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Effects of Alpha- and Beta-Adrenergic Receptor Blockade upon Inflammatory Responses to Acute And Chronic Sleep Fragmentation

Nicholas David Wheeler
Western Kentucky University, nicholas.wheeler101@gmail.com

EFFECTS OF ALPHA- AND BETA-ADRENERGIC RECEPTOR BLOCKADE UPON INFLAMMATORY RESPONSES TO ACUTE AND CHRONIC SLEEP FRAGMENTATION

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EFFECTS OF ALPHA- AND BETA-ADRENERGIC RECEPTOR BLOCKADE UPON INFLAMMATORY RESPONSES TO ACUTE AND CHRONIC SLEEP FRAGMENTATION

Date Recommended June 29, 2020

Ashley, Noah
Digitally signed by Ashley, Noah
DN: cn=Ashley, Noah, o=Western Kentucky University, ou=Dept. of Biology, email=noah.ashley@wku.edu, c=US
Date: 2020.07.16 21:17:40 -04'00'

Noah T. Ashley, Ph.D., Primary Advisor

Banga, Simran
Digitally signed by Banga, Simran
DN: cn=Banga, Simran, o, ou, email=simran.banga@wku.edu, c=US
Date: 2020.07.16 09:52:03 -05'00'

Simran Banga, Ph.D., Committee Member

Smith, Michael
Digitally signed by Smith, Michael
DN: cn=Smith, Michael, o=Western Kentucky University, ou=Department of Biology/Mahurin Honors College, email=michael.smith1@wku.edu, c=US
Date: 2020.07.15 11:55:15 -05'00'

Michael E. Smith, Ph.D., Committee Member

Ranjit T. Koodali
Digitally signed by Ranjit T. Koodali
Date: 2020.07.27 13:41:23 -05'00'

Associate Provost for Research and Graduate Education

I dedicate this thesis to David R. Wheeler, MSW.

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EFFECTS OF ALPHA- AND BETA-ADRENERGIC RECEPTOR BLOCKADE UPON INFLAMMATORY RESPONSES TO ACUTE AND CHRONIC SLEEP FRAGMENTATION

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Directed by: Noah T. Ashley, Simran Banga, and Michael E. Smith

Department of Biology Western Kentucky University

Generally, sleep is viewed as a recuperative process and its dysregulation has cognitive, metabolic, immunological, and inflammatory implications that are largely deleterious to human health. Epidemiological and empirical studies have suggested that sleep fragmentation (SF) as result of obstructive sleep apnea (OSA) and other sleep abnormalities leads to pronounced systemic inflammatory responses, which are influenced by the sympathetic nervous system (SNS). However, the underlying molecular mechanisms contributing to SNS regulation of SF-induced inflammatory states are not fully understood. To assess the effects of the SNS system, C57BL/6j female mice were placed in automated SF chambers (12L:12D) and subjected to either control conditions, acute sleep fragmentation (ASF), or chronic sleep fragmentation (CSF) and injected with either a pharmacological adrenergic receptor antagonist or saline. ASF comprised of an automated bar sweep every 120sec for 24h (during 12L:12D; both resting and active periods), whereas CSF comprised of one automated bar sweep every 120sec for 12h (during the 12L; resting period) over a period of 4 weeks. Adrenergic blockade was achieved through an intraperitoneal injection of either phentolamine (5mg/kg BW), an α (alpha)-receptor blocker, or propranolol (5mg/kg BW), a β (beta)-receptor blocker. Serum corticosterone concentration, brain and peripheral cytokine (IL-1β, TNFα, and

TGFβ) mRNA expression, and body mass were assessed. Both ASF and CSF significantly elevated serum corticosterone concentrations as well as cytokine mRNA expression levels, and mice subjected to CSF experienced a significant decrease in body mass. Mice subjected to CSF and treated with phentolamine or propranolol had a greater propensity for a decrease in cytokine gene expression compared with ASF, but effects were tissue-specific. Taken together, these results suggest that both α- and beta-adrenergic receptors contribute to the SNS mediation of inflammatory responses, and adrenergic antagonists can effectively mitigate inflammation from SF in some tissues.

INTRODUCTION

Sleep is a conserved recuperative process and its dysregulation has cognitive, metabolic, immunological, and inflammatory implications that are largely deleterious to human health (Faraut et al., 2012; Zhu et al., 2012). Sufficient sleep is vital for cardiovascular health, and decreased sleep duration has been shown to be specifically correlated with increased cardiovascular morbidity (Wolk et al., 2005; Ferrie et al., 2007; Meier-Ewert et al., 2009). Additionally, a recent study in mice has discovered that the flow of cerebrospinal fluid increases during sleep, facilitating the removal of metabolic waste products from the brain (Xie et al., 2013). If not removed, the accumulation of metabolic waste due to chronic sleep loss could adversely affect the immune system, thus predisposing individuals towards disease. With the increased prevalence of chronic sleep loss, it is essential to understand sleep-immune interactions due to the important public health implications. Many of these sleep abnormalities have been linked to a higher occurrence of obstructive sleep apnea (OSA) as a result of the obesity epidemic (Peppard et al., 2000; Young et al., 2002).

Epidemiological and empirical studies indicate that OSA induces sleep fragmentation (SF), promoting pronounced inflammatory responses in the brain and periphery (Frey et al., 2007; Wisor et al., 2011). Thus, this pro-inflammatory phenotype could provide a link between OSA and adverse health outcomes. For example, the accumulation of inflammatory insults due to cardiovascular disease can lead to a low-grade, chronic inflammatory state fostering the rupture of plaques and endothelial dysfunction (Mullington et al., 2009). Furthermore, dysregulated sleep and OSA is associated with increased circulating concentrations of proinflammatory cytokines,

including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α (Faraut et al., 2012). Pro-inflammatory cytokines attract and upregulate mediators of the inflammatory development, stimulating the production of hepatic acute phase proteins such as C-reactive protein (CRP). In contrast to cytokines, CRP does not display diurnal variation (Meier-Ewert et al., 2001), and elevated levels have been associated with individuals predisposed to cardiovascular disease (Mullington et al., 2009; Nagai et al., 2010).

There is evidence that hormonal and vascular responses regulate inflammatory responses; however, there are few empirical studies investigating these responses within the context of sleep loss. The activation of the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS) are commonly identified as physiological stress responses (Suchecki et al., 1998; Meerlo et al., 2008), and can be triggered by sleep deprivation or restriction. Sleep curtailment can increase SNS activity and subsequently an elevation of norepinephrine (NE) released through noradrenergic neurons and NE and epinephrine (E) from the adrenal medullae. In minutes following SNS activation, glucocorticoids are released from adrenal cortices via HPA activation (Suchecki et al., 1998; Meerlo et al., 2008). While effects of acute sleep loss on SNS and HPA activity can be considered mild or adaptive, chronic sleep loss caused by OSA, shift work, and modern lifestyles can contribute to more deleterious effects such as cardiovascular and metabolic disease, obesity and neurological disorders (Schwartz et al., 1999; Mavanji et al., 2012; Zielinksi et al., 2013). In both mice and humans, there is accumulating evidence that sleep abnormalities increase production of pro-inflammatory cytokines in neural and peripheral tissues (Irwin et al., 2006; Chennaoui et al., 2011; Kim et al., 2011; Ashley et al., 2016), thus providing a connection between sleep loss and chronic disease.

The production of catecholamines by the SNS has been shown to occur in response to various forms of sleep loss (Dismdale et al., 1995; Tiemeier et al., 2002; Mishra et al., 2020). Furthermore, previous research from our lab confirms that catecholamines play a modulatory role upon inflammatory phenotypes induced by acute and chronic SF (Mishra et al., 2020). The aim of this study is to determine whether α- or beta-adrenergic receptors, or both, mediate inflammatory responses to SF through measurement of cytokine gene expression in brain and peripheral tissues. To test the influence of the sympathetic nervous system on inflammatory responses to sleep loss, female C57BL/6 mice were subjected to ASF or CSF conditions and injected with a non-selective beta-adrenergic receptor antagonist, propranolol (5mg/kg BW), a non-selective α-adrenergic receptor antagonist, phentolamine (5mg/kg BW), or vehicle (saline). In the immune system, myeloid cells typically express α- and β-adrenergic receptors, whereas lymphocytes largely express β-adrenergic receptors. Given that the majority of immune cells in the periphery contain β-adrenergic receptors (Liu and Hong, 2003; Kolmus, 2015), we hypothesized that propranolol will more effectively reduce inflammatory mediators than phentolamine from SF. An alternative hypothesis is that phentolamine will suppress inflammatory mediators more than propranolol, suggesting α-adrenergic receptors have a greater influence upon pro-inflammatory responses to SF. Lastly, the finding that both drugs have no effect upon inflammatory responses (the null hypothesis) would suggest that SNS activity has little or no effect upon mediating inflammation from SF.

MATERIALS AND METHODS

Animals

Female C57BL/6j mice (n = 120) were housed in our colony room (12:12-h light-dark cycle, lights on at 0800, 21℃ ± 1℃) at Western Kentucky University. After weaning at 21 days of age, mice were separated into polypropylene cages with same-sex littermates and provided with corncob bedding, and food and water *ad libitum*. This study was conducted under the approval of the Institutional Animal Care and Use Committee at Western Kentucky University (#19-14), and the procedures followed the National Institutes of Health's "Guide for the Use and Care of Laboratory Animals" and international ethics standards.

Experimental Protocol

Female mice >8 weeks of age were selected for experiments and placed in automated sleep fragmentation chambers (Lafayette Instrument Company; Lafayette, IN; model 80390). Female mice were used in consideration of the NIH Notice Number: NOT-OD-15-102, which highlights the over-reliance on male animals and cells in basic and clinical research. This may obscure the understanding of key sex influences on health processes and outcomes; therefore, it is essential to incorporate female organisms into future studies. Each cage contained no more than 5 mice, were provided corncob bedding, and food and water *ad libitum*. Mice were tagged with numbered ear tags and then acclimated to the automated sleep fragmentation chamber for 72 hours prior to initiating the sleep fragmentation experiments.

Experiment 1: Acute Sleep Fragmentation (ASF)

Fifty-three mice received the following pharmacological treatments at 7:30am using a single intraperitoneal injection: 18 mice were treated with phentolamine (5.0 mg/kg BW; an α-adrenergic receptor blocker), 18 mice were treated with propranolol (5.0 mg/kg BW; a β-adrenergic blocker), and 17 mice were treated with vehicle (0.9% NaCl solution). Dosages of phentolamine and propranolol were based upon previous studies that effectively inhibited signaling of the sympathetic nervous system while under varying types of stressful stimuli (Allison et al. 1969, Hermansen and Hyttel 1971, Fabian et al. 1973, Hall et al. 1987, Sim et al. 2012, Jun et al. 2014). Thirty minutes following injections (8:00am, lights on), experimental mice (n = 27; n = 9 injected with phentolamine, n = 9 injected with propranolol, and n = 9 injected with vehicle) were subjected to ASF; i.e. the sweeping bar set to move horizontally at an interval of 120 seconds for 24 hours. This rate is comparable to sleep disruptions that occur in humans with severe sleep apnea (Ramesh 2009, Goyal and Johnson 2017). Control mice (n = 26; n = 9 injected with phentolamine, n = 9 injected with propranolol, and n = 8 injected with vehicle) were not subjected to any bar sweeps, but were still housed in the sleep fragmentation chamber.

Experiment 2: Chronic Sleep Fragmentation (CSF)

To induce CSF, experimental mice (n = 30) were subjected to a horizontal sweeping bar set to move every 120 seconds (30 swipes/h) during the light phase (i.e. from 8:00am to 8:00pm) every day for 4 weeks (28 days), while the control mice (n = 30) were not subjected to any bar sweeps. On the 27th day of CSF, 24 hours prior to tissue collection, experimental mice (n = 30; n = 10 injected with Phentolamine, n = 10 injected with Propranolol, and n = 10 injected with Vehicle) and control mice (n = 30; n = 10

injected with Phentolamine, *n* = 10 injected with Propranolol, and *n* = 10 injected with vehicle) received their respective pharmacological injections at 7:30 am. Mice were weighed on an electronic scale (to the nearest 0.1 g) every week to track changes in body mass in response to CSF. A schematic of the overall experimental design is presented in Figure 1.

Sample Collection

In both experiments, 24 hours following drug administration (8:00 am), mice were deeply anesthetized using isoflurane vapors (< 2min) and rapidly decapitated in < 3min of initial handling for tissue gene expression studies and measurement of baseline corticosterone concentration. Trunk blood from decapitated mice was collected, kept on ice for < 20min and then spun at 3000×g for 30 min at 4°C. The serum was drawn out and stored at -20°C for later ELISA analyses. For gene expression studies, the brain, extra-oviductal white adipose tissue (EOWAT), heart, liver, and spleen were dissected from mice and stored in RNAlater solution (ThermoFischer Scientific). Brain samples were later dissected and hypothalami, hippocampi, and pre-frontal cortexes were collected, and placed in RNAlater. All tissue samples were stored at 4°C no more than 30 days before RNA extraction (see below).

ELISA

Serum levels of corticosterone (*n* = 6-10/group) were measured as per the manufacturer's protocols (Catalogue number- ADI-900-097, EnzoLife Sciences; Abcam). Average intra- and inter-assay coefficients of variation were 4.629% and 7.204% respectfully.

Measurement of gene expression

RNA was extracted from EOWAT, heart, liver, spleen, and brain tissue using RNeasy mini kits (Qiagen). RNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (ThermoScientific). Total RNA was reverse transcribed into cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies, Cat number:1384368813). The prepared cDNA was used as template for determining relative cytokine gene expression using an ABI 7300 RT-PCR system. Cytokine probes (IL-1β: Mm00434228 , TNFα: Mm00443258 , TGFβ: Mm00447500; Applied Biosystems) labelled with fluorescent marker 5-FAM at the 5' end and quencher MGB at the 3'end was used for genes of interest along with 18S (VIC-labelled probe) as the endogenous control according to the manufacturer's instructions. Samples were run in duplicate and the cycle threshold (Ct) obtained by fluorescence exceeding background levels was used to calculate the relative expression in mRNA levels of the genes of interest relative to the endogenous control using a standard curve. The standard curve was created by injecting a mouse with 100μL lipopolysaccharide (1 mg/kg BW) to induce a severe pro-inflammatory response, extracting RNA from the liver, and then reverse transcribing the RNA into cDNA. The cDNA was used to create a ten-fold series dilution (1:1, 1:10, 1:100, 1:1000, 1:10000) to generate the standard curve plot points. Outliers were removed based upon a two-sigma analysis.

Statistical Analysis

Data are presented as mean (±SE) (v.1.1.463, R Development Core Team, Boston, MA). A two-way ANOVA assessed the effect of sleep fragmentation (ASF and CSF) alone, the effect of the adrenergic blocks (propranolol and phentolamine) alone, and

the interaction effect of ASF and CSF with the pharmacological blocks on the serum levels of corticosterone and mRNA expression of cytokines. The interaction term was removed from the model if it was nonsignificant to preserve degrees of freedom. Tukey's HSD test was used for post-hoc analysis. Logarithmic transformation was used to satisfy the requirement of homogeneity of variances. A repeated measures two-way ANOVA assessed the effect of CSF on body mass, the effect of the repeated measure (time) on body mass, and their interaction effect. Tukey's HSD test was used for post-hoc analysis. Results are presented as means ±1 SE, and $p < 0.05$ was considered statistically significant.

RESULTS

Acute Sleep Fragmentation (ASF)

Serum Corticosterone

ASF increased serum corticosterone (Cort) concentration ($F_{1,34}$=5.66, p=0.0023, Fig. 2A) while drug treatment had a trending effect on serum Cort ($F_{2,34}$=2.778, p=0.0763, Fig. 2A). An interaction effect between drug and sleep treatments was present ($F_{2,34}$=4.577, p=0.0174, Fig. 2A); ASF Propranolol (Pro) exhibited significantly elevated serum Cort relative to Control (CON) Vehicle (Veh) and ASF Phentolamine (Phe) groups (Tukey's HSD, p<0.05).

Brain Response

Prefrontal Cortex

ASF significantly increased gene expression of IL-1β ($F_{1,48}$=161.524, p<0.001, Fig. 3A) and TGFβ ($F_{1,52}$=1620.917, p<0.001, Fig. 3G), but did not affect the expression

of TNFα ($F_{1,43}$=0.679, p=0.414, Fig. 3D) compared to controls. There was no significant effect of drug treatment upon expression of any of the cytokines (IL-1β, $F_{2,48}$=2.015, p=0.144, Fig. 3A; TNFα, $F_{2,43}$=2.403, p=0.102 Fig. 3D; TGFβ, $F_{2,52}$=0.968, p=0.386, Fig. 3G).

Hippocampus

ASF significantly increased cytokine gene expression in hippocampus (IL-1β: $F_{1,45}$=55.294, p<0.001, Fig. 3B; TNFα: $F_{1,46}$=94.886, p<0.001, Fig. 3E; TGFβ: $F_{1,49}$=1055.799, p<0.001, Fig. 3H) compared to controls. There was a significant effect from drug treatment on TNFα expression ($F_{2,48}$=4.813, p=0.014, Fig. 3E), but not on IL-1β expression ($F_{2,45}$=2.417, p=0.10071, Fig. 3A) or TGFβ ($F_{2,49}$=0.618, p=0.5434, Fig. 3H). Tukey's HSD showed that Pro increased TNFα expression levels compared to Phe and Veh. There was an interaction effect between drug and sleep treatments for IL-1β expression ($F_{2,45}$=5.392, p=0.00796, Fig. 3B) and TGFβ expression ($F_{2,45}$=5.012, p=0.0105, Fig. 3H); however, post-hoc tests revealed only a significant difference between sleep treatments for each gene (p>0.05).

Hypothalamus

In hypothalamus, ASF significantly increased cytokine gene expression (IL-1β: $F_{1,47}$=275.748, p<0.001, Fig. 3C; TNFα: $F_{1,45}$=49.538, p<0.001, Fig. 3F; TGFβ: $F_{1,47}$=1373.839, p<0.001, Fig. 3I). Drug treatment also had an effect on cytokine gene expression (IL-1β: $F_{2,47}$=18.225, p<0.001, Fig. 3C; TNFα: $F_{2,45}$=6.827, p=0.00257, Fig. 3F; TGFβ: $F_{2,47}$=1373.839, p<0.001, Fig. 3I). Specifically, Pro and Phe decreased IL-1β and TGFβ expression relative to Veh while TNFα expression was reduced by just Phe.

There was an interaction effect between drug and sleep treatments influencing IL-1β expression ($F_{2,47}$=0.9715, p<0.001, Fig. 3C); post-hoc tests revealed that ASF groups had higher expression levels than CON treated groups and that CON Veh had higher expression levels than CON Phe and Pro.

Peripheral Response

EOWAT

ASF significantly increased gene expression of IL-1β ($F_{1,45}$=156.499, p<0.001, Fig. 4A), TNFα ($F_{1,46}$=, p=23.14, Fig. 4E), and TGFβ ($F_{1,43}$=1048.367, p<0.001, Fig. 4I) compared to controls. Drug treatment significantly altered the expression of IL-1β ($F_{2,45}$=6.311, p=0.00384, Fig. 4A) and TGFβ ($F_{2,43}$=4.831, p=0.0128, Fig. 4I) but not TNFα ($F_{2,46}$=0.58, p=0.564, Fig. 4E) relative to Veh. Post-hoc Tukey's test revealed that Phe decreased IL-1β expression when compared to Pro whereas Pro increased TGFβ expression compared with Veh.

Heart

In cardiac tissue, ASF significantly increased gene expression of IL-1β ($F_{1,38}$=12.698, p=0.001006, Fig. 4B) and TGFβ ($F_{1,48}$=805.935, p<0.001, Fig. 4J) compared with controls, however, TNFα ($F_{1,45}$=356.664, p<0.001, Fig. 4F) expression levels were significantly lower than that of the controls. There was a drug effect on the expression of IL-1β ($F_{2,38}$=8.421, p<0.001, Fig. 4B) and TGFβ ($F_{2,48}$=7.227, p=0.0018, Fig. 4J). Post-hoc tests showed that Pro suppressed the expression levels of IL-1β and TGFβ relative to Veh. There was no effect of the pharmacological blockade on the expression of TNFα ($F_{2,43}$=0.189, p=0.828, Fig. 4F). There was an interaction effect on

IL-1β gene expression ($F_{2,38}$=3.417, p=0.043204, Fig. 4B); post-hoc tests revealed that ASF Pro had IL-1β expression levels equal to that of CON groups, and that ASF Pro expression was significantly lower than ASF Veh, but not ASF Phe (Fig. 4B). A trending interaction effect was found in the expression of TGFβ ($F_{2,48}$=2.725, p=0.0757, Fig. 4J) where ASF Pro reduced expression levels of TGFβ relative to ASF Phe and ASF Veh, yet was more elevated than the controls.

Liver

ASF treatment significantly increased hepatic gene expression of each cytokine assessed (IL-1β: $F_{1,48}$=212.249, p<0.001, Fig. 4C; TNFα: $F_{1,42}$=2153.849, p<0.001, Fig. 4G; TGFβ: $F_{1,47}$=947.400, p<0.001, Fig. 4K) relative to controls. There was a trending drug effect on the expression of TGFβ ($F_{2,47}$=2.782, p=0.0721, Fig. 4K), yet no effect on IL-1β expression of ($F_{2,48}$=0.323, p=0.725, Fig. 4C) or TNFα ($F_{2,42}$=1.856, p=0.169, Fig. 4G).

Spleen

In spleen, ASF increased cytokine gene expression (IL-1β: $F_{1,46}$=65.294, p<0.001, Fig. 4D; TNFα: $F_{1,44}$=189.456, p<0.001, Fig. 4H; TGFβ: $F_{1,45}$=575.832, p<0.001, Fig. 4L) compared with controls. Drug treatment significantly decreased the expression of TNFα ($F_{2,44}$=5.648, p=0.00656, Fig. 4H) and TGFβ ($F_{2,45}$=4.936, p<0.0115, Fig. 4L) with Pro and Phe, respectively, relative to Veh injection. There was a trending effect caused by drug treatment on IL-1β expression ($F_{2,46}$=3.188, p=0.0505, Fig. 4D), but post-hoc tests revealed no significant differences ($p>0.05$). A trending interaction effect between ASF and drug treatment on TNFα ($F_{1,42}$=2.465, p=0.0505, Fig. 4H) was also detected,

however, post-hoc tests revealed no significant differences ($p>0.05$). Table 1 provides a summary of results for ASF effects, drug effects, and interaction effects on cytokine gene expression in brain and peripheral tissues.

Chronic Sleep Fragmentation (CSF)

Serum Corticosterone

CSF increased serum Cort concentration ($F_{1,53}$=14.106, p=0.000431, Fig. 2B) relative to controls, while drug treatment also altered circulating serum corticosterone concentrations ($F_{2,53}$=8.198, p=0.000791, Fig. 2B). The interaction effect between drug and sleep treatments also had a significant effect on corticosterone ($F_{2,34}$=4.577, p=0.0174, Fig. 2B); post-hoc tests revealed that corticosterone concentrations of CSF Pro was not significantly different from controls while CSF Phe and CSF Veh had significantly higher circulating concentrations.

Brain Response

Prefrontal Cortex

In prefrontal cortex, CSF significantly increased cytokine gene expression (IL-1β: $F_{1,44}$=30.70, p<0.001, Fig. 5A; TNFα: $F_{1,49}$=39.393, p<0.001, Fig. 5D; TGFβ: $F_{1,44}$=66.60, p<0.001, Fig. 5G) relative to controls. There was also a significant effect of drug treatment on cytokine gene expression (IL-1β, $F_{2,44}$=30.70, p<0.001, Fig. 5A; TNFα, $F_{2,49}$=7.336, =0.00163, Fig. 5D; TGFβ, $F_{2,44}$=18.05, p<0.001, Fig. 5G). Furthermore, an interaction effect between sleep and drug treatments altered cytokine gene expression (IL-1β, $F_{2,44}$=3.51, p=0.0385, Fig. 5A; TNFα, $F_{2,49}$=4.408, p=0.01736, Fig. 5D; TGFβ, $F_{2,44}$=11.57, p<0.001, Fig. 5G). Post-hoc tests showed that Pro and Phe groups

significantly decreased IL-1β expression levels compared to Veh among CSF mice. In addition, CSF Phe treatment significantly decreased TNFα and TGFβ expression levels relative to CSF Phe and CSF Veh mice.

Hippocampus

CSF significantly increased the gene expression of each cytokine in the hippocampus (IL-1β: $F_{1,47}$=40.943, p<0.001, Fig. 5B; TNFα: $F_{1,49}$=56.787, p<0.001, Fig. 5E; TGFβ: $F_{1,42}$=58.056, p<0.001, Fig. 5H) compared with controls. Drug treatment had a significant effect on TNFα expression ($F_{2,49}$=13.967, p<0.001, Fig. 5E), a trending effect on TGFβ ($F_{2,49}$=0.618, p=0.5434, Fig. 5H), and no effect on IL-1β ($F_{2,47}$=0.327, p=0.723, Fig. 5B). Tukey's HSD showed Phe and Pro- treated groups had lower TNFα expression levels than Veh, while there were no significant differences in drug treatments for TGFβ ($p>0.05$). There was an interaction effect between drug and sleep treatments in TNFα expression ($F_{2,49}$=9.002, p=0.00468, Fig. 5E); post-hoc tests revealed that TNFα expression levels in CSF Phe were significantly less than CSF Pro and Veh, and not significantly different from controls.

Hypothalamus

In hypothalamus, CSF had an effect on the gene expression of IL-1β ($F_{1,50}$=47.92, p<0.001, Fig. 5C) and TNFα ($F_{1,51}$=11.744, p=0.00121, Fig. 5F) but not on TGFβ ($F_{1,47}$=0.207, p=0.65060, Fig. 5I) relative to controls. Drug treatment, however, altered the gene expression of each cytokine assessed (IL-1β: $F_{2,50}$=22.04, p<0.001, Fig. 5C; TNFα: $F_{2,51}$=16.382, p<0.001, Fig. 5F; TGFβ: $F_{2,47}$=1373.839, p<0.001, Fig. 5I). Post-hoc tests showed Phe and Pro treated groups had higher IL-1β and TNFα expression

levels than Veh, and that TGFβ was higher in Pro than in Phe or Veh. There was an interaction effect between drug and sleep treatments influencing TNFα expression ($F_{2,51}$=3.748, p=0.03208, Fig. 5F); however, post-hoc tests revealed that only CON Veh was statistically different from all other treatment groups.

Peripheral Response

EOWAT

In adipose tissue, CSF significantly increased cytokine gene expression (IL-1β: $F_{1,46}$=40.026, p<0.001, Fig. 6A; TNFα: $F_{1,49}$=102.074, p<0.001, Fig. 6E; TGFβ: $F_{1,44}$=79.15, p<0.001, Fig. 6I) compared with controls. Drug treatment had a significant effect on the expression of IL-1β ($F_{2,45}$=6.311, p=0.00384, Fig. 6A) and TNFα ($F_{2,49}$=3.375, p=0.0423, Fig. 6E), and had a trending effect on TGFβ ($F_{2,37}$=2.75, p=0.077012, Fig. 6I) expression. Post-hoc Tukey's revealed that Phe resulted in a lower IL-1β and TNFα expression than when treated with Pro. A significant interaction effect in TGFβ expression ($F_{1,37}$=10.00, p<0.001, Fig. 6I) was present, and post-hoc tests show CSF Phe had lower expression levels than CSF Pro, which was not significantly different from CON Phe.

Heart

In cardiac tissue, CSF significantly increased cytokine gene expression (IL-1β: $F_{1,42}$=27.748, p<0.001, Fig. 6B; TNFα: $F_{1,41}$=27.47, p<0.001, Fig. 6F; TGFβ: $F_{1,47}$=92.809, p<0.001, Fig. 6bJ) compared with controls. Drug treatment had a significant effect on the expression of each cytokine assessed (IL-1β: $F_{2,45}$=8.085, p=0.00107, Fig. 6B; TNFα: $F_{2,41}$=12.21, p<0.001, Fig. 6F; TGFβ: $F_{2,37}$=7.493,

p=0.00150, Fig. 6J). Post-hoc tests showed Phe and Pro reduced expression of IL-1β, and Phe reduced TNFα expression compared with Pro and Veh. There was an interaction between sleep and drug treatments on TGFβ expression ($F_{2,47}$=8.158, p<0.001, Fig. 6J) and post-hoc tests show that CSF Veh and CSF Pro exhibited high TGFβ expression levels while CSF Phe had expression levels equal to CON Phe and Pro.

Liver

In liver, CSF treatment significantly increased the gene expression of IL-1β ($F_{1,44}$=5.721, p=0.0211, Fig. 6C) and TGFβ ($F_{1,49}$=101.727, p<0.001, Fig. 6K), but not TNFα ($F_{1,44}$=0.020, p=0.888, Fig. 6G), relative to controls. There was a trending drug effect on the expression of TGFβ ($F_{2,49}$=2.603, p=0.0843, Fig. 6K), and no effect on the expression of IL-1β ($F_{2,44}$=1.121, p=0.3349, Fig. 6C) or TNFα ($F_{2,44}$=1.463, p=0.243, Fig. 6G). There were no significant differences between treatment groups using Tukey's post-hoc tests (all $p>0.05$).

Spleen

In spleen, CSF increased cytokine gene expression (IL-1β: $F_{1,39}$=22.939, p<0.001, Fig. 6D TNFα: $F_{1,38}$=129.488, p<0.001, Fig. 6H; TGFβ: $F_{1,45}$=44.173, p<0.001, Fig. 6L) compared with controls. Drug treatment significantly altered the expression of IL-1β ($F_{2,39}$=4.652, p=0.0154, Fig. 6D) and TNFα ($F_{2,38}$=4.133, p=0.02376, Fig. 6H) but had no effect on TGFβ ($F_{2,45}$=1.448, p=0.246, Fig. 6L). Post-hoc test revealed that Pro reduced IL-1β expression compared with Phe or Veh, and Phe reduced TNFα expression compared to Pro and Veh. There was an interaction effect between sleep treatment and drug treatment on TNFα expression ($F_{2,38}$=5.732, p=0.00667, Fig. 6H), and post-hoc tests

revealed that expression levels in CSF Phe were significantly less than CSF Pro or Veh. Table 2 provides a summary of results for CSF effects, drug effects, and interaction effects on cytokine gene expression in brain and peripheral tissues.

Body Mass

There was an effect of sleep treatment alone ($F_{1,51.481}$=52.3236, p<0.001, Fig. 7), an effect of time ($F_{4,246.029}$=4.4742, p=0.01656, Fig. 7), as well as an interaction effect between CSF and time ($F_{4,246.029}$=24.3706, p<0.001, Fig. 7) on body mass. Post-hoc tests revealed that the percent change in body mass for both control (CON) and chronic sleep fragmented (CSF) mice diverged at Week 2 and continued to do so at Weeks 3 and. Body mass for CON Week 4 (2.61%) was significantly higher than CON Week 0 (0.0%) and 1 (-0.42%), while CSF Week 2 (-2.41%) was significantly lower than CSF Week 0 (0.0%), and CSF Weeks 3 (-5.26%) and 4 (-6.53%) are significantly less than CSF Week 2 (-2.41%).

DISCUSSION

These results show that treatment with adrenergic receptor antagonists, phentolamine and propranolol, largely mitigated the inflammatory phenotype in response to acute and chronic sleep fragmentation (ASF and CSF), but these effects were tissue-dependent. These results are consistent with a previous study from our lab showing that chemical denervation of sympathetic nerve terminals reduces inflammatory responses in peripheral tissues of female mice (Mishra et al. 2020). Additionally, in some tissues, opposing effects were observed where pharmacological blockade actually increased pro-inflammatory gene expression (e.g., increased TNF expression in hippocampus from

propranolol treatment in the ASF experiment, increased IL-1β and TNFα in hypothalamus from propranolol and phentolamine treatments in CSF experiment). These data are not surprising considering that catecholamines can exert both pro-inflammatory and anti-inflammatory responses (Barnes, Carson, and Nair 2015).

SF Effects

This study confirms previous studies (Dumaine and Ashley 2015, Mishra et al. 2020) that acute and chronic SF leads to elevated pro-inflammatory gene expression in peripheral and brain tissues. ASF increased expression of IL-1β and TNF in peripheral tissues (fat, liver, spleen) compared with control mice receiving no SF. In heart, ASF elevated IL-1β expression while decreasing TNFα expression compared with controls. A similar finding was reported in a previous study in female mice (Mishra et al. 2020). In our study, ASF increased IL1 and TNFα expression in hypothalamus and hippocampus compared with controls, and in the pre-frontal cortex, ASF increased IL-1β expression, but not TNFα. For CSF studies, there were marked increases in pro-inflammatory gene expression (IL-1β and TNFα) in all brain and peripheral tissues studied, except for liver which showed an increase in IL-1β, but not TNFα, in response to CSF. These findings suggest that CSF is a more potent inducer of inflammation than ASF, as reported in other studies (Mullington et al., 2010, Mishra et al., 2020).

TGFβ is generally regarded as an anti-inflammatory cytokine that downregulates inflammatory pathways (Marie et al., 1996; Sanjabi et al., 2009; Zhou et al., 2012), suggesting that the TGFβ expression could have increased in response to SF as a homeostatic mechanism to inhibit the action of pro-inflammatory cytokines. Alternatively, this cytokine has also been shown to have pro-inflammatory effects

(reviewed in Morikawa, 2016; see also empirical study Yan et al., 2014). TGFβ has been shown to promote the differentiation of T helper 17 cells in conjunction with IL-6, which stimulates inflammation and amplifies autoimmune conditions (Korn et al., 2009). There is also a synergistic relationship between TGFβ and IL-4 that encourages the development of T cells which produces cytokines IL-9 and IL-10. These IL-9 and IL-10 producing T cells incite tissue inflammation and do not engage in suppressive activity (Zhou et al., 2008). Further research is needed to determine exact role that TGFβ plays in regulating the immune response to SF.

Phentolamine Effects

Phentolamine is a non-selective α-adrenergic receptor antagonist that has been commonly used to treat hypertension by acting on blood vessels to induce dilation. It competitively blocks both α-1 and α-2 receptors and can cross the blood-brain-barrier (Richards, 1978; Auer, 1981; Limberger et al., 1989; Antunes-Rodrigues et al. 1993). Alpha-1 receptors are typically found in vascular smooth muscle while α-2 receptors are detected in the brain and periphery and are thought to modulate sympathetic outflow in the brainstem (Reid 1986). In the ASF experiment, phentolamine was effective at decreasing pro-inflammatory gene expression in hypothalamus of ASF and control mice, while having no effect upon pre-frontal cortex or hippocampus. The same effect was observed in EOWAT with a reduction in IL-1β. In contrast, in the CSF experiment, the effect of phentolamine on cytokine gene expression was more widespread, and there were more interaction effects in comparison to the ASF Experiment. Among CSF mice, phentolamine decreased IL-1β expression in prefrontal cortex and TNFα expression in hippocampus compared to vehicle. In addition, phentolamine reduced TNFα expression

in spleen and decreased TGFβ expression in heart of CSF mice relative to vehicle. These findings suggest that mice experiencing CSF respond differently to an α-adrenergic receptor blockade than mice only experiencing 24 h of SF. Previous research has shown that increased sympathetic tone induced by chronic stressors, including chronic sleep loss, diminishes α-adrenergic receptor quantity and sensitivity in the brain and peripheral vasculature (Grote, 2000; Kim et al., 2013; Schmidt et al., 2019). In relation to the immune system, a number of immune cells are regulated by α-adrenergic receptor stimulation including cell proliferation, cytokine production, lytic activity and antibody production (Grisanti, 2011). In this study, phentolamine affects inflammatory responses could involve either changes in blood flow to various target tissues or direct interactions on immune cells, although further study is warranted.

Propranolol Effects

Propranolol is a non-selective competitive β-adrenergic receptor antagonist that also crosses the blood-brain barrier. It blocks the action of catecholamines from binding to both β_1 and β_2 adrenergic receptors. Sleep loss stimulates nerve fibers from the SNS to release the neurotransmitter norepinephrine and bind to leucocyte adrenergic receptors (Irwin and Opp, 2017), which leads to expression of pro-inflammatory cytokines. In the immune system, myeloid cells typically express α- and β-adrenergic receptors, whereas lymphocytes largely express β-adrenergic receptors. Because of this fact, we originally predicted that propranolol would have a greater effect upon inflammatory responses than phentolamine. However, this hypothesis was not clearly supported. In the ASF experiment, propranolol inhibited IL-1β gene expression in heart of ASF mice compared with vehicle. In addition, TGFβ expression was also inhibited. In spleen, propranolol

decreased TNFα expression in control and ASF mice. However, propranolol increased TNFα expression in the hippocampus in ASF and control mice. In the CSF experiment, propranolol decreased IL-1β and TNFα expression in prefrontal cortex of CSF mice relative to vehicle. In addition, propranolol reduced IL-1β gene expression in control and CSF mice, while increasing IL-1β and TNFα expression in hypothalamus. These data suggest that these tissues are sensitive to β-adrenergic receptor blockade, and that catecholamines play a role in mediating inflammation in these tissues. Past research has also shown evidence that catecholamines influence the distribution and activity of β-adrenergic receptors due to chronic sleep loss in the brain (Radulacki and Micovic, 1982; Kim et al., 2013). However, some tissues exhibited differential responses to propranolol, which highlights the complexity of the effect that catecholamines have upon regulating inflammatory responses. To help explain these disparate effects, it has been postulated that the net effect of stimulating or inhibiting adrenergic receptors on immune cells is not straightforward, as there are a variety of factors at play that can alter the outcome, such as the activation state of the target cell, the proximity of the cell to the drug, and the pattern of expression of adrenergic receptors (Pongratz and Straub, 2014). To control these variables, it would be ideal to examine the effects of these adrenergic antagonists upon cultured cells, or even single cells.

Serum Corticosterone & Body Mass

Serum corticosterone concentration was elevated as a result of CSF and an interaction effect of sleep and drug treatments reduced the circulating concentration of corticosterone in CSF mice receiving propranolol. In response to both ASF and CSF, the hypothalamic-pituitary-adrenal axis (HPA axis) was affected as evidenced by elevated

serum corticosterone (Cort) levels. The elevation in Cort is thought to act in an anti-inflammatory manner in response to a stressor, i.e. sleep fragmentation, to suppress the action of pro-inflammatory cytokines produced by the innate immune system (reviewed in Besedovsky and del Rey, 1996; but also see empirical studies Glover et al., 2009; Lima et al,. 2014; Mileya et al., 2017). As seen in previous experiments from our lab, both acute and chronic sleep fragmentation resulted in elevated Cort levels (Dumaine and Ashley 2018; Mishra et al., 2020). However, Cort concentration was lower in CSF receiving vehicle than ASF receiving vehicle; another finding duplicated from our lab (Mishra et al., 2020). We report female mice subjected to ASF given phentolamine exhibited a reduced production of Cort, whereas treatment with CSF and propranolol reduced Cort concentrations, indicating that α- and β-adrenergic receptors, respectively, are integral in regulating the HPA axis in acute and chronic settings. Both α- and β-adrenergic receptors have been implicated in HPA axis regulation (Bugajski et al., 1995), and the reduction of circulating Cort concentrations has been attributed to the down regulation of SNS activity (Lowrance et al., 2016; Mishra et al., 2020). We suggest that reduction of Cort in CSF mice receiving propranolol is the product of a combined effect of an adaptive neurologic response to chronic stress, CSF, and the antagonistic action of the β-adrenergic blocker, propranolol. The abundance of hypothalamic β-adrenergic receptors decreases in response to chronic stress (Stone and Platt, 1982; Thorsdottir et al., 2019), therefore increasing the efficacy of propranolol and inhibiting catecholamine binding at the hypothalamus (Tuross and Patrick, 1986), which in turn reduces adrenocorticotropic hormone (ACTH) release (Spiga and Lightman, 2015) and consequently, Cort secretion.

Body mass was also affected by sleep treatment, in which control mice (CON) increased body mass from Week 2 (0.65%) to Week 4 (2.61%) while CSF decreased body mass from Week 2 (-2.41%) to Week 4 (-6.53%). These findings are in contrast to male mice, where 8 weeks of CSF leads to body mass gain (Carreras et al., 2015). This sexual difference in body mass regulation in response to CSF needs to be explored further but could be due to gonadal steroids.

This study systematically evaluated the influence of SNS manipulation upon the SF-associated hormonal, morphological, and neural and peripheral inflammatory responses. The changes observed in inflammatory responses appear to be representative of the activation of stress-axes and were correlated with SF duration. Additionally, there was a tissue-dependent response to phentolamine and propranolol, suggesting that both types of adrenergic receptors play a role in regulating inflammatory responses to SF. Our findings in SF-associated inflammatory responses in female mice corroborates previous findings from our lab and establishes that exposing mice to four weeks of CSF achieves comparable inflammatory effects seen in eight weeks of CSF. To our knowledge, this is the first study assessing the effects of adrenergic receptor blockade upon inflammatory responses to both ASF and CSF. Lastly, this study provides evidence that both α- and β-adrenergic receptors are involved in the SNS regulation of inflammatory responses to SF.

APPENDIX A: FIGURES

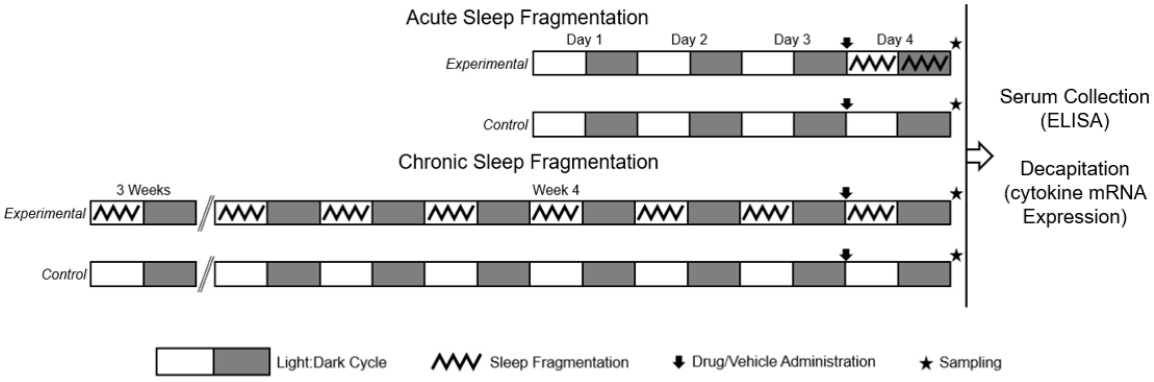

Figure 1. Experimental Protocol for ASF and CSF Studies

Experimental protocol for the two experiments performed. Experimental mice subjected to sleep fragmentation (SF) in an automated SF chamber for 24 hours (experiment 1: Acute SF) or 4 weeks (experiment 2: Chronic SF). An automated horizontally sweeping bar moved across the bottom of the chamber every 2 minutes to ensure that sleep was regularly disrupted. The control (CON) groups of each experiment were also contained in their own SF chambers, however, the bars inside the chambers remained stationary. Mice were acclimated to the SF chambers for 72 hours (days 1-3) prior to the initiation of the experiments. In the Acute SF experiment (ASF), control and experimental mice received an intraperitoneal injection of 0.9% saline (vehicle), phentolamine, or propranolol 30 minutes before initiating their respective sleep treatments. After 24 hours of ASF or CON treatment, mice (SF: n = 9/group; CON: n = 10/group) were decapitated for tissue gene expression quantification. In the Chronic SF (CSF) experiment, control and experimental received an intraperitoneal injection of 0.9% saline (vehicle), phentolamine, or propranolol 24 hours before the conclusion of the chronic sleep treatments. After 4 weeks of CSF or CON sleep treatment, mice (SF: n = 10/group; CON: n = 10/group) were decapitated for tissue gene expression quantification. All mice were >8 weeks of age, were subjected to 12 hours light: 12 hours dark cycles with lights on at 8:00am and lights off at 8:00pm, and were provided food and water *ad libitum*.

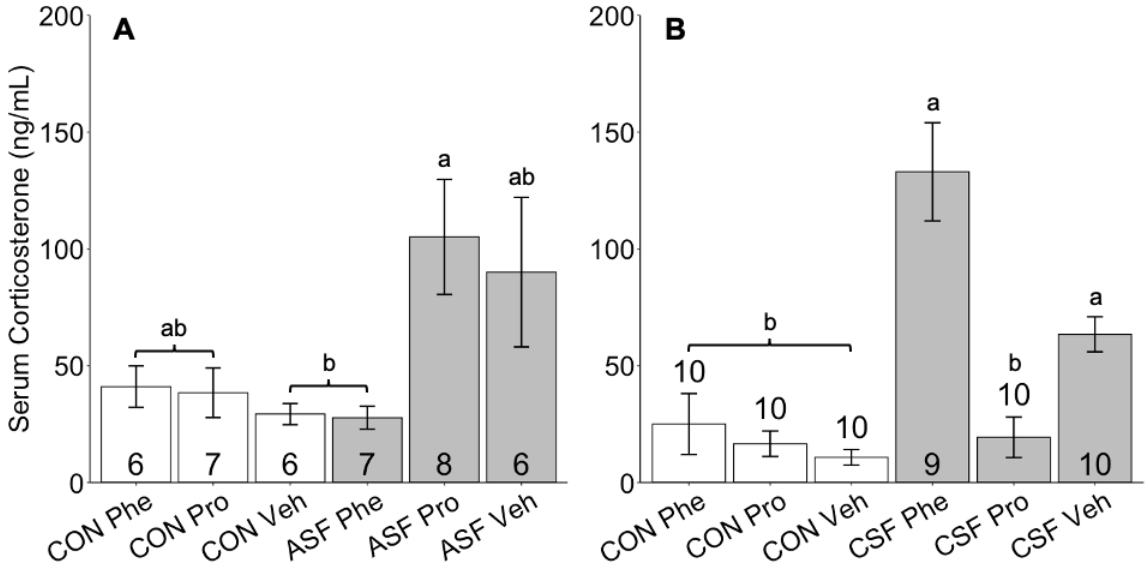

Figure 2. ASF and CSF Experiments – Serum Corticosterone Concentration

Effects of acute sleep fragmentation (ASF, Fig. 4A) and chronic sleep fragmentation (CSF, Fig. 4B), adrenergic blockade (phentolamine (Phe) or propranolol (Pro)) or vehicle (Veh), and their interaction on serum corticosterone levels. Sample sizes of each treatment group are listed with their respective bar graph and were analyzed using a two-way ANOVA and Tukey's HSD post hoc tests. Data shown as means $\pm$ 1 SE for each group and differing letters denotes p <0.05.

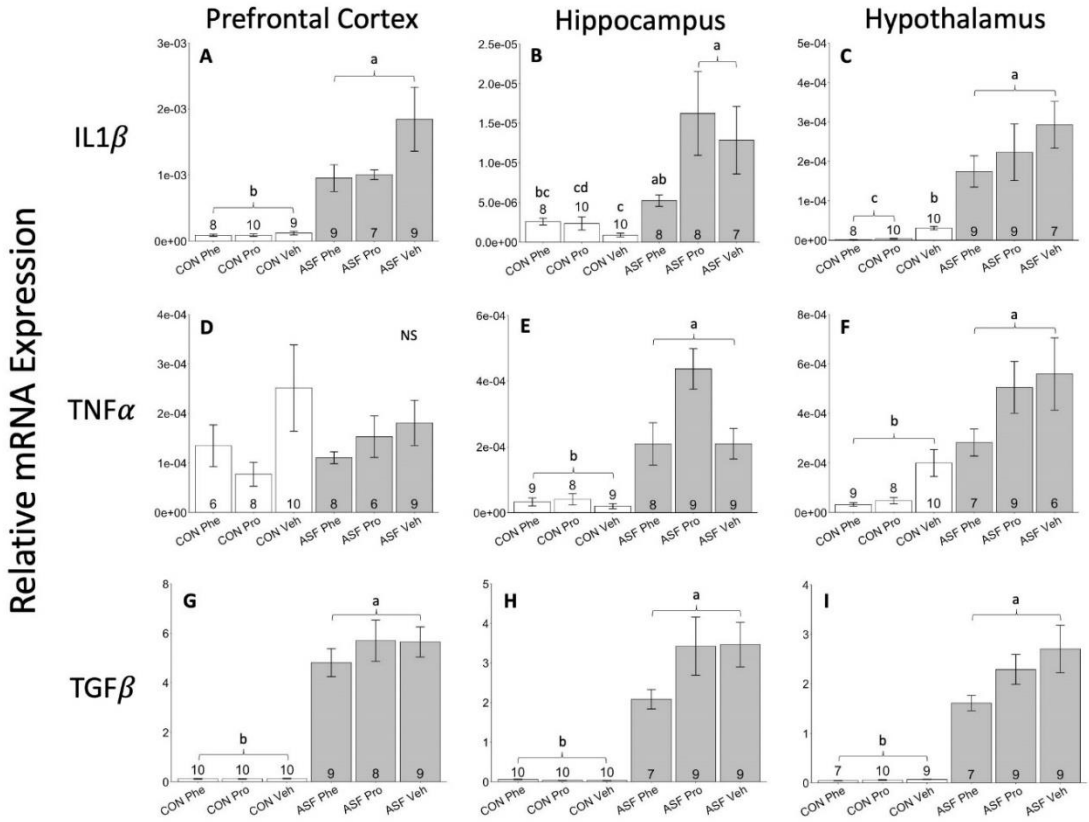

Figure 3. ASF Experiment – Cytokine mRNA Expression in Brain

Effects of acute sleep fragmentation (ASF), adrenergic blockade, and their interaction on cytokine (IL-1β, TNFα, and TGFβ) mRNA expression in prefrontal cortex (A, D, G), hippocampus (B, E, H), and hypothalamus (C, F, I) of mice injected with a pharmacological adrenergic block (phentolamine (Phe) or propranolol (Pro)) or vehicle (Veh) and were either subjected to control (CON) or acute SF (ASF). Sample sizes of each treatment group are listed with their respective bar graph and were analyzed using a two-way ANOVA and Tukey's HSD post hoc tests. Data shown as means $\pm$ 1 SE for each group and differing letters denotes p <0.05.

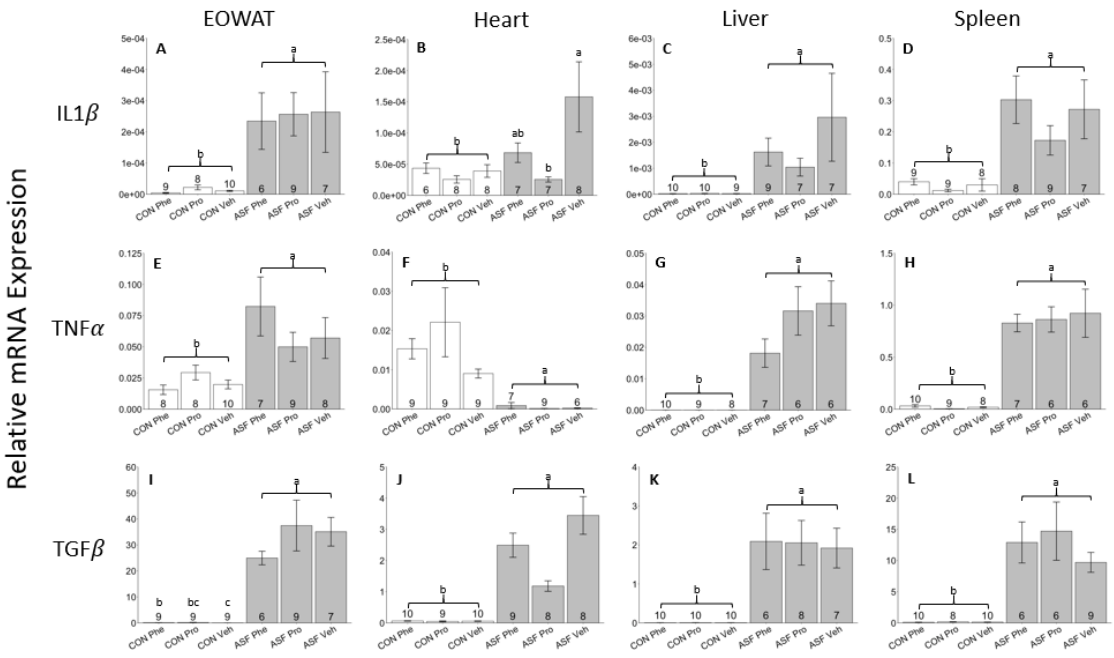

Figure 4. ASF Experiment – Cytokine mRNA Expression in Peripheral Tissues

Effects of acute sleep fragmentation (ASF), adrenergic blockade, and their interaction on cytokine (IL-1β, TNFα, and TGFβ) mRNA expression levels in EOWAT (A, E, I), heart (B, F, J), liver (C, G, K), and spleen (D, H, L) of mice injected with a pharmacological adrenergic block (phentolamine (Phe) or propranolol (Pro)) or vehicle (Veh) and were either subjected to control (CON) or acute SF (ASF). Sample sizes of each treatment group are listed with their respective bar graph and were analyzed using a two-way ANOVA and Tukey's HSD post hoc tests. Data shown as means $\pm$ 1 SE for each group and differing letters denotes $p < 0.05$.

Summary of ASF Results for Cytokine Gene Expression in Brain and Peripheral Tissues.				
Tissue	**Sleep Fragmentation**	**Phentolamine**	**Propranolol**	**Interaction**
Prefrontal Cortex	Increases: IL1β & TGFβ	No Effect	No Effect	No Effect
Hippocampus	Increases: IL1β, TNFα, & TGFβ	No Effect	Increases: TNFα	No Effect
Hypothalamus	Increases: IL1β, TNFα, & TGFβ	Decreases: IL1β, TNFα, & TGFβ	No Effect	No Effect
EOWAT	Increases: IL1β, TNFα, & TGFβ	Decreases: IL1β	Increases: TGFβ	No Effect
Heart	Increases: IL1β & TGFβ Decreases: TNFα	No Effect	Decreases: IL1β & TGFβ	Decreases: IL1β (ASF Pro) & TGFβ (ASF Pro)
Liver	Increases: IL1β, TNFα, & TGFβ	No Effect	No Effect	No Effect
Spleen	Increases: IL1β, TNFα, & TGFβ	Decreases: TGFβ	Decreases: TNFα	No Effect

Table 1. ASF Experiment – Summary of ASF effects, drug effects, and interaction effects on cytokine gene expression in brain and peripheral tissues.

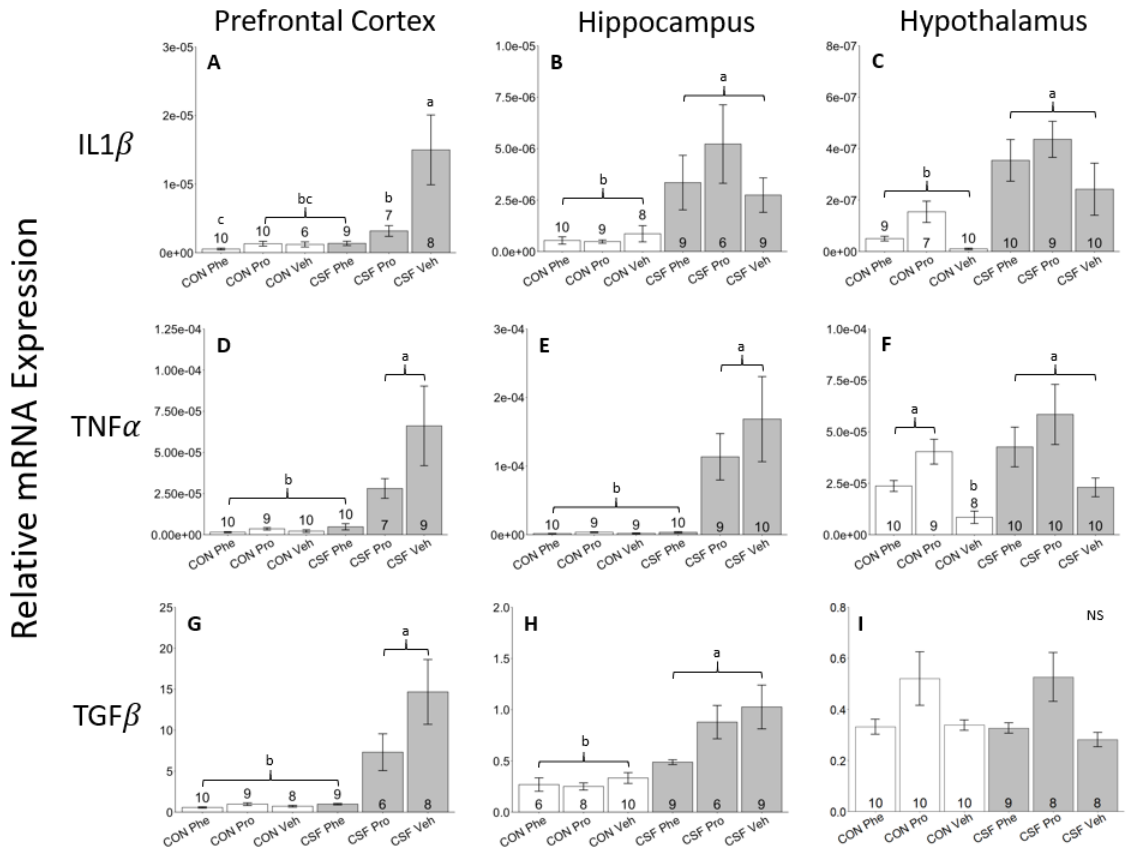

Figure 5. CSF Experiment – Cytokine mRNA Expression in Brain

Effects of chronic sleep fragmentation (CSF), adrenergic blockade, and their interaction on cytokine (IL-1β, TNFα, and TGFβ) mRNA expression levels in prefrontal cortex (A, D, G), hippocampus (B, E, H), and hypothalamus (C, F, I) of mice injected with a pharmacological adrenergic block (Phentolamine (Phe) or Propranolol (Pro)) or vehicle (Veh) and were either subjected to control (CON) or chronic SF (CSF). Sample sizes of each treatment group are listed with their respective bar graph and were analyzed using a two-way ANOVA and Tukey's HSD post hoc tests. Data shown as means $\pm$ 1 SE for each group and differing letters denotes p <0.05.

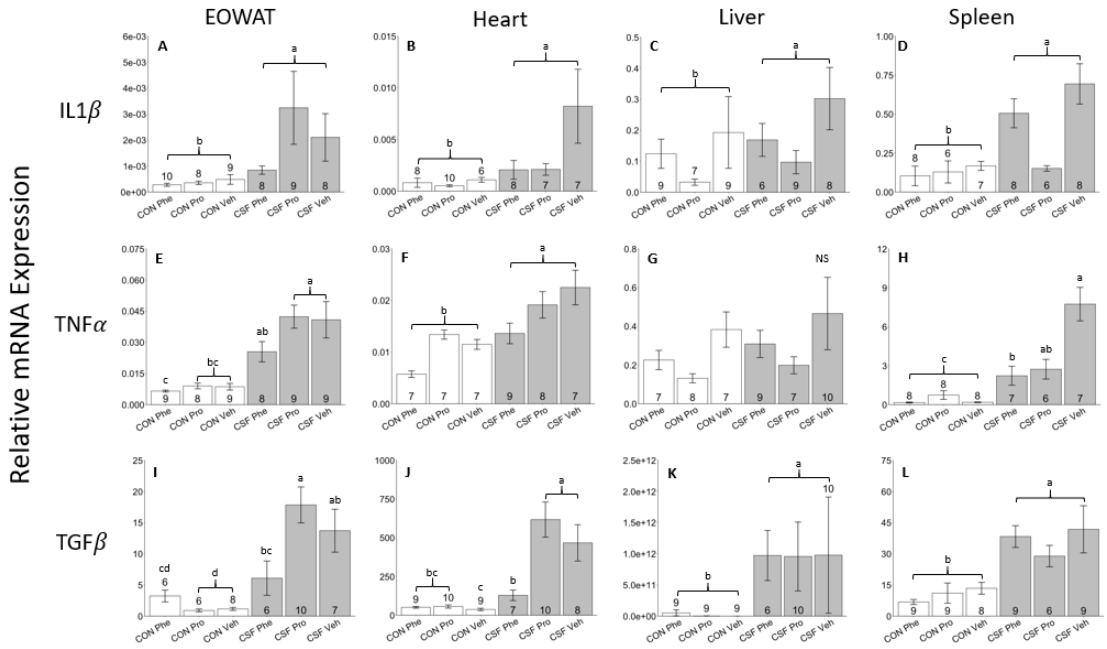

Figure 6. CSF Experiment – Cytokine mRNA Expression in Peripheral Tissues

Effects of chronic sleep fragmentation (CSF), adrenergic blockade, and their interaction on cytokine (IL-1β, TNFα, and TGFβ) mRNA expression levels in EOWAT (A, E, I), heart (B, F, J), liver (C, F, K), and spleen (D, H, L) of mice injected with a pharmacological adrenergic block (phentolamine (Phe) or propranolol (Pro)) or vehicle (Veh) and were either subjected to control (CON) or chronic SF (CSF). Sample sizes of each treatment group are listed with their respective bar graph and were analyzed using a two-way ANOVA and Tukey's HSD post hoc tests. Data shown as means $\pm$ 1 SE for each group and differing letters denotes p <0.05.

Summary of CSF Results for Cytokine Gene Expression in Brain and Peripheral Tissues.				
Tissue	**Sleep Fragmentation**	**Phentolamine**	**Propranolol**	**Interaction**
Prefrontal Cortex	Increases: IL1β, TNFα, & TGFβ	Decreases: IL1β, TNFα, & TGFβ	Decreases: IL1β	Decrease: IL1β (CSF Phe & Pro), TNFα (CSF Pro), & TGFβ (CSF Phe)
Hippocampus	Increases: IL1β, TNFα, & TGFβ	Decreases: TNFα	No Effect	Decreases: TNFα (CSF Phe)
Hypothalamus	Increases: IL1β & TNFα	Increases: IL1β & TNFα	Increases: IL1β, TNFα, & TGFβ	No Effect
EOWAT	Increases: IL1β, TNFα, & TGFβ	Decreases: IL1β & TNFα	No Effect	No Effect
Heart	Increases: IL1β, TNFα, & TGFβ	Decreases: IL1β, TNFα, & TGFβ	Decreases: IL1β	Decreases: TGFβ (CSF Phe)
Liver	Increases: IL1β & TGFβ	No Effect	No Effect	No Effect
Spleen	Increases: IL1β, TNFα, & TGFβ	Decreases: TNFα	No Effect	Decreases: TNFα (CSF Phe)

Table 2. CSF Experiment – Summary of CSF effects, drug effects, and interaction effects on cytokine gene expression in brain and peripheral tissues.

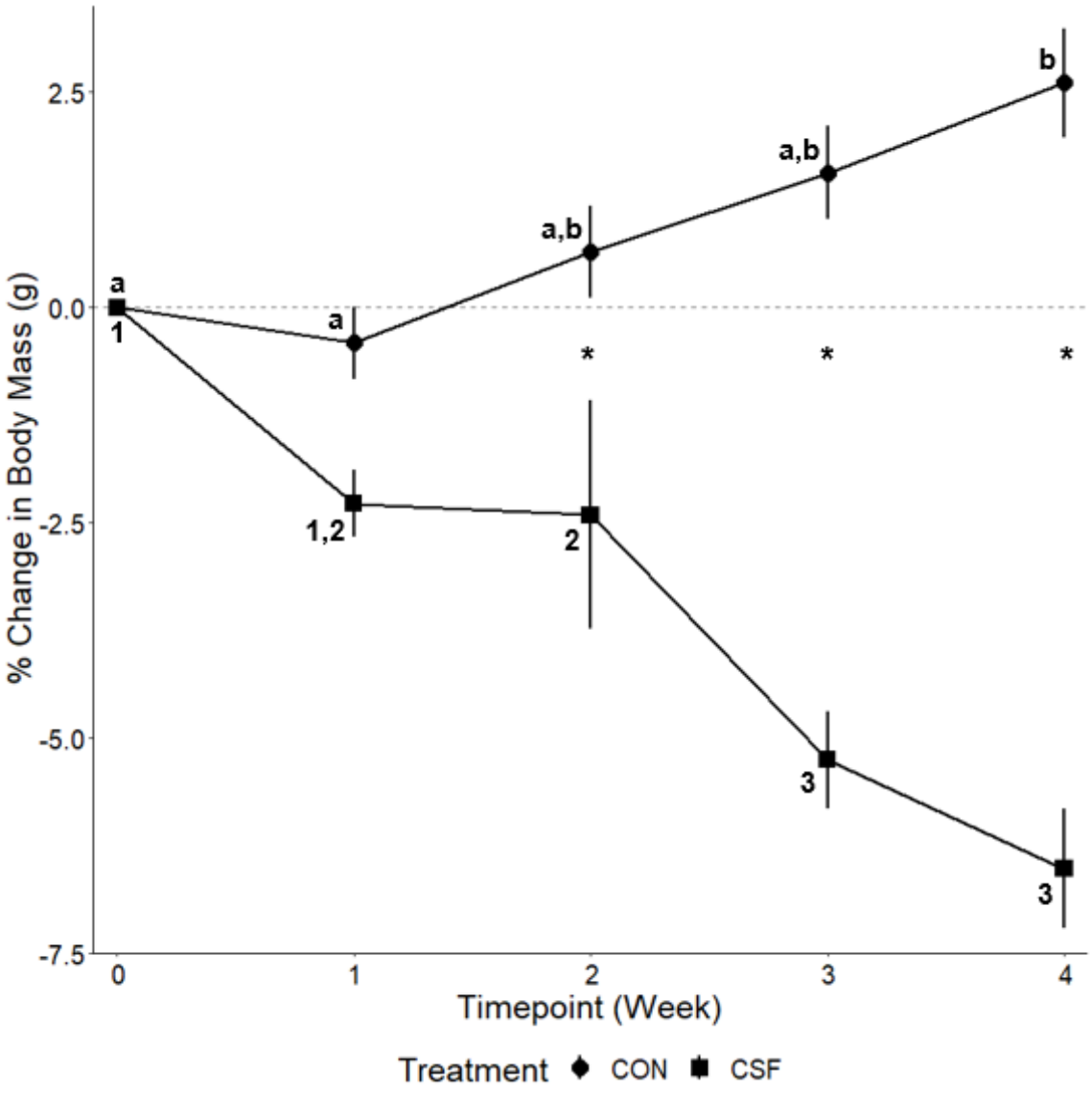

Figure 7. CSF Experiment – Percentage Change in Body Mass

Effects of chronic sleep fragmentation (CSF) on weekly body mass. Timepoint "Week 0" represents the start of the experiment. Sample sizes are CON n = 60 and CSF = 60, and were analyzed using a repeated-measures ANOVA. Data are shown as means ± 1 SE for each group and asterisks (*****) denotes a significant difference between treatments at each timepoint, letters denote differences within CON, and numbers denote difference within CSF (p<0.05).

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