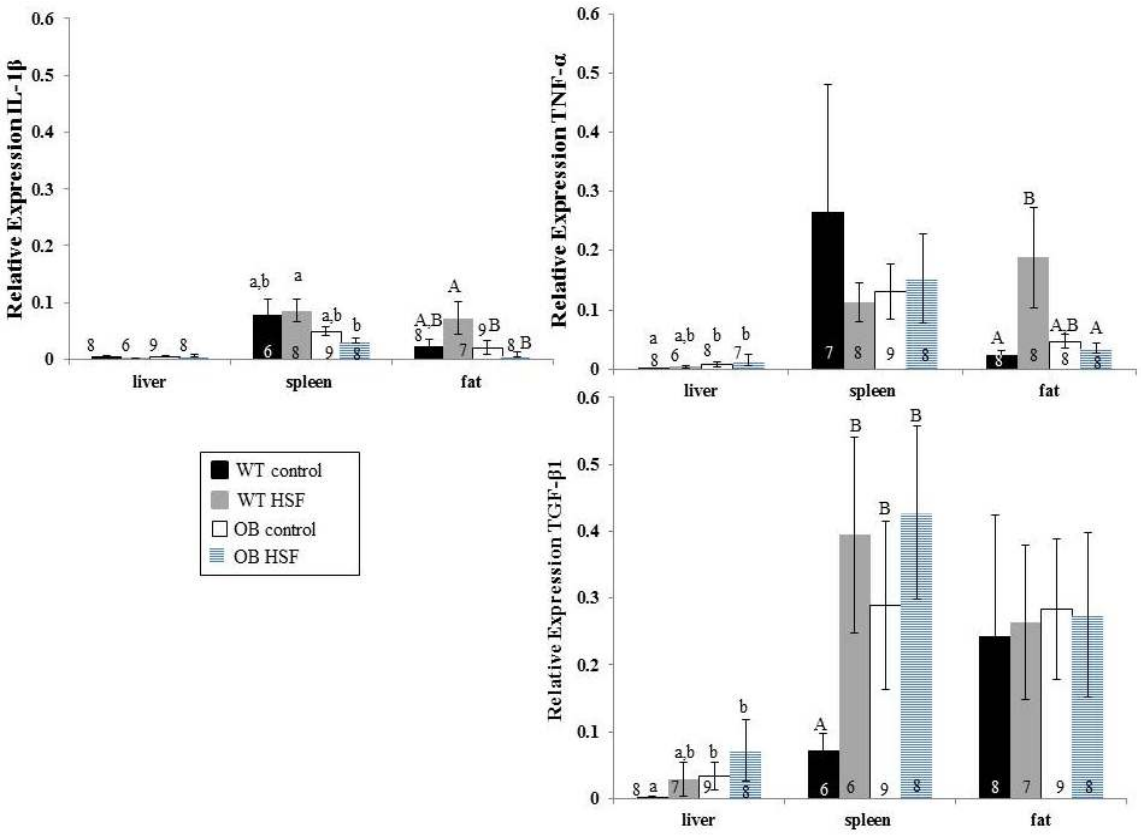

Figure 9. Effects of fragmented sleep, obesity, and the interaction between the two factors on cytokine gene expression in the liver, spleen, and inguinal adipose tissue. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissue samples, resulting in a decreased sample size in these groups. Shared symbols indicate no significant differences between groups. Column capitalization corresponds with the groups being compared. The numbers at the base of the column indicate the sample size of the group.

Cytokine gene expression in cardiac muscle tissue is different for lean and obese mice

Obesity decreased IL-1β gene expression in cardiac muscle tissue (two-way ANOVA; log-transformed, $F_{1,29} = 50.719$; $p < 0.001$; Fig.10). Post-hoc analysis revealed

differences between the control and OB control groups ($p = 0.0005$; Fig. 10), between the control and OB HSF groups ($p < 0.0001$; Fig. 10), between the HSF and OB control group ($p < 0.0001$; Fig. 10), and between the HSF and OB HSF groups ($p < 0.0001$; Fig. 10). No other factors affected IL-1βgene expression (two-way ANOVA; log-transformed: sleep: $F_{1,29} = 0.003$, $p = 0.9555$; interaction: $F_{2,29} = 2.876$, $p = 0.1006$; Fig.10). Additionally, both highly fragmented sleep (two-way ANOVA; log-transformed, $F_{1,29} = 16.106$, $p = 0.0004$; Fig.10) and obesity (two-way ANOVA; log-transformed, $F_{1,29} = 28.964$, $p < 0.001$; Fig.10) decreased TNF- α gene expression in the heart, but there was no interaction between the two factors (two-way ANOVA; log-transformed, $F_{2,29} = 1.660$, $p = 0.2078$; Fig.10) Post-hoc analysis indicated that there was significant difference between the control and OB control groups ($p = 0.0064$, Fig.10), between the control and OB HSF group ($p < 0.0001$; Fig. 10), between the HSF and OB HSF groups ($p < 0.0001$, Fig.10), and between the OB control and OB HSF group ($p < 0.0001$; Fig. 10). Furthermore, a significant increase in TGF-β1 gene expression was detected in cardiac muscle tissue due to obesity (two-way ANOVA; $F_{1,29} = 77.344$, $p < 0.001$; Fig.11). Post-hoc analysis revealed differences in TGF-β1 gene expression in cardiac muscle between both the control and OB control groups ($p < 0.0001$; Fig. 11), between the control and OB HSF group ($p < 0.0001$; Fig. 11), between the HSF and OB control ($p < 0.0001$; Fig. 11), and between the HSF and OB HSF groups ($p < 0.0001$; Fig.11). There were no differences in TGF-β1 gene expression due to any of the other factors (two-way ANOVA; sleep: $F_{1,29} = 3.275$, $p = 0.0807$; interaction: $F_{2,29} = 1.014$, $p = 0.3223$; Fig.11).

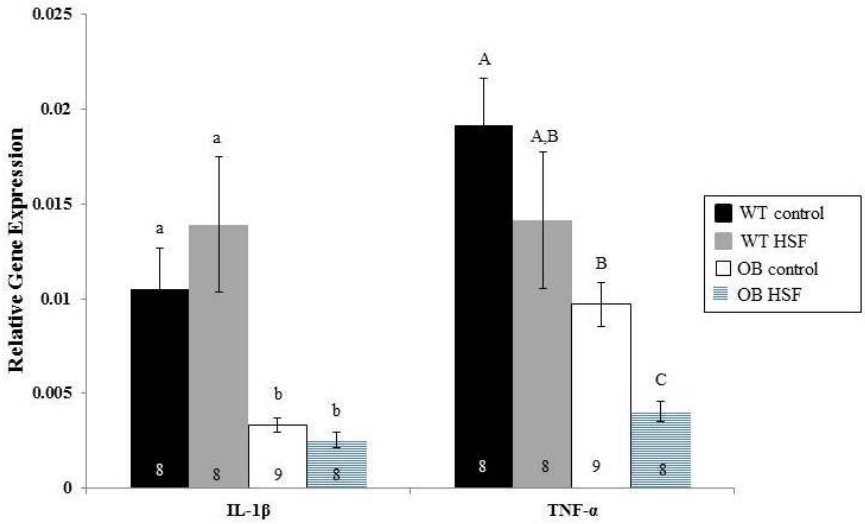

Figure 10. Obesity decreases pro-inflammatory cytokine gene expression profile in cardiac muscle tissue. Data are shown as mean ± SE for each group. Shared symbols indicate no significant differences between groups. Column capitalization corresponds with the groups being compared. The numbers at the base of the column indicate the sample size of the group.

Obesity increases pro-inflammatory cytokine gene expression in the hypothalamus

IL-1β gene expression was significantly elevated in the hypothalamus due to obesity (two-way ANOVA; log-transformed; $F_{1,26} = 11.637$, $p = 0.0021$; Fig.12). Post-hoc tests revealed that IL-1β gene expression was different between control and OB control groups ($p = 0.0195$, Fig. 12), between the control and OB HSF group ($p = 0.0177$, Fig.12), between the HSF and OB control ($p = 0.0296$, Fig.12) and between the HSF and OB HSF groups ($p = 0.0275$, Fig.12). However, no other factors affected IL-1β gene expression in the hypothalamus (two-way ANOVA; log-transformed, sleep: $F_{1,26} = 0.002$, $p = 0.9619$; interaction: $F_{2,26} = 0.012$, $p = 0.9138$; Fig.12). Additionally, obesity increased TNF-α

gene expression in the hypothalamus (two-way ANOVA; log-transformed, $F_{1,25}$ = 29.267, $p < 0.0001$; Fig.12). Post-hoc analysis revealed significant differences between the control and OB control ($p = 0.0038$; Fig.12), between the control and OB HSF group ($p = 0.0035$; Fig.12), between the HSF group and the OB control ($p = 0.0002$; Fig.12), and between the HSF and OB HSF groups ($p < 0.0001$; Fig.12). However, no other factors affected TNF-α gene expression in the hypothalamus (two-way ANOVA; log-transformed; sleep: $F_{2,25} = 1.196$, $p = 0.2845$; interaction: $F_{1.25} = 0.655$, $p = 0.4260$; Fig.12). Additionally, there were no significant differences in TGF-β1 gene expression in the hypothalamus due to any of the factors tested (two-way ANOVA; log-transformed; sleep: $F_{1,25} = 2.294$; $p = 0.1424$; obesity: $F_{1,25} = 0.035$; $p = 0.8541$; interaction: $F_{2,25}$ = 0.214; $p = 0.6474$; Fig.12).

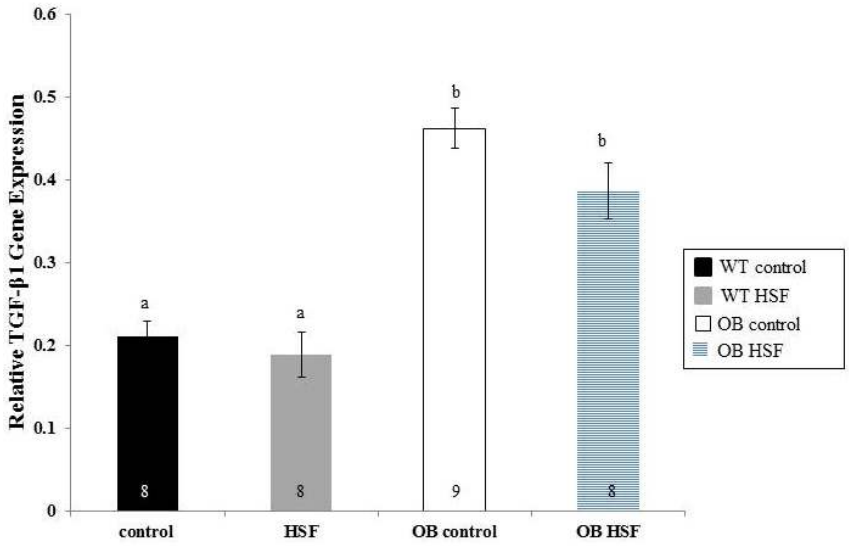

Figure 11. Obesity increases anti- inflammatory cytokine gene expression profile in cardiac muscle tissue. Data are shown as mean ± SE for each group. Shared symbols indicate no significant differences between groups. The numbers at the base of the column indicate the sample size of the group.

Obesity increases pro-inflammatory cytokine gene expression in the hypothalamus

IL-1β gene expression was significantly elevated in the hypothalamus due to obesity (two-way ANOVA; log-transformed; $F_{1,26} = 11.637$, $p = 0.0021$; Fig.12). Post-hoc tests revealed that IL-1β gene expression was different between control and OB control groups ($p = 0.0195$, Fig.12), between the control and OB HSF group ($p = 0.0177$, Fig.12), between the HSF and OB control ($p = 0.0296$, Fig.12) and between the HSF and OB HSF groups ($p = 0.0275$, Fig.12). However, obesity was the only factor affecting this increase (two-way ANOVA; log-transformed, sleep: $F_{1,26} = 0.002$, $p = 0.9619$; interaction: $F_{2,26} = 0.012$, $p = 0.9138$; Fig.12). Additionally, obesity increased TNF-α gene expression in the hypothalamus (two-way ANOVA; log-transformed, $F_{1,25} = 29.267$, $p < 0.0001$; Fig.12). Post-hoc analysis revealed significant differences between the control and OB control ($p = 0.0038$; Fig.12), between the control and OB HSF group ($p = 0.0035$; Fig.12), between the HSF group and the OB control ($p = 0.0002$; Fig.12), and between the HSF and OB HSF groups ($p < 0.0001$; Fig.12). However, sleep and the interaction between sleep and obesity did not affect the changes TNF-α gene expression in the hypothalamus (two-way ANOVA; log-transformed; sleep: $F_{2,25} = 1.196$, $p = 0.2845$; interaction: $F_{1.25} = 0.655$, $p = 0.4260$; Fig.12). Furthermore, there were no significant differences in TGF-β1 gene expression in the hypothalamus for any of the factors tested (two-way ANOVA; log-transformed; sleep: $F_{1,25} = 2.294$; $p = 0.1424$; obesity: $F_{1,25} = 0.035$; $p = 0.8541$; interaction: $F_{2,25} = 0.214$; $p = 0.6474$; Fig.12).

Obesity increases IL-1β gene expression in the prefrontal cortex

IL-1β gene expression was significantly elevated in the prefrontal cortex as a result of obesity (two-way ANOVA; log-transformed; obesity: $F_{1,27} = 6.899$; $p = 0.0140$; Fig.12). Post-hoc tests indicated that the difference was between the control and OB control ($p =$ 0.0.0372; Fig.12) and between the control and OB HSF group ($p = 0.0108$; Fig.12). However, no other factors affected IL-1β gene expression (two-way ANOVA; log-transformed; sleep: $F_{1,27} = 1.432$; $p = 0.2419$; interaction: $F_{2,27} = 0.264$; $p = 0.6115$; Fig.12). Additionally, there were no significant changes in TNF-α gene expression (two-way ANOVA; log-transformed; sleep: $F_{1,27} = 0.319$; $p = 0.5769$; obesity: $F_{1,27} = 0.225$; p = 0.6394; interaction: $F_{2,27} = 0.118$; $p = 0.7340$; Fig.12) or TGF-β1 gene expression (two-way ANOVA; sleep: $F_{1,28} = 0.006$; $p = 0.9388$; obesity: $F_{1,28} = 0.066$; $p = 0.7985$; interaction: $F_{2,28} = 1.276$; $p = 0.2682$; Fig.12) for any of the factors tested in the prefrontal cortex.

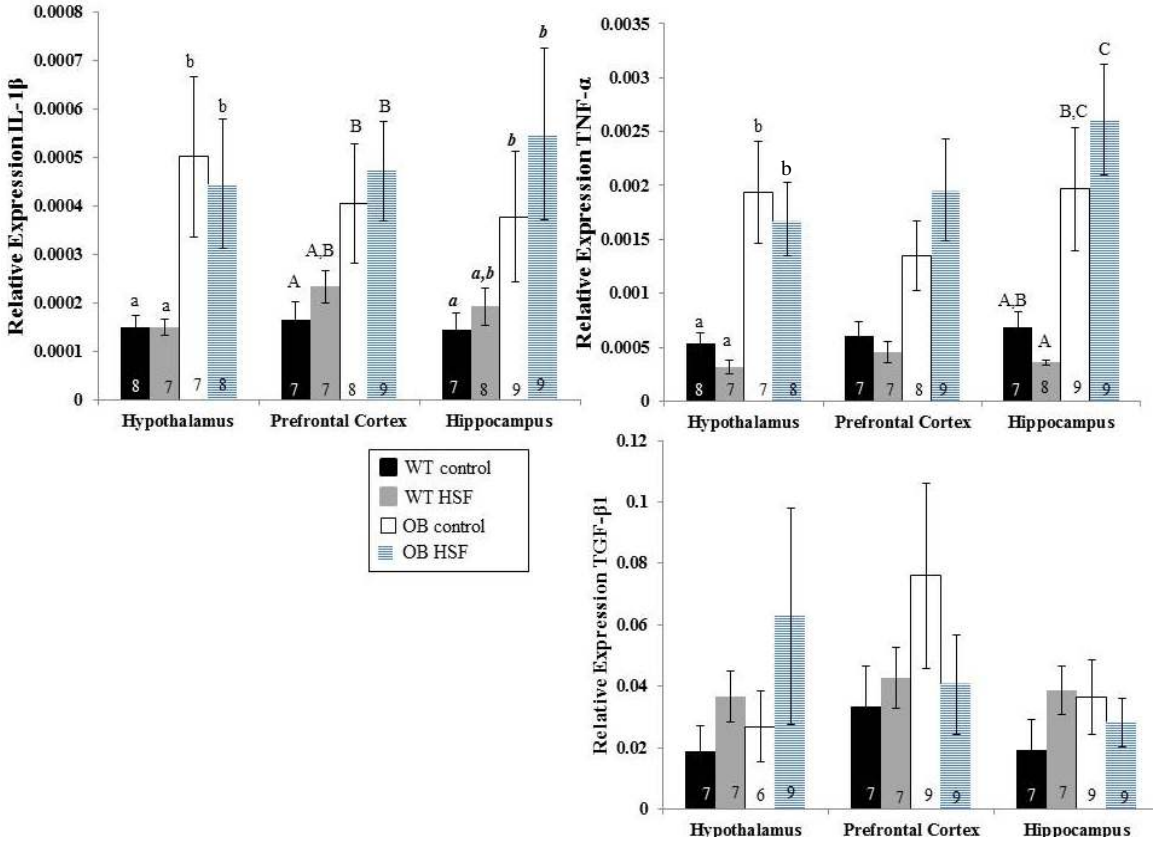

Figure 12. Obesity increases pro-inflammatory cytokine gene expression in the brain. Data are shown as mean ± SE for each group. Gene expression was undetectable by RT-PCR in some tissues, which resulted in a decreased sample size in these groups. Shared symbols indicate no significant differences between groups. Column capitalization corresponds with the groups being compared. The numbers at the base of the column indicate the sample size of the group.

Obesity increases pro-inflammatory cytokine gene expression in the hippocampus

Obesity increased IL-1β gene expression in the hippocampus (two-way ANOVA; log-transformed, $F_{1,28} = 9.876$, $p = 0.0039$; Fig.12). Post-hoc tests revealed the difference was between the control and OB control ($p = 0.0182$; Fig.12) and between the control and OB

HSF group ($p = 0.00281$; Fig.12). However, no other factors affected IL-1β gene expression in the hippocampus (two-way ANOVA; log-transformed, sleep: $F_{1,28} = 2.218$, $p = 0.0.1476$; interaction: $F_{2,28} = 0.165$, $p = 0.6880$; Fig.12). Additionally, obesity increased TNF-α gene expression in the hippocampus (two-way ANOVA; log-transformed, $F_{1,29} = 24.539$, $p < 0.0001$; Fig.12). Post-hoc analysis revealed significant differences between the control and OB HSF group ($p = 0.0019$; Fig.12), between the HSF and OB control ($p = 0.0012$; Fig.12), and between the HSF and OB HSF groups ($p < 0.0001$; Fig.12). However, no other factors affect TNF-α gene expression in the hippocampus (two-way ANOVA; log-transformed; sleep: $F_{1,29} = 0.001$, $p = 0.9799$; interaction: $F_{2,29} = 4.123$, $p = 0.0516$; Fig.12). Similarly, there was no significant difference in TGF-β1 gene expression in the hippocampus due to any of the factors (two-way ANOVA; sleep: $F_{1,27} = 0.074$; $p = 0.7873$; obesity: $F_{1,27} = 0.127$; $p = 0.7240$; interaction: $F_{2,27} = 1.116$; $p = 0.3002$; Fig.12).

Sleep fragmentation increases serum corticosterone

Serum corticosterone concentration varied significantly among treatments due to sleep (two-way ANOVA; $F_{1,30} = 41.101$; $p < 0.0001$; Fig.13). Post-hoc tests revealed that lean mice exposed to HSF (200.58 ng/mL ± 30.02; mean ± SE) had significantly increased serum corticosterone ($p = 0.0310$, Fig.13) when compared with control mice (112.36 ng/mL ± 16.89; mean ± SE). Additionally, the OB HSF mice had significantly higher serum corticosterone concentrations than the lean control mice ($p = 0.0083$, Fig.13) and also higher levels than the OB control mice (140.71 ng/mL ± 20.65; mean ± SE) ($p = 0.0404$, Fig.13). However, serum corticosterone concentration did not differ significantly

due to obesity (two-way ANOVA; $F_{1,30} = 0.777$; $p = 0.3850$; Fig.13) or as a result of the interaction between sleep and obesity (two-way ANOVA; $F_{2,30} = 0.031$; $p = 0.8609$; Fig.13).

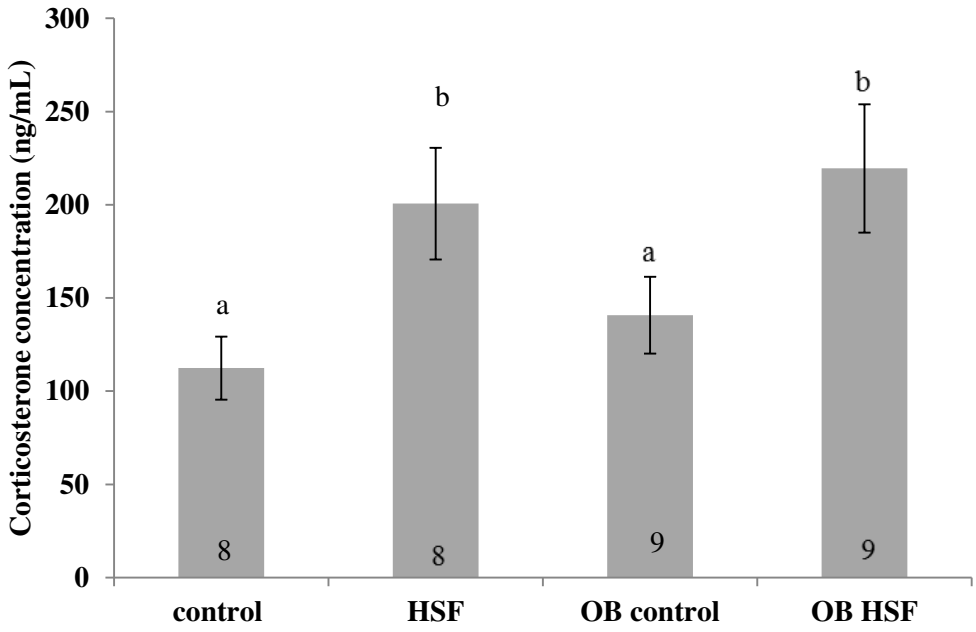

Figure 13. Serum corticosterone concentrations increase following 24 hours of sleep fragmentation in both lean and obese mice. Data are shown as mean (ng/ mL) ± SE for each group. Shared letters indicate there is no significant difference between groups. Numbers at the base of the column indicate sample size.

DISCUSSION

Obesity and sleep fragmentation are often times concurrent conditions in patients with OSA (Shahar et al., 2001). Independently, both are known to be pro-inflammatory conditions, but there is limited information regarding the endocrine and immune consequences of simultaneous occurrence of both obesity and sleep curtailment. Currently, the only published experiment examining the interplay between obesity and sleep fragmentation is a behavioral experiment investigating the two within the context of pain sensitivity and anxiety (He et al., 2015). As a result, the findings of this experiment are novel in the field. Previous literature indicates that obesity increases the cytokine gene expression profile overall, with both pro-inflammatory (IL-1β, TNF-α, IFN-γ, IL-6, IL-12) and anti-inflammatory cytokine (IL-4, IL-10) levels being elevated in the serum of obese humans, chimpanzees, and mice (Fjeldborg et al., 2014; Nehete et al., 2014; Unnikrishnan et al., 2015). Additionally, it has also been demonstrated that sleep fragmentation results in augmented expression of IL-1β, TNF-α, CRP, IL-6, IL-8 in both the serum and adipose tissue of humans and rodents (Ciftci et al., 2004; Kent et al., 2011; Venancio and Suchecki, 2014). The effects of chronic low-grade inflammation, due to either obesity or OSA, have been demonstrated to contribute to the development of conditions such as metabolic syndrome, diabetes, hypertension, and cardiovascular disease (McNicholas et al., 2007; Ryan et. al, 2009; Zhang et al., 2014). Because obesity is a pro-inflammatory state, and because disruptions during sleep in patients with OSA further exacerbate inflammation, the summative effect of these two conditions has the potential to be increasingly detrimental on the physiological integrity of the organism.

In this study, obese and lean mice exhibited different immunological responses to acute sleep fragmentation. Obesity was the primary factor that increased the inflammatory environment, possibly creating a ceiling effect, by which inflammatory responses to SF were overwhelmed by the effects from obesity. Changes in cytokine gene expression were observed in all tissues of interest and the pro-inflammatory versus anti-inflammatory expression profile was heterogeneous in various tissue locations. Simultaneous alterations in pro-inflammatory and anti-inflammatory cytokine gene expression were found in the liver (obesity), spleen (obesity and sleep), and cardiac muscle tissue (obesity). The inguinal adipose tissue (interaction) was dominated by a decrease in pro-inflammatory gene expression, while all 3 regions of the brain were characterized by a pro-inflammatory response attributable to obesity.

Interestingly, in the spleen, fat, and heart, the gene expression of pro-inflammatory cytokines (IL-1β and TNF-α) decreased with obesity. This result was surprising given the previous literature describing elevated circulating TNF-α, IL-6, CRP, IL-1β, and IFN-γ associated with both OSA and obesity independently (Kent et al., 2011; Nehete et al., 2014). However, despite this difference in the peripheral tissue, the immune response of the brain was dominated by increases in pro-inflammatory cytokines in hypothalamus, prefrontal cortex, and hippocampus, as a result of obesity. This increase supported previous literature detailing obesity induced states of neuroinflammation and association with psychopathologies (Aguilar-Valles et al., 2015; He et al., 2015). As a result, it can be concluded that in addition to having different responses between obese and lean individuals, our data seem to indicate a heterogeneous response in the brain and periphery, as a result of obesity. Previous literature suggests that the effects of OSA and

obesity on cytokine gene expression would be additive, due to the pro-inflammatory nature of the two factors independently (Unnikrishnan et al., 2015). However, based upon the decreased pro-inflammatory cytokine gene expression in the obese mice when compared to the lean in the spleen, fat, and heart, the results of this experiment support the hypothesis that neuroendocrine pre-adaptions exist in obese mice as a result of chronic exposure to the low-grade inflammation associated with obesity (He et al., 2015; Pak et al., 2014).

Table 2. The effect of obesity on cytokine gene expression following SF.

Tissue	Effect	Factor
Liver	Increase: TNF-α and TGF-β1	Obesity
Spleen	Decrease: IL-1β	Obesity
	Increase: TGF-β1	Sleep
Fat	Decrease: IL-1β	Obesity and Interaction
	Increase: TNF-α (sleep)	Interaction
	Decrease: TNF-α (OB)	Interaction
Heart	Decrease: IL-1β and TNF-α	Obesity
	Increase: TGF-β1	
Hypothalamus	Increase: IL-1β and TNF-α	Obesity
Prefrontal Cortex	Increase: IL-1β	Obesity
Hippocampus	Increase: IL-1β and TNF-α	Obesity

Chronic SF has been shown to increase orexigenic behavior, while caloric expenditure remains unchanged, ultimately leading to weight gain from excess food intake (Carreras et al., 2015; Wang et al., 2014). Previous literature has indicated that these changes in weight gain are mediated by alterations in the expressions of leptin and ghrelin, satiety and orexigenic hormones, respectively, that act antagonistically on the

hypothalamus to maintain energy balance (Garcia-Garcia et al., 2014). Although the obese mice were leptin gene knockouts, both the sleep fragmented obese mice and lean mice lost significant body mass when compared to the control mice following acute sleep fragmentation. Consequently, this suggests that the alterations in orexigenic behavior following sleep curtailment are multifaceted and cannot just be explained by a simultaneous increase in ghrelin and decrease in leptin, at least in the short-term (Pak et al., 2014). Alternatively, the mass fluctuations following acute sleep fragmentation may also be impacted by the physiological stress response or increased locomotion during the pronged period of wakefulness. Furthermore, the amount of body mass lost due to acute sleep fragmentation did not depend upon the initial body weight, as the lean and obese mice lost similar amounts of body mass. The only factor affecting this observed difference was sleep. While weight gain is typically observed in chronic sleep fragmentation experiments involving mice, this weight gain is time dependent. The progression towards metabolic syndrome, characterized by reduced glucose clearance and reduced insulin sensitivity, does not impact body weight until after 2 weeks of experimentation (Carreras et al., 2014). As a result of the acute nature of this experiment, a body weight loss is observed in both the obese and lean HSF groups following sleep fragmentation.

Chronic exposure to sleep fragmentation and the hypoxic intervals associated with OSA result in the accumulation of cell damage (Pak et al., 2014). Cells in the pancreas, liver, heart, and adipose tissue have been hypothesized to be damaged due to oxidative stress and inflammation. Taken together, this damage explains the reduced insulin sensitivity, reduced glucose clearance, and disruption of elastic fibers in cardiac

vessel endothelium that have been observed with chronic sleep fragmentation (Carreras et al., 2014 ; Jia et al., 2014; Youn et al., 2014; Zhang et al., 2014). To counteract the negative effects of inflammation in the tissues most impacted by metabolic dysfunction, there must be some type of physiological anti-inflammatory response. It has been demonstrated that both pro-inflammatory and anti-inflammatory cytokines have increased expression in the tissue of obese individuals compared to lean individuals (Fjeldborg et al., 2014). The propensity toward an anti-inflammatory response could be protective in this case, as obese individuals would be at the greatest risk for the development of metabolic abnormalities (Youn et al., 2014). The simultaneous increase of pro-inflammatory and anti-inflammatory cytokines in the liver tissue of obese mice supports previous literature that indicates an overall elevation in cytokine levels in the tissue of obese individuals compared to the lean counterparts (Fjeldborg et al., 2014). Because the liver is an essential insulin response tissue, the increase in anti-inflammatory cytokines in obese mice could be protective in preventing tissue damage due to chronic inflammation from obesity. Helping to further underscore the need to protect insulin-sensitive hepatocytes from inflammatory damage is the fact that it has been demonstrated that TLR-4 expression in the liver is important in regulating metabolic disorders, including inflammation and insulin resistance (Jia et al., 2014).

In contrast to the increases in pro-inflammatory cytokine gene expression associated with both obesity and sleep fragmentation detailed in the literature, IL-1β gene expression decreased in both the spleen and inguinal adipose tissue as a result of obesity (Kent et al., 2011; Nehete et al., 2014). This finding directly opposes the increased IL-1β gene expression found in the fat in Chapter 1 and previous observations that have

characterized obesity as a pro-inflammatory state, with increases in both the potent pro-inflammatory cytokines TNF-α and IL-6 (Pak et al., 2014). However, this discrepancy may be explained by the gene KO that was chosen for this experiment. Under normal physiological conditions, leptin is a pro-inflammatory signal that helps to support immune cell activation, proliferation, differentiation, and the production of pro-inflammatory cytokines, including IL-1β and TNF-α (Besedovsky et al. 2012). Because the leptin gene is absent in the ob/ob KO mice, the normal effect of leptin on gene expression of IL-1β and TNF-α has been eliminated. Alternatively, the decrease in pro-inflammatory cytokines following sleep fragmentation could also be a neuroendocrine adaptation in the obese mice to minimize tissue damage from the pre-existing pro-inflammatory condition associated with obesity (Pak et al., 2014). As a potential protective mechanism, the obese mice could have reduced pro-inflammatory cytokine gene expression to protect important metabolic tissues, as previously mentioned. The interaction between sleep and obesity also altered TNF-α gene expression in the adipose tissue. The effect observed due to sleep was similar to the results of previous experiments (Makki et al., 2013; Neto et al., 2010). The only observed alteration in cytokine gene expression as a result of sleep was observed in the spleen with TGF-β1. This result is surprising, given the previous literature implicating sleep fragmentation alone as a pro-inflammatory condition (Ciftci et al., 2004; Kent et al., 2011). Again, because TGF-β1 is an anti-inflammatory cytokine, this increase suggests a potential preventative response to combat inflammatory damage due to acute sleep loss occurring in the spleen.

In addition to the risk associated with the development of metabolic dysfunction resulting from sleep disruption, chronic sleep fragmentation can also lead to

cardiovascular alterations (Carreras et al., 2014; Kheirandish-Gozal et al., 2010; Zhang et al., 2014). Shortened sleep intervals unfavorably alter protein expression profiles, such that increases in the production of white blood cells, C - Reactive protein, IL-6 are observed, along with a simultaneous decrease in the anti-inflammatory cytokine IL-10 (Gozal et al., 2008; Kheirandish-Gozal et al., 2010; Mullington et al., 2009). Taken together with endothelial dysfunction and thickening and disruption of elastic fibers in the aortic arch and thoracic aorta as a result of sleep fragmentation, these changes unfavorably compound the risk for the development of cardiovascular disease (Carreras et al., 2014). Furthermore, left ventricle hypertrophy, pulmonary hypertension, diastolic dysfunction, and cardiac dilation attributed to obesity pose an additional independent risk for the development of cardiovascular disease (Otto et al., 2007). As a result, obese individuals with untreated OSA likely have a hightened risk for the development of cardiovascular disease, due to chronic exposure to inflammatory compounds. However, because this risk increases with cumulative exposure to inflammatory compounds over time, the results of an acute experiment may not be a direct link to replicate is occurring in these individuals. In lieu of this noted shortcoming, an acute model does provide insight into the initiation of the inflammatory response, which is vital in understanding the transition towards chronic pathology. Ultimately the results of the acute model are connected to what is occurring in a chronic model, but are not a direct link.

Previous literature indicates that a combination of OSA and previous cardiovascular events results in increased inflammatory cytokine expression (Testlemans et al., 2013). Similarly, it is also reported that obesity alone increases inflammation and hypertrophy in the heart (Unsold et al., 2015). However, in contrast to the increase in

pro-inflammatory cytokines that would be expected, there was a decrease in IL-1β and TNF-α in cardiac muscle tissue, observed with a simultaneous increase in TGF-β1, all attributable to obesity. The increase in the anti-inflammatory cytokine supports previous findings in which the entire cytokine gene expression profile was elevated in the tissue of obese individuals compared to that of lean individuals and also supports modulation of the inflammatory response to prevent damage to vital tissue (Fjeldborg et al., 2014). After a chronic period of sleep loss, the onset of cardiovascular disease is proposed to be a consequence of damage due to a pathway involving oxidative stress induced by the products of the NOX-2 enzyme of macrophages (Carreras et al., 2014; Zhang et al., 2014). The heightened TGF-β1 gene expression in the cardiac muscle tissue of obese individuals again suggests the presence of a protective response against cardiac inflammation. Additionally, the decrease in pro-inflammatory cytokines can be attributed to the absence of leptin in these animals and is therefore a consequence of the gene KO that was chosen. The observed alterations in cytokine gene expression in this experiment were detected at the mRNA level. Previous experimentation has shown that mRNA and protein expression levels of TNF-α are correlated following experimental sleep loss and vaccine stimulation. However, experiments involving IL-1β have produced conflicting results and TGF-β1 has not been studied within this context (Irwin et al., 2006, Shebl et al., 2010). Consequently, it is equivocal whether protein levels correlate directly with mRNA expression, but nonetheless, increased mRNA expression still provides insight into the rapid temporal dynamics of cytokine activation at the transcriptional level.

Sleep fragmentation altered serum corticosterone concentration independently of initial body mass. Both the lean and obese HSF groups exhibited increased serum

glucocorticoid concentrations, but there was no difference detected between these two groups. This result supports the findings of the previous experiment in Chapter 1 and also replicates previously reported increases in serum corticosterone following varying times of sleep deprivation (Ashely et al., 2013; Bonnavion et al., 2015; Neto et al., 2010). Although the ob/ob mice are known to exhibit hypercorticosteronemia, the serum corticosterone concentrations of the obese mice were not found to be different from those of the lean mice in this experiment. The corticosterone concentrations may be biologically elevated in the obese mice, but there was no statistically significant difference between the two mouse strains.

In rodents, it has been demonstrated that leptin inhibits release of stress hormones by means of the HPA-axis and that deficiency in leptin affects HPA signaling, resulting in HPA-hyperactivation (Bonnavion et al., 2015). Because the ob/ob mice are leptin knockouts, it would be expected that excess circulating corticosterone could impact the experimental results, through the anti-inflammatory action of glucocorticoids. However, because there is no difference between the lean and obese serum corticosterone concentrations, hypercorticosteronemia cannot fully explain the decreased cytokine gene expression due to obesity, indicating that these observed differences are due to obesity or other physiological variables associated with obesity, such as insulin concentration. Furthermore, the increase in serum corticosterone concentration and mass loss as a result of HSF in both the lean and obese mice suggest the animals are experiencing a physiological stress response. Alternatively, increases in serum corticosterone concentration could have been attributed to a shift in natural biological rhythms from forced arousals during the light phase of the LD cycle or to an increase in activity levels

in the SF groups as a result of tactile stimulation from the swipe bar. The combination of body mass loss and simultaneous increase in serum corticosterone concentrations suggest that HSF mice were experiencing a physiological stress response, including activation of the HPA axis, as a result of sleep interruptions occurring so frequently. However, it is unclear whether HPA activation occurred because of the method of sleep fragmentation used or the direct effect of disrupted sleep *per se*.

The majority of previous experiments investigating the effect of sleep loss on the immune system have utilized experimental models simulating either OSA or obesity, but have not used a model that combines the two conditions. Chronic sleep interruptions from OSA influence overall the restfulness of sleep and have been shown to induce an acute inflammatory response (Gozal et al., 2008; Kent et al., 2011; Pulixi et al. 2014). Similarly, obesity is also known to be a pro-inflammatory condition, with adipose tissue exhibiting an endocrine function and secreting pro-inflammatory cytokines and other adipokines into the bloodstream (Ryan et al., 2009). Due to the pro-inflammatory nature of both conditions, it can reasonably be concluded that when both conditions exist concurrently, there will be some type of inflammatory response initiated; however, this response has yet to be characterized.

It is known that sleep fragmentation has differential effects on the behavior of lean and obese mice, but there has been no investigation into effects upon immune function (He et al., 2015). Because obesity and sleep fragmentation associated with OSA often times occur simultaneously, there is a need to understand the interplay between the pro-inflammatory responses resulting from both conditions. Furthermore, if the resulting inflammatory response is different within the context of obesity, this may suggest that

obese and lean individuals suffering from OSA may be responding to the disease and treatments differently. In the present study, we provide compelling evidence in support of differential cytokine gene expression between lean and obese mice and found that this response was heterogeneous based upon tissue location. Because the results of this experiment suggest that the absence of leptin may be affecting cytokine gene expression in the obese mice, this experiment should be replicated within the context of a diet induced model of obesity to tease out the effects of leptin. Despite this, the current results suggest that a pro-inflammatory environment was induced in the brain of obese mice, while a protective anti-inflammatory environment was produced in the periphery of the same animals, all within the context of heightened serum corticosterone concentrations.

APPENDIX A: SUPPLEMENTAL FIGURES

Figure 14. The Model 80391 Sleep Fragmentation Chamber that was used for this experiment (Lafayette Industries).

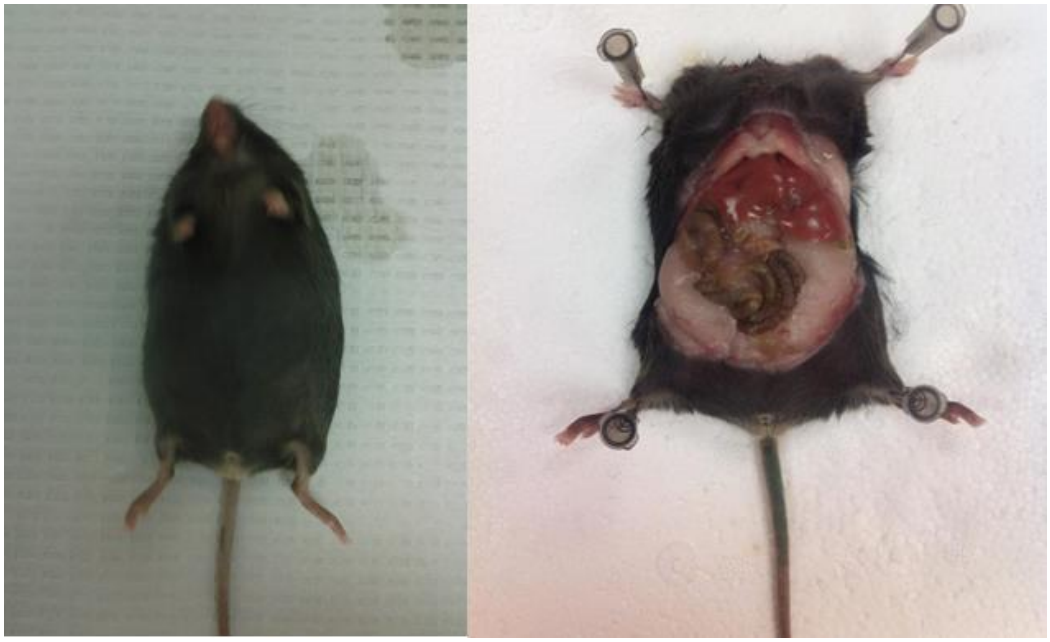

Figure 15. 8 week old male ob/ob KO mouse before and after dissection.

Figure 16. Male littermates from breeding of heterozygous ob mice at 8 weeks of age. From left to right, the mice are homozygous dominant, heterozygous ob, homozygous recessive ob/ob knock out (obese mouse).

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