THE IMPACT OF EARLY LIFE STRESS ON INFLAMMATION IN ADULTHOOD

By

John W. Figg

Submitted in partial fulfillment of the

requirements for Departmental Honors in

the Department of Biology

Texas Christian University

Fort Worth, Texas

May 6th, 2019

THE IMPACT OF EARLY LIFE STRESS ON

INFLAMMATION IN ADULTHOOD

Project Approved:

Michael Chumley, Ph.D.
Department of Biology
(Supervising professor)

Gary Boehm, Ph.D.
Department of Psychology

Marlo Jeffries, Ph.D.
Department of Biology

Abstract

Stress has been linked to altering acute and long-term inflammatory responses. Stress has been shown to activate inflammatory responses, specifically microglial activation in the brain. While acute inflammation is one of the first responses to fighting disease and infection, prolonged inflammation has been associated with neurogenerative disease such as Alzheimer's disease. Stress at critical periods of development, known as early life stress (ELS) has been linked to chronic dysregulation of the hypothalamic-pituitary adrenal (HPA) axis, depression and alterations to microglial cells. The goal of this study is to investigate the effect of stress in mice during early development through maternal stress during pregnancy and the impact on neuroinflammation in adult offspring. In utero, offspring are vulnerable to the harmful effects of pro-inflammatory cytokines due to stress experienced by adult mice, following an ELS timeline. Three conditions were utilized: (1) mice undergoing stress during the entire pre-natal period and with the early postnatal period, (2) mice undergoing stress during the early postnatal period, and (3) mice undergoing no additional stress at any point. For mice in the combination-stress condition, there was an immunosuppressive effect through downregulation of pro-inflammatory cytokines. These data support existing publications that indicate an immunosuppressive role of prenatal stress, leaving the host less protected against chronic disease.

Acknowledgement

I would like to extend my upmost gratitude to Dr. Michael Chumley, and graduate students Chris Hagen. Two years ago, Dr. Michael Chumley invited me to participate in his laboratory and extended an open door for me to engage in undergraduate research. Looking back, undergraduate research has been one of the most impactful, if not the most impactful experience that I have had during my time at TCU. I have grown tremendously and my passion to pursue research in a clinical setting has only become stronger as a result of my experience in this lab. I would like to thank Chris Hagen for allowing me to assist him with this project, which is also the cumulation of work that he has done for his thesis. Chris shared with me many papers on stress and how it affects the body's regulation of inflammation which drew my interest toward this project. Chris devoted lots of time with me in reading my thesis, listening to me practice my honors thesis and SRS poster. I also want to thank Dr. Marlo Jeffries and Dr. Gary Boehm. Both professors have positively impacted my education at TCU. I have found their classes to be challenging, but highly meaningful in terms of building skills such as inquiry, problem-solving and becoming more curious about physiology. Both professors have helped me better understand why I want to become a physician and shaped my interests in medicine.

Table of Contents

Introduction

The global prevalence of dementia is estimated to be as high as 24 million people, and this prevalence is expected to double by 2040 (Reitz, 2011). Alzheimer's disease (AD) is the leading cause of dementia and presents with a gradual decline is cognitive abilities, often in memory deficits. Globally dementia has exerted immense pressure on healthcare cost, and in the US AD-related healthcare costs 172 billion dollars every year to manage (Reitz, 2011). The increasing burden of AD on healthcare systems and taxpayer dollars can be attributed to a growth of the aging population in the US. Over the next few decades, aging "baby boomers" will add an additional 10 million patients to this cohort. In the US, AD is the 6th leading cause of death and the 5th leading cause of death in patients 65 or older (Reitz, 2011). Relative to other diseases that are associated with aging, the proportion of deaths due to AD have increased drastically. For example, between 2000 and 2008, deaths attributed to heart disease decreased by 13% and deaths attributed to stroke decreased by 20%. In contrast, from 2000 to 2015 deaths due to AD increased 123% (Reitz, 2011). Aside from the taxpayer cost and increasing deaths due to AD, what is not always observed are the caretaker burdens placed upon family members. Time, wages lost from time off, and stress deeply affect people who are supporting patients and time for care often goes unpaid. In 2011, family members and unpaid caretakers worked 17.4 billion hours in support of AD patients and patients suffering from other forms of dementia. Even worse, some 800,000 AD patients live alone and half do not have an identifiable caretaker (Reitz, 2011). The lack of support in this patient population increases the risk for other preventable healthcare costs such as accidental death, falls, proper nutrition, and self-care. There is

substantial need to continue research in AD and other dementia in order to generate new therapies that alleviate healthcare, societal and personal costs.

Research conducted over the past 30 years has revealed a causal link between improperly folded amyloid beta and tau protein in plaques and neurofibrillary tangles, leading to neurodegenerative processes in the brain. Amyloid beta peptide is roughly 42 amino acids in length and is produced as a breakdown product of amyloid precursor protein (APP) cleavage (Kumar 2015). The family of enzymes responsible for APP cleavage is the secretase family, specifically beta-secretase which will cleave APP into the amyloid beta peptide. Once amyloid beta is released following cleavage, the peptide can bind to amyloid beta receptor on neurons and are internalized into cells where aggregates can form between multimers (Kumar 2015). The accumulation of this peptide into plaques generates disruption in neurotransmission and eventually, death of neurons. As neurons die, memory suffers and this marks the beginning of a slow, degradative process. Neurofibrillary tangles are the result of hyperphosphorylated tau protein accumulating in the extracellular space and lead to disruptions in communication between neurons (Kumar 2015). Important evidence in the elucidation of the role of amyloid beta in AD has been the genes implicated in the production of APP. In familial AD, there can be mutations in several genes such as *APP*, *PSEN1*, or *PSEN2*. In the *APP* gene, mutations affect amyloid beta cleavage and aggregation. The *PSEN1* and *PSEN2* genes provide a subunit that has catalytic activity involved in the cleavage of APP.

Onset of AD occurs over a long period of time, and due to chronic development, the exact reasons behind what processes are going wrong remains uncertain. Certain genes are associated with developing AD, but not all cases of AD have genetic alterations. Some of the few genetic risk factors include *APOE*. For patients who are homozygous for *APOE4*, the lifetime

risk for AD is greater than 50%. Heterozygous patients have a lifetime risk of 20-30% for *APOE3* and *APOE4* (Scheltens 2016). Familial AD represents a mere 5% of the cases, a relatively small portion inherited in an autosomal dominant manner. With sporadic AD accounting for the other 95% of cases, there is likely a complicated interplay of environmental and genetic factors. Other types of diseases and syndromes also elevate the risk for developing AD such as vascular diseases and stroke, hypertension, atherosclerosis, obesity and many others (Scheltens 2016). These diseases can be used to identify certain lifestyle patterns such as poor diet, alcohol and drug use, and smoking as contributing to this neurodegenerative process. More recently, research has found a connection in the immune system and systemic inflammation and the production of amyloid beta. Inflammation is considered a normal immune response when a pathogen breaches the physical protection provided by the human body, but prolonged inflammation can lead to negative consequences. Inflammation will lead to the activation of a variety of cell types including neutrophils and macrophages. As a result of immune cell activation, tissue around the infection will get damaged since immune cells are releasing cytotoxic chemicals and enzymes to destroy pathogens. Systemic inflammation can lead to neuroinflammation and result in activation of immune cells in the brain known as microglia. Many of the signaling molecules synthesized and secreted during an inflammatory response are tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β). In our lab, previous experiments have demonstrated that inflammation initiated by lipopolysaccharide (LPS) can lead to production of amyloid beta in the brain (Kahn 2012). In mice, LPS was injected daily for a week (Kahn 2012). LPS is a component of membrane in gram-negative bacteria and will initiate an immune response upon injection in a mouse. LPS binds to CD14 receptors and activates Toll-

like receptor 4 (TLR) on macrophages, leading to the production of pro-inflammatory cytokines such as TNF-α and IL-1β.

According to epidemiological research, stress-related disorders have been implicated as a risk factor in the development of AD and inflammation. For example, in patients with major depression (MD), the risk is twice as high in developing AD, exhibiting cognitive decline and presenting with more amyloid beta plaques and neurofibrillary tangles (Sierksma 2012). These disorders have been implicated in decreases in neuronal plasticity, changes in neurogenesis, changes in morphology of granular neurons in the dentate gyrus and other aspects of the brain. The HPA axis is a crucial regulatory mechanism by which the brain uses to lead to a proper behavioral response as a result of a stressor in the environment. When stress is experienced by an organism, corticotropin releasing hormone (CRH) is secreted from the hypothalamus and released into the hypothalamic-hypophysial portal system where it travels through the blood and targets receptors on the anterior lobe of the pituitary gland. CRH stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) which will be secreted into systemic circulation and target receptors in the adrenal cortex. ACTH stimulates the synthesis and release of glucocorticoids into systemic circulation and these steroids have numerous target effects including inhibiting inflammatory responses. Stress can be advantageous or disadvantageous depending on the context. During early critical developmental periods, stress can help prepare an organism for future obstacles. On the other hand, stress can hyperactivate the system to something that is not present in the environment and this mismatching of stressor and environment can contribute to increased risk of acquiring psychopathology (Bodegom 2017).

Early life stress (ELS) is defined as exposure to stress during critical periods of development including pre-natal and postnatal periods of time. ELS can lead to permanent

changes in certain areas of the brain which affects networks and functionality, and increases overall risk for psychopathology. Pre-natal stressors such as bedding restriction, maternal separation and fragmented maternal care have been shown to increase HPA-axis hyperreactivity in adulthood, depressive symptomatology and increased immune and inflammatory responses to certain stimuli (Roque 2016). Maternal separation has been observed to reduce synaptic connectivity in the hypothalamus as well as enhance activation of vasopressinergic neurons and neurons that release corticotropin releasing hormone (Roque 2016). These changes are not only in the hypothalamus, but the hippocampus contributes to HPA dysfunction too. Stress has been shown to activate inflammatory processes mediated by microglial cells in the brain (Roque 2016). Patients who suffer from major depression experience chronic stress and on a cellular level, there is a higher number of activated microglia in the brain, supported by post mortem evidence (Sierksma 2012). Activated microglial cells in conjunction with changes in the concentration of neurotrophic factors in the brain create a dynamic that favors loss of neurons, loss of plasticity and greater cognitive dysfunction in a particular area of the brain responsible for learning and memory. Upon activation, microglial cells undergo a series of morphological changes: thickening of processes, elongation, and reorienting toward neural and astrocytic signaling molecules (Bollinger 2016). Activated microglial cells release neurotoxic compounds and function to prune dendritic spines. Chronic activation of microglial cells has been associated with heightened microglial density and deficits in working memory (Bollinger 2016). An important neurotrophic factor known as brain-derived neurotrophic factor (BDNF) promotes neural plasticity, survival and differentiation which all contribute to learning and memory processes (Roque 2016). Patients with major depressive disorder present with low BDNF, providing a possible explanation for cognitive declines and atrophy.

The environment, associated cellular actors and molecular signals that the mother and fetal offspring develop with have major implications on the molecular patterns of stress and inflammation because fetus mirrors the mother's responses to stress and relaxation. Prenatal stress has been linked with greater risk of acquiring diseases later in life, suggesting a possible immune-deficiency component of stress on development (Dis-Chavez 2012). Inflammatory responses are exacerbated into offspring of stressed-pregnant mothers and this hyperactivity of HPA responses have been attributed to various psychiatric disorders, namely major depressive disorder. There appears to be a cycle of stress leading to aberrant changes in regulation of inflammation and the players involved, producing pathological changes in neuronal connection and structure. Neurological changes manifest phenotypically as psychiatric disorders and could eventually manifest as the chronic accumulation of amyloid beta plaques. However, further research is needed to investigate the differential effects of pre and postnatal stressors and if adversity during these early developmental stages can impact health later in life, especially with regard to markers of Alzheimer's disease-like pathologies such as inflammation. The goal of the present study is to analyze the effects of stress at various time points in development on long term alterations in inflammation and the development of AD. It is hypothesized that early life stress during pre-natal and postnatal time points will have differential effects on adulthood inflammation, BDNF, anxiety-like behaviors and cognition.

Materials and Methods

Animals

C57BL/6 mice were housed in a temperature and light controlled vivarium with free access to food and water. Mice are housed depending on the condition to which they were assigned. For each of the three conditions, there will be 2 mothers and approximately 8 offspring. This protocol was reviewed by IACUC and received approval.

Pre-natal stress

Female mice that were 2-3 months in age were paired with a sexually experienced male. Daily examination of the females was conducted to look for a vaginal plug. The presence of a vaginal plug indicated that a mating had occurred and this would be embryonic day 0 (E0). On E3, pregnant mice were moved to a cage without bedding, known as bedding deprivation. Pregnant mice remained in this condition until birth of offspring. Females in the control condition will remain in their cage with normal bedding and will not be transferred to a cage without bedding.

Postnatal stress

Postnatal day 0 (PND0) is the day of birth. The mother and pups will be transferred to a cage with bedding to decrease the risk of pup mortality. The animals will remain in this cage until PND3. On PND3, pups will be transferred to a cage without bedding until weaning. For mice in the control condition (no stress), no cage changes will occur.

Behavior

Contextual Fear Conditioning (CFC) used automatic fear conditioning chambers (Coulbourn Instruments, Whitehall, Pennsylvania) and FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The chamber and software together allowed for the measurement of freezing behavior. Each chamber contained a grid floor, providing a means for the electrical, adverse

stimulus to be administered. At the top of the chamber was a camera that monitored all the movements of the animals, allowing measurement of freezing behavior. Along with a shock, dotted walls and an olfactory cue were utilized as the contexts. CFC training began 24 hours after the final injection of either LPS or saline, followed by testing an additional 24 hours after training. A 120 second acclimation period began the training session. After this was complete, a 2 second 0.7mA shock was administered. Once the stimulus was delivered, animals remained in the boxes for another 60 seconds. During testing day, there were no shocks. Animals were placed in the boxes and animal movements and freezing behavior were monitored via the computer system for 90 seconds. The amount of time in seconds animals spent freezing was then collected and analyzed.

Elevated zero is a behavioral test employed to assess anxiety-like behavior in mice. The platform is about 1 meter high and has areas that are enclosed and areas that are open. Naturally, mice prefer enclosed spaces rather than open spaces. Stressed mice have been shown to prefer enclosed spaces to open spaces (Bailey 2009). Noldus Ethnovision program (Noldus, Leesburg, VA) is used to track the movement of mice while on the maze. The amount of time spent in the open versus closed segments is recorded. Each session is 5 minutes with lights turned off. After the mouse is removed, a student cleaned up the maze with water.

Novel object placement (NOP) is used to assess recognition memory. Mice tend to explore objects that are new to an environment more than objects that are familiar. NOP consists of an open field, and 50 mL conical tubes filled with silver beads. The first day of NOP is referred to a habituation which contains no objects and lasts for 5 minutes per session for three training sessions. After each session, objects and arena are cleaned with water and ethanol. During testing days five hours after training, conical tube will be placed in its original spot and

the other tube will be placed in a new spot. Between trials, students use water to clean the arena and conical tubes.

LPS administration and tissue collection

At adulthood (4 months), stressed and non-stressed mice were randomly assigned to LPS (serotype O55B5) or saline treatment conditions. Animals in the LPS condition will be administered one injection of LPS (Sigma Aldrich) to evoke an immune response. The dosage was 250 mg/kg. The other group will receive saline and act as a control. LPS is stored in -80 C. Mice were administered an intraperitoneal injection (i.p). Deviations in injection were noted.

Four hours after injection of either saline or LPS, animals were sacrificed. 2/3 of dorsal hippocampus were removed from the brain under RNase free conditions and stored in Invitrogen RNAlater stabilization solution.

RNA isolation and RT-PCR

RNA is isolated on the Maxwell 16 Total RNA Purification Kit and mRNA expression of TNF-α, IL-1β, IL-6 and BDNF will be quantified utilizing reverse transcriptase polymerase chain reaction (RT-PCR). I-Script cDNA Synthesis Kit from Bio-Rad will be utilized to convert cDNA from mRNA and study gene expression of TNF-α, IL-1β, IL-6 and BDNF. RNA was isolated according to an established protocol.

Samples were thawed and kept over ice. Homogenization solution was prepared, PCR tubes were labeled and each tube receive 40 µL of nuclease free water. RNase free microfuge tubes were labeled and received 200 µL of lysis buffer. Samples were prepared and added to cartridges. One plunger was added to each cartridge. On the Maxwell 16 Total RNA Purification Kit, the program “simply RNA” was run. Samples RNA samples were then placed the -80 °C freezer or were nano-dropped to measure the amount of RNA in each sample.

Statistical analysis

A 2 (treatment; LPS, saline) x 3 (condition; combination stress, postnatal stress, no stress) univariate analysis of variance (ANOVA) will be used following RT-PCR to determine significant differences.

Results

Males and females did not exhibit anxiety-like behavior

Behavioral testing occurred four months after the conclusion of bedding restriction in order to assess how stress impacted inflammation in adulthood, and behavioral alterations could be expected as a result of early life stress. Elevated zero is a behavioral measure of anxiety-like behavior. The more time that mice spend in the closed arms of the platform indicates an increased level of anxiety. The amount of time spent in the open arm was assessed for male and female mice. For female mice, no significant differences were seen in comparing control mice, postnatal stress mice and combination stress mice ($p = 0.631$; Figure 1). For male mice, no significant differences were seen in comparing control mice, postnatal stress mice and combination stress mice ($p = 0.398$; Figure 2).

Female mice in the combination stress condition demonstrated locomotor deficits

Another metric that was assessed from elevated zero was total distance traveled around the platform. Female mice in the combination stress condition traveled significantly less around the platform compared to control and postnatal stress mice ($p<0.05$; Figure 3). Female mice may have been less active due to depressive symptoms. Male mice did not achieve significant differences between any of the conditions for total distance traveled ($p = 0.424$; Figure 4).

Male mice in the combination stress condition demonstrated learning and memory deficits

Contextual fear conditioning is a behavioral test to assess memory of a context in which an adverse stimulus was administered. One behavior that is correlated with memory is freezing response and mice that freeze for longer amounts of time demonstrate memory of a context. During the first batch of males, freezing response was highest in the control animals and lowest in the combination stress animals. However, this trend did not achieve any significant differences among the three conditions ($p = 0.125$; Figure 5). Another batch of male mice was run, but a different series of behavioral tests were conducted. Control animals and combination stress animals were repeated and combination stress animals had a significant reduction in time freezing compared to the control ($p<0.05$; Figure 6). For female mice, no significant relationships were demonstrated among the three conditions ($p = 0.381$; Figure 7).

Different stress conditions did not demonstrate significant changes in IL-1β, but approached significance

The treatment group of mice that received LPS demonstrated significantly elevated levels of IL-1β compared to saline treatment group of mice ($p<0.001$; Figure 8). This effect is expected since LPS is an inflammatory stimulus, and thus cytokines should be produced in response. No significant changes in IL-1β production were observed in comparing the three conditions for both LPS and saline, but combination stress mice in the LPS treatment approached a significant reduction in LPS ($p = 0.077$; Figure 8). While this is not a significant compared to LPS control and postnatal mice, this trend points to an immunosuppressive effect as a result of combination stress.

Treatment and condition produced significant alterations in TNF-α production

LPS-treated mice had significantly higher levels of TNF-α produced compared to production by saline-treated mice ($p<0.001$; Figure 9). This effect is expected due to LPS being an established stimulator of inflammation. In comparing each condition within the LPS treatment, combination stress mice demonstrated significant reduction in TNF-α production compared to postnatal stress mice ($p<0.05$). LPS-treated control mice were neither significantly different from the postnatal stress mice nor the combination stress mice. In analyzing the saline-treated mice, TNF-α was significantly reduced for combination stress animals compared to control and postnatal stress mice. Mice that received combination stress demonstrated an immunosuppressive effect.

Treatment and condition produced significant alterations in IL-6 production

LPS treatment produced significantly higher levels of IL-6 compared to the saline treatment ($p<0.001$; Figure 10). Again, supporting previous changes in cytokine production, when an inflammatory stimulus is administered, cytokine production is naturally going to increase. For LPS-treated mice, IL-6 was significantly reduced in combination stress mice compared to control and postnatal stress mice ($p<0.05$). For saline-treated mice, no significant changes in IL-6 were observed. Similar to the other two inflammatory cytokines, reductions in IL-6 indicate involvement of immunosuppression.

Treatment and condition produced significant alterations in BDNF production

In comparing LPS-treatment to saline-treatment, LPS-treatment had significantly lower levels of BDNF compared to BDNF production in the saline treatment ($p<0.01$; Figure 11). Initiation of inflammation will naturally decrease levels of BDNF and this supports the effects of LPS on inflammation in mice. Elevated inflammation will naturally decrease BDNF regardless of the presence of various stressors. For LPS-treated animals, no differences were seen among any of the three conditions. For saline-treated animals, combination stressed animals had a significantly

reduced level of BDNF compared to control and postnatal stress animals ($p<0.05$). Given that saline animals were not exposed to an inflammatory stimulus, BDNF levels would be altered by the different stress conditions. Combination stress may have an effect on levels of BDNF in the brain. In comparing levels of BDNF in combination stress animal in LPS and saline treatments, no significant differences are seen, which may suggest that BDNF reached a bottoming-out effect, and could not be lowered further by the stress condition.

Discussion

In the present study, we sought to determine the effect of stress at different developmental time points on inflammation in adult mice. Previously, ELS has been shown to induce changes in neural morphology and is associated with an increased risk for psychopathology. When mice experience pre-natal stress, the HPA axis is observed to be more hyperreactive in adulthood, and mice exhibit more depressive behaviors as well as elevated immune responses to stimuli (Roque 2016). When maternal separation was used to stress mice postnatally, neurons did not form as many synapses compared to animals that did not experience a postnatal stressor (Roque 2016). Within the hippocampus, microglia active inflammatory processes and are more active in mice that experienced early life stress (Roque 2016). Other studies have demonstrated that ELS can increase the vulnerability of mice to infection later in adulthood (Dis-Chavez 2012). While many studies have demonstrated alterations to inflammation, and behavior as a result of ELS either administered in a postnatal window or pre-natal window, more research is required to understand the effects of a combination stress paradigm on inflammation in adulthood. To assess the effects of stress on both development in utero and after birth, combination stress was utilized in conjunction with a control and postnatal condition.

To test our hypothesis, three conditions were proposed: control group (no stressor), postnatal stress mice and combination stress mice. During the pre-natal window, stress was applied after a mating had occurred and continued until birth. During the postnatal window, stress was applied on postnatal day 3 and continued until pups were weaned. Pups grew to adulthood and at 4 months, behavior and responses to inflammatory stimuli were assessed. Mice underwent several behavioral tests including elevated zero, contextual fear conditioning and novel object placement. To assess inflammation, expression levels of three inflammatory cytokines were assessed and a cytokine related to neurogenesis and synaptogenesis was also measured to monitor changes in neuronal markers in the dorsal hippocampus. Mice received an injection of either LPS or saline, and several hours after injections, mice were sacrificed and brain was harvested for gene expression analysis.

We hypothesized that inflammatory responses in adulthood would be altered as a result of early life stress. Evidence from the literature indicates that various regulatory systems in mice would be altered, but the direction that these alterations occurred was more variable. Some studies found HPA hyperreactivity, some studies found reduced responses from immune cells, but hyperreactive immune cells in the brain. Based on analysis of the literature, it was difficult to predict whether inflammation would be suppressed or elevated, thus suggesting that some alteration would occur was a stronger hypothesis. Inflammatory cytokines were expected to be significantly different from control animals as a result of the administration of stress. Behavior was expected to be altered as well, and evidence from the literature suggested the presence of depressive symptoms and learning and memory deficits. These effects would result from data gathered from elevated zero and contextual fear conditioning. Mice that were more depressed may have decreased locomotor activity. For CFC, mice that spent less time freezing

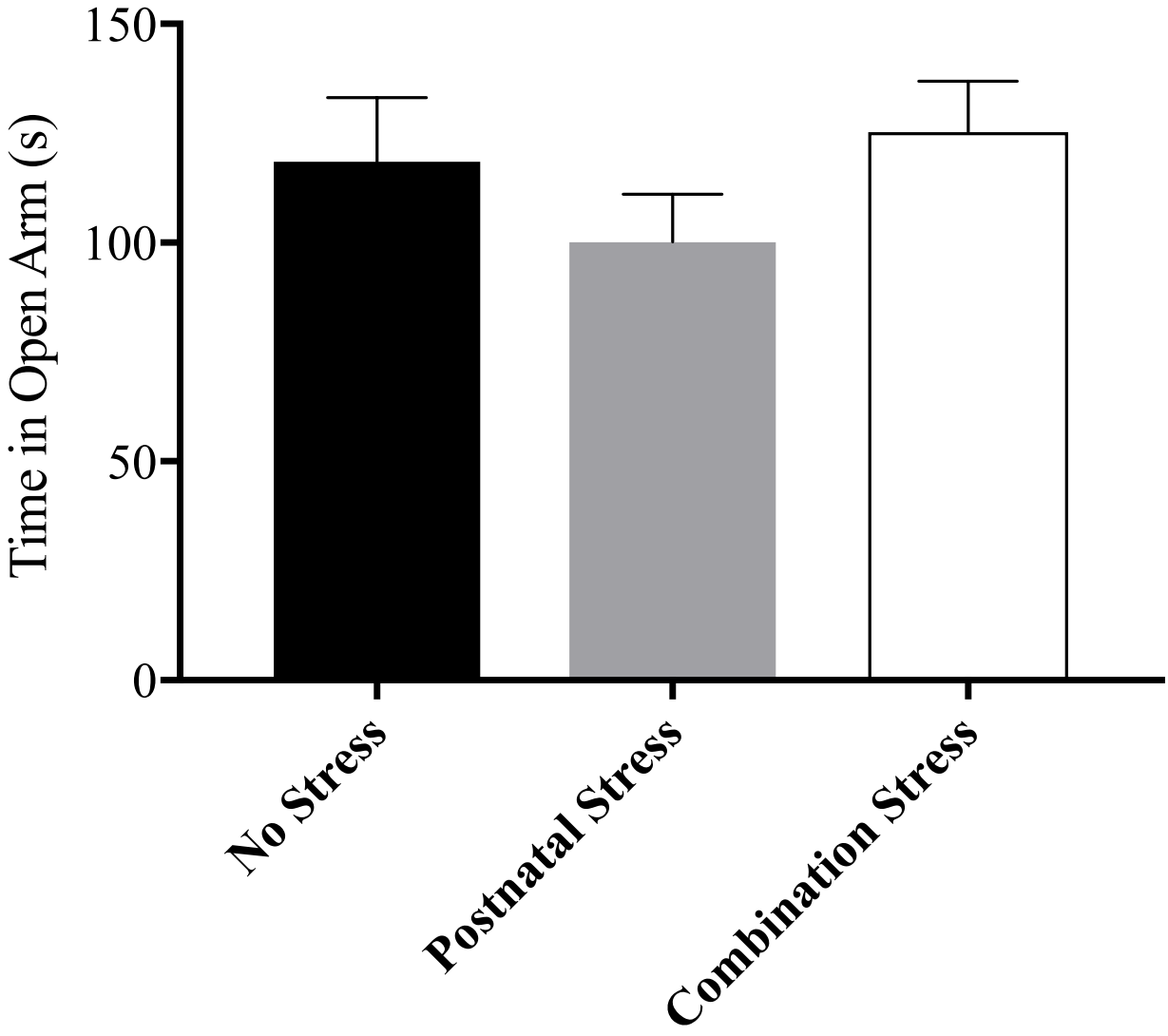
demonstrated deficits in learning and memory. If a mouse had memory of a context where an adverse stimulus was received, it would freeze. Mice that do not freeze lack proper memory formation and learning about the stimulus and do not recall their context that they received the stimulus in.

Our results indicate that mice showed sex-specific behavioral alterations as a result of combination stress. Females demonstrated significantly reduced locomotor activity, suggesting depressive behavior whereas males showed no significant differences. In CFC, only males demonstrated learning and memory deficits. Evidence from rat studies have found that female rats are more sensitive to HPA activation following a pre-natal stressor. Female guinea pigs demonstrated impaired HPA-associated glucose tolerance and insulin sensitivity whereas male guinea pigs did not demonstrate these effects (Verma 2011). Differences in male and female sex response to stress can be observed across species and understanding why there is a sex-specific response requires greater investigation. Related to the behavioral learning and memory deficits experienced by male mice, decreases in BDNF may point to a molecular mechanism of losses in learning. Lower BDNF is an indicator that neurons are not forming as many synapses, and are not dividing as much. Since stress is occurring at time points when brain development would be occurring, changes in brain morphology would alter the ability of mice to learn. Stress has been previously shown to alter brain morphology and cellular players in the brain. In mice experiencing stress, microglia produce deficits in working memory, decrease the number of neurons in the brain, decrease plasticity and result in greater levels of cognitive dysfunction (Bollinger 2016). Lower levels of BDNF would be predicted to produce cognitive deficits in the ability of mice to form memory about a context in which they received an adverse stimulus. Lastly, while an immunosuppressive effect was observed, cytokines were differentially affected

by stress. For IL-1β, no significant differences were observed among conditions, but there was a trend toward a significant reduction in IL-1β for the combination stress group. TNF-α and IL-6 did achieve significant reductions for mice experiencing combination stress. However, more interestingly is the fold-changes that occurred. IL-6 decreased roughly 3-fold for LPS-treated mice in the combination stress condition compared to postnatal mice and control mice. TNF-α was much less sensitive to LPS compared to IL-6 and IL-1β. TNF-α for combination stress had a relative expression near 1, compared to post-stress mice which hovered around 2. For IL-1β, relative expression hovered around 10 for postnatal stress mice, but decreased to a relative expression of around 5-6 for combination stress. All three cytokines are involved in inflammatory, but were different based on their sensitivity. Based on these sensitivities, the immune response to infection in adulthood may be biased toward a particular response. IL-6 is relevant for the T helper cell 2 response and since it was significantly reduced, T helper cell 2 response may be inhibited, leading to a predominance of other T cell subtypes. However, more exploration and research are necessary to understand how T cells differentiate as a result of stress applied to different developmental time points.

Overall, these results suggest that stress at various developmental time points produces alterations in inflammation in adulthood, specifically an immunosuppressive effect on the molecular level. Behaviorally, alterations are observed, but appear to be distinct to a particular sex.

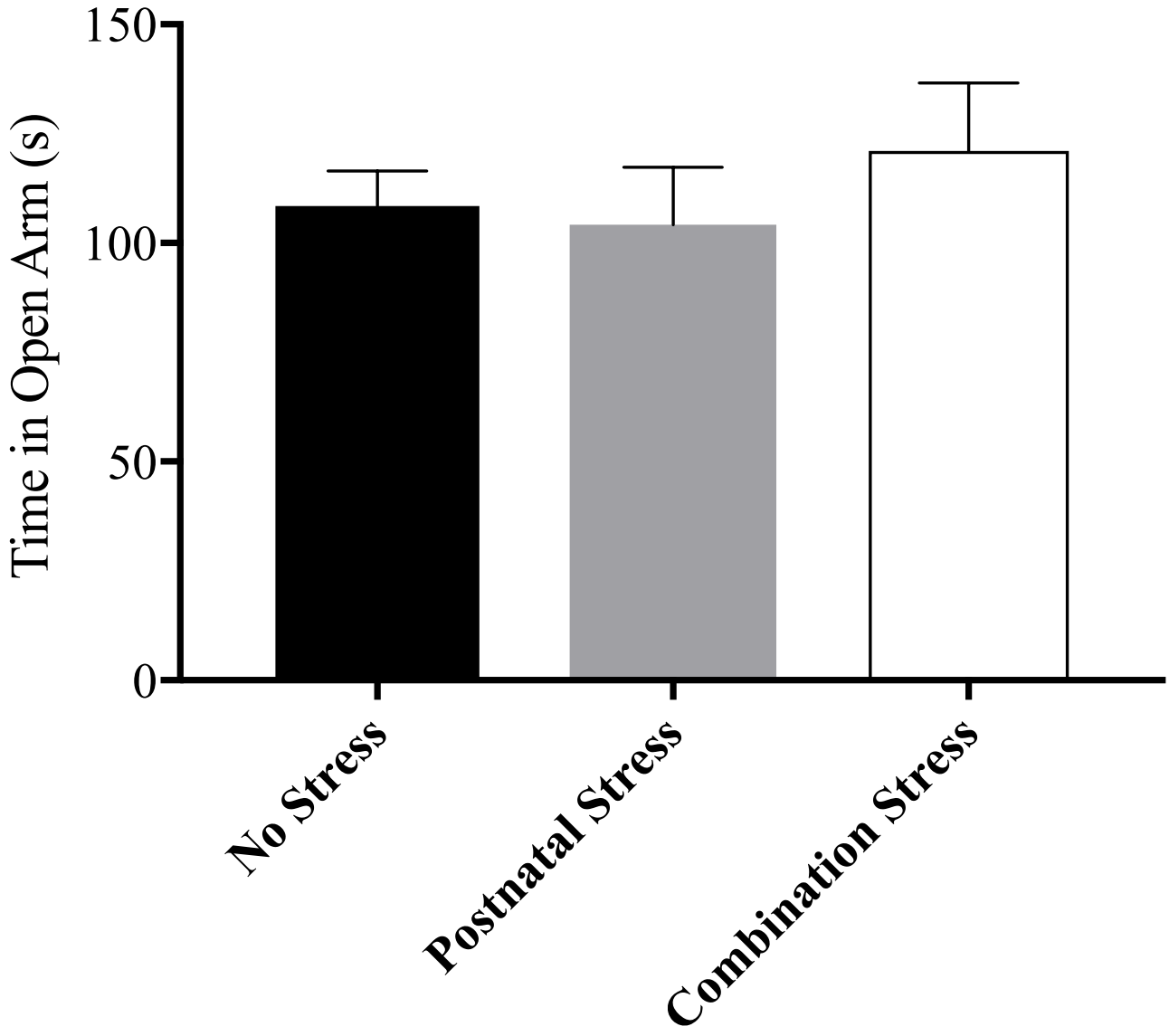
Figure 1



Elevated 0 in Males After Bedding Restriction

One-way ANOVA shows no significant difference in time spent in the open arm ($p=0.398$) N's 12-15

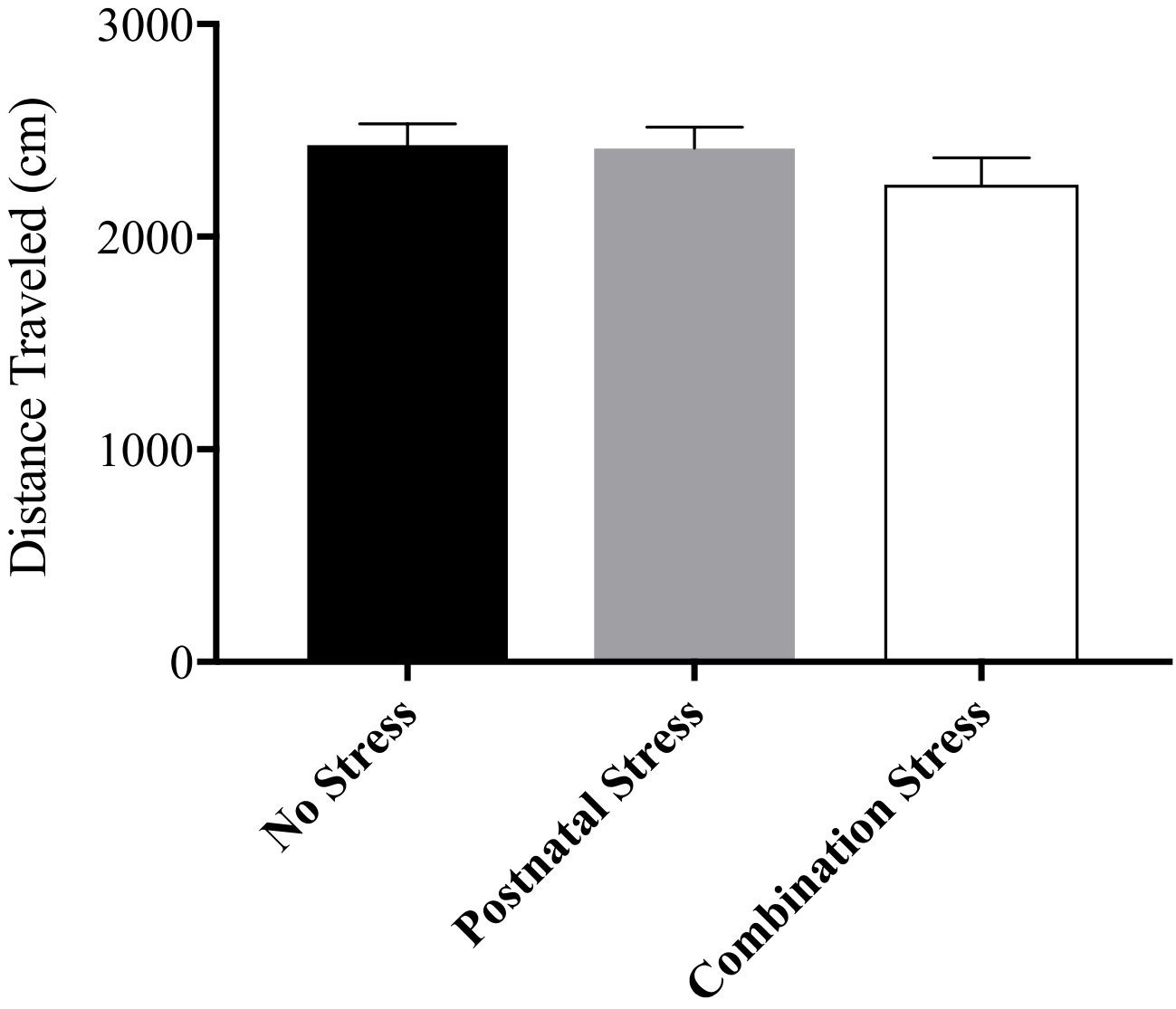
Figure 2



Elevated 0 in Females After Bedding Restriction

One-way ANOVA shows no significant difference in time spent in the open arm ($p=0.631$) N's 9-14

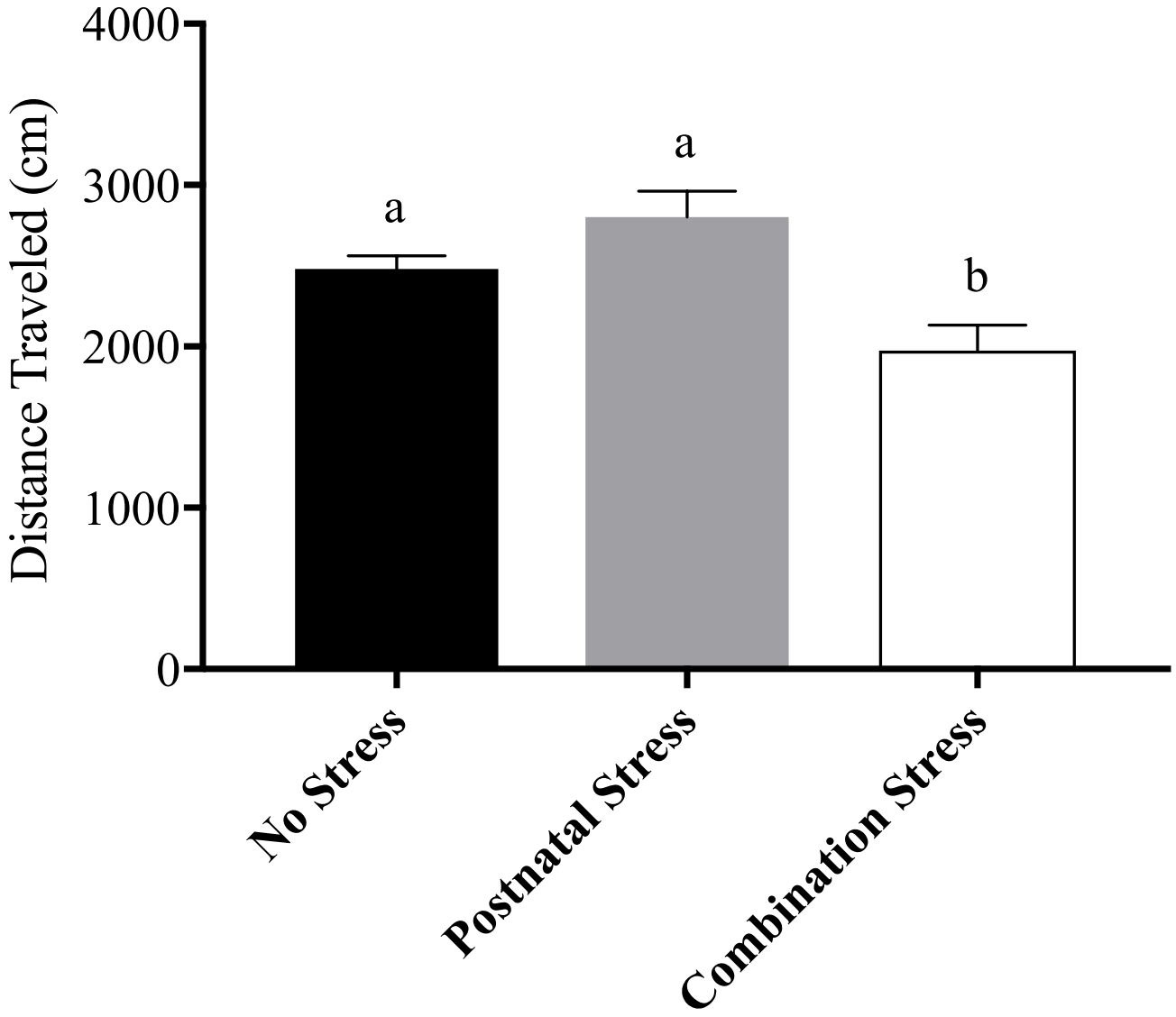
Figure 3



Elevated 0 in Males After Bedding Restriction

One-way ANOVA shows no significant differences in distance traveled ($p=0.424$) N's 12-15

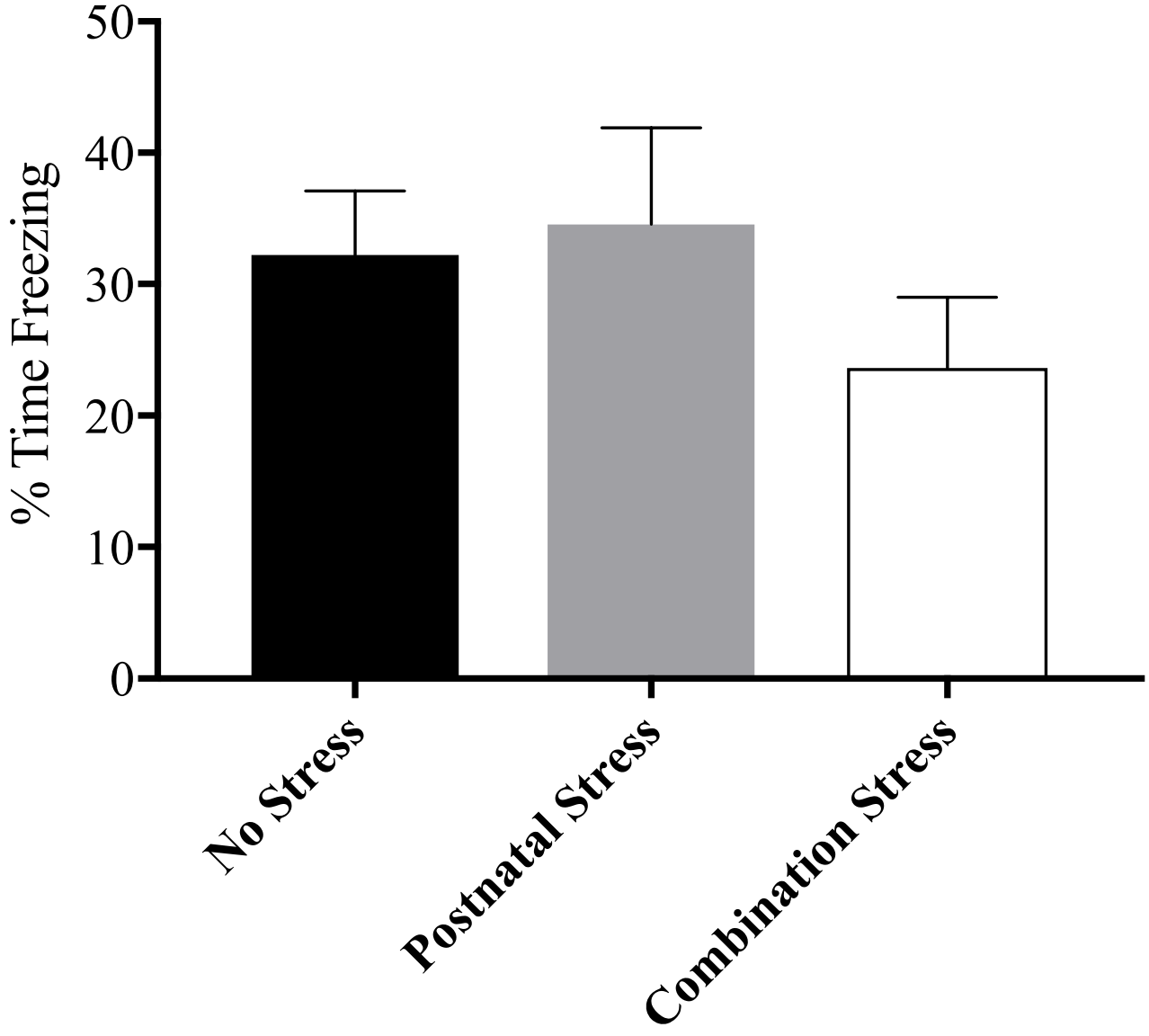
Figure 4



Elevated 0 in Females After Bedding Restriction

One-way ANOVA shows significant difference between the postnatal and combination stress groups for distance traveled ($p<0.05$) N's 9-14

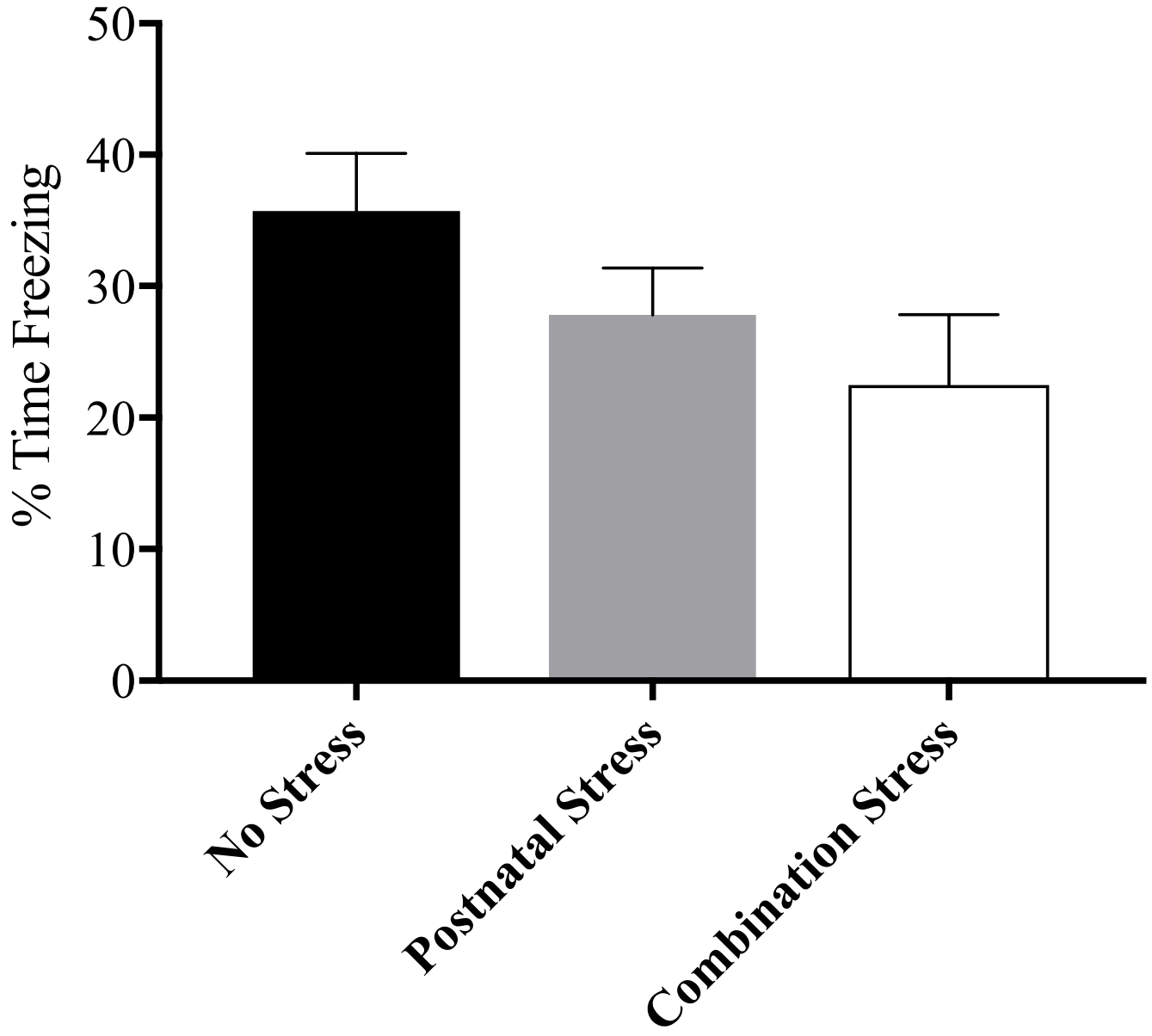
Figure 5



CFC in Females after Bedding Restriction

One-way ANOVA shows no significant differences in percent freezing ($p=0.381$) N's 7-14

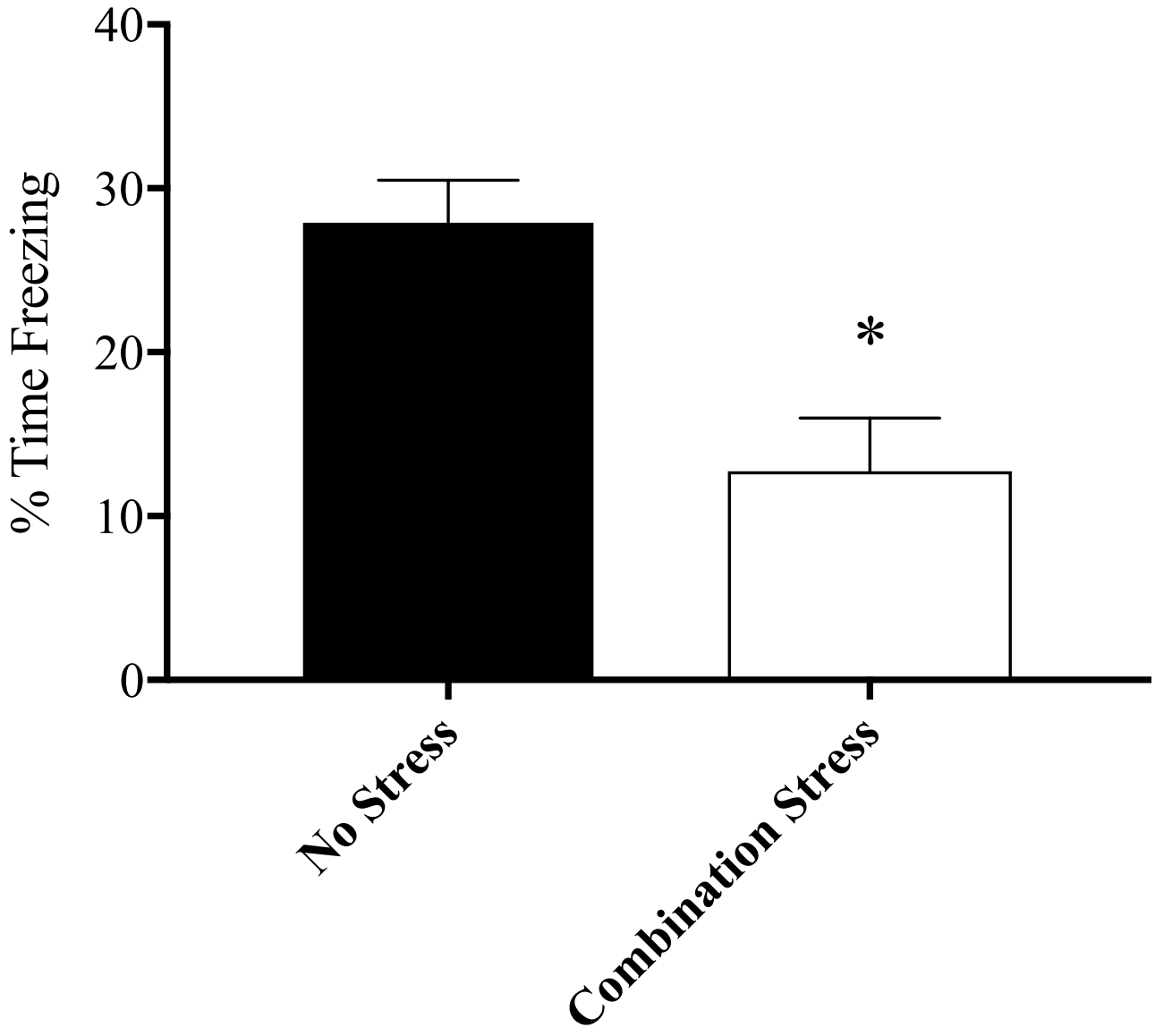
Figure 6



CFC in Males after Bedding Restriction

One-way ANOVA shows no significant differences in percent freezing ($p=0.125$) N's 11-14

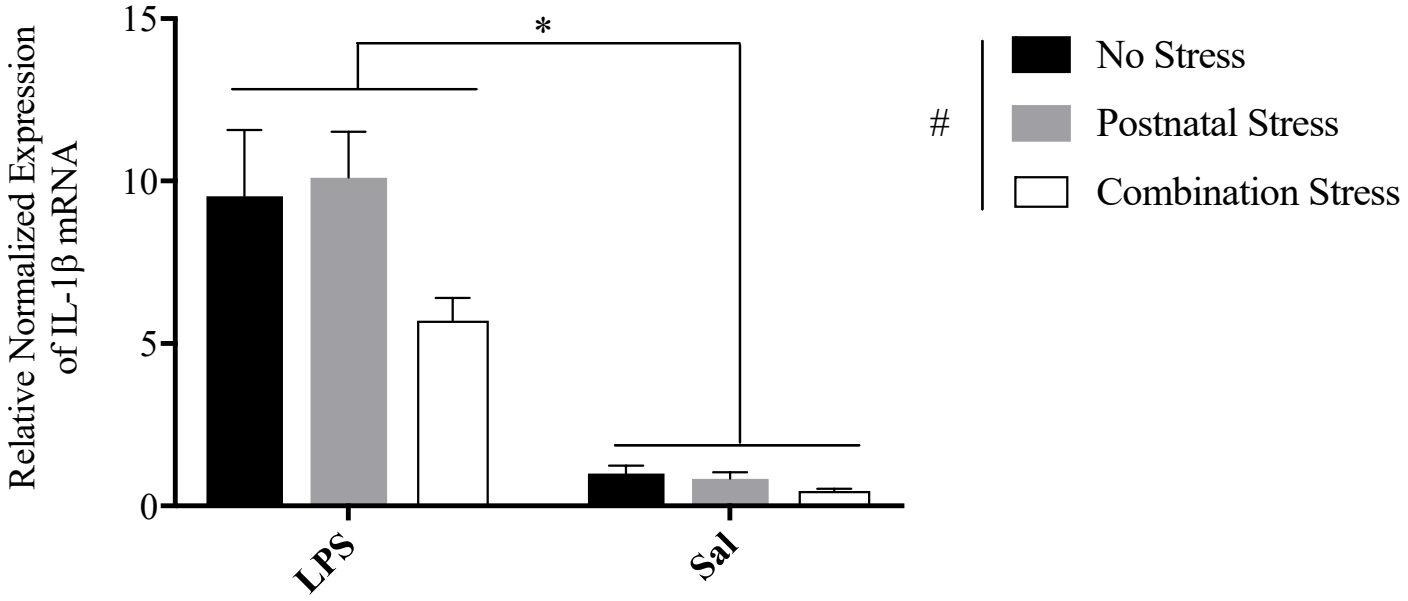
Figure 7



CFC in Males after Bedding Restriction

One-way ANOVA shows significant difference in time freezing between no stress mice and combination stress mice

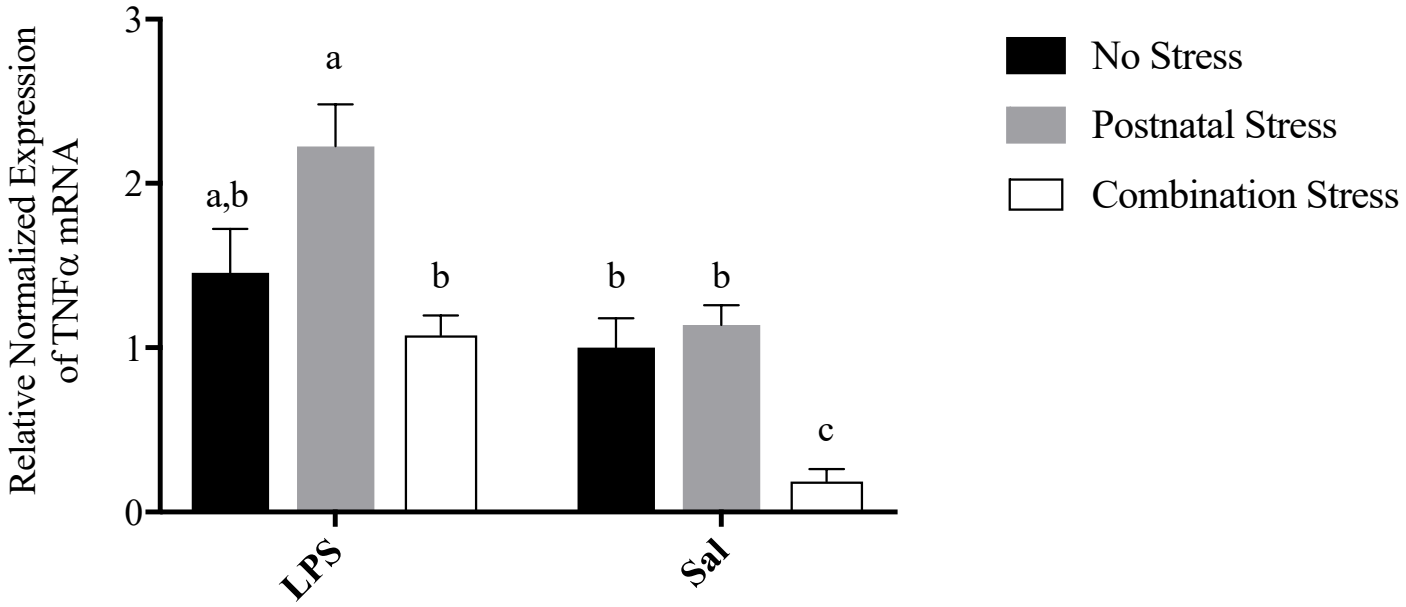
Figure 8



Hippocampal mRNA Expression of IL-1β After Bedding Restriction

Results from RT-PCR were normalized to β-actin prior to being normalized to control group (No Stress/Saline). No significant differences in stress effect ($p=0.077$) * represents significant differences ($p<0.001$) by 2 Way ANOVA N's 5-7

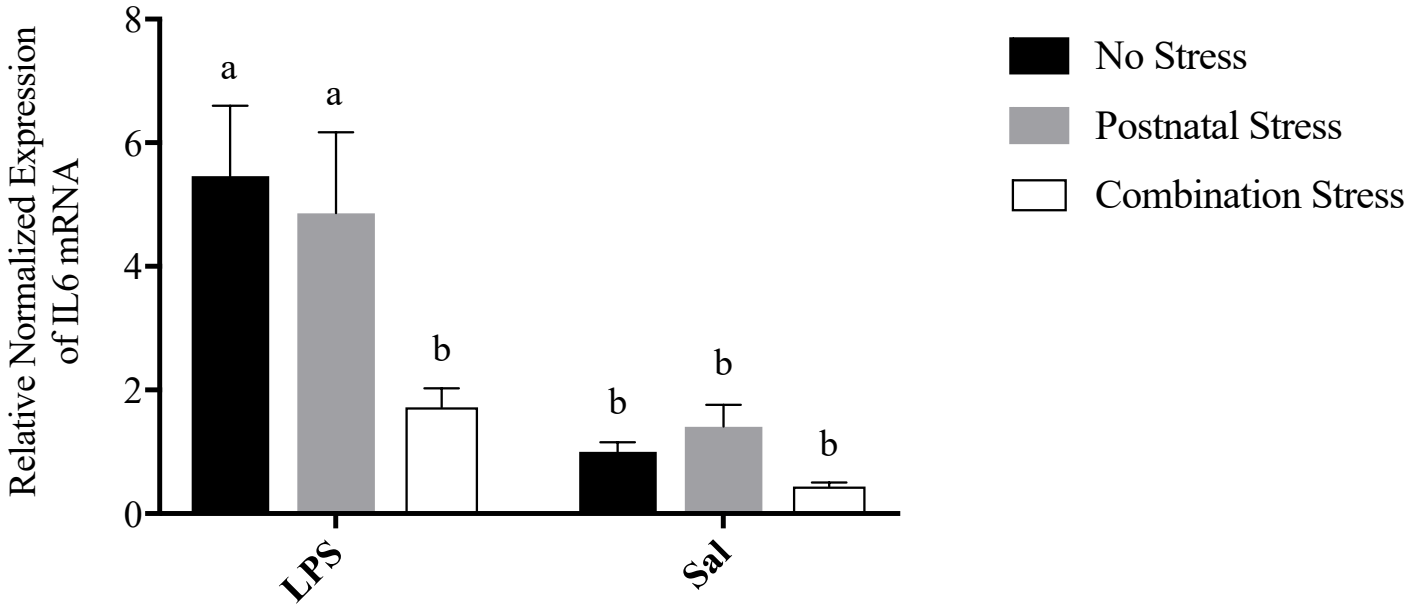
Figure 9



Hippocampal mRNA Expression of TNFα After Bedding Restriction

Results from RT-PCR were normalized to β-actin prior to being normalized to control group (No Stress/Saline). Significant differences in stress condition and LPS and saline treatment ($p<0.001$) Different letters represents significant differences ($p<0.05$) by 2 Way ANOVA N's 5-7

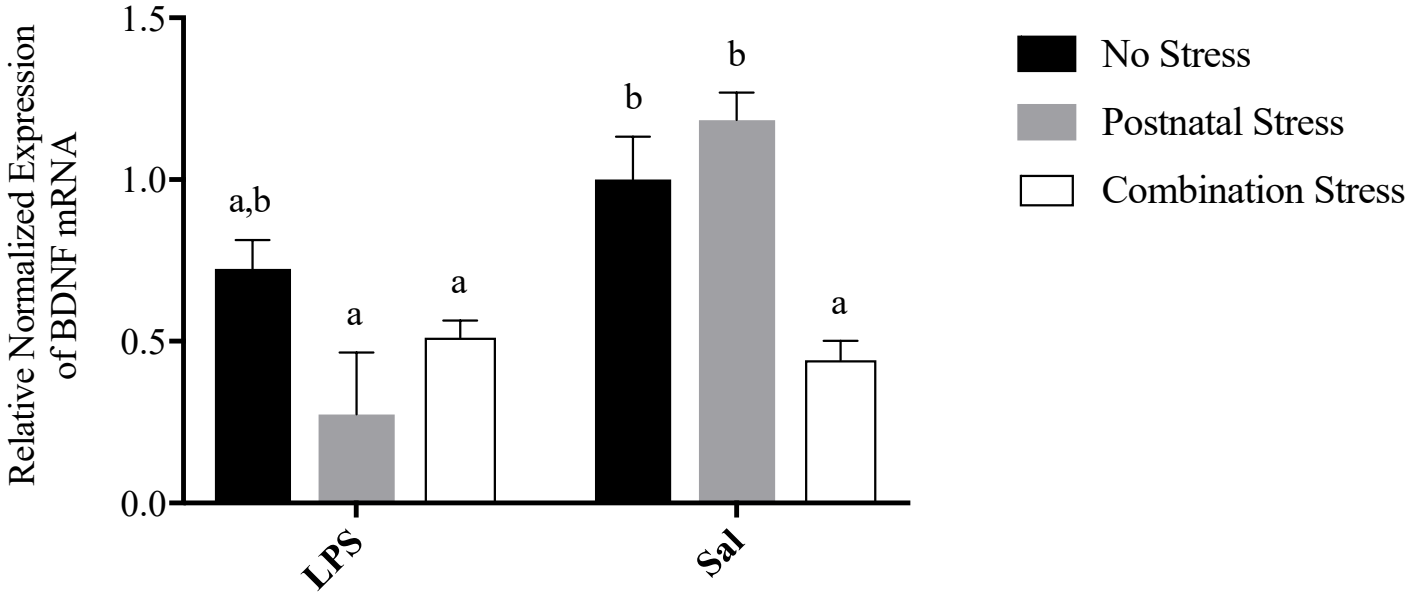
Figure 10



Hippocampal mRNA Expression of IL6 After Bedding Restriction

Results from RT-PCR were normalized to β-actin prior to being normalized to control group (No Stress/Saline). Significant differences in stress condition ($p<0.05$) and LPS and saline treatment ($p<0.001$) Different letters represents significant differences ($p<0.05$) by 2 Way ANOVA N's 5-7

Figure 11



Hippocampal mRNA Expression of BDNF After Bedding Restriction

Results from RT-PCR were normalized to β-actin prior to being normalized to control group (No Stress/Saline). Significant differences in stress condition and LPS and saline treatment as well as interaction ($p<0.01$). Different letters represent significant differences ($p<0.05$) by 2 Way ANOVA N's 4-7

References

Bailey KR, Crawley JN. Anxiety-Related Behaviors in Mice. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2009. Chapter 5.Available from: https://www.ncbi.nlm.nih.gov/books/NBK5221/

Bodegom, M., Homberg, J., Henckens, M., Modulation of the Hypothalamic-Pituitary-Adrenal Axis by Early Life Stress Exposure. *Frontiers in Cellular Neuroscience*. 11:87

Bollinger, J., Burns, C., Wellman, Cara. Differential effects of stress on microglial cell activation in male and female medial prefrontal cortex. (2016) *Brain, Behavior and Immunity*, 52 88-97

Curzon P, Rustay NR, Browman KE. Cued and Contextual Fear Conditioning for Rodents. In: Buccafusco JJ, editor. Methods of Behavior Analysis in Neuroscience. 2nd edition. Boca Raton (FL): CRC Press/Taylor & Francis; 2009. Chapter 2. Available from: https://www.ncbi.nlm.nih.gov/books/NBK5223/

Diz-Chaves, Y., Pernia, O., Carrero, P., Garcia-Segura, L. Prenatal Stress causes alterations in morphology of microglia and the inflammatory response of the hippocampus of adult female mice. *Journal of Neuroinflammation* 2012, 9:71

Kahn, M.S., Kranjac, D., Alonzo, C.A., Haase, J.H., Cedillos, R.O., McLinden, K.A…Chumley, M.J. (2012). Prolonged elevation in hippocampal Aβ and cognitive deficits followed repeated endotoxin exposure in the mouse. *Behavioral Brain Research*, 229 (1) 176-184.

Kumar, A., Singh, Arti. A review on Alzheimer's disease pathophysiology and its management: an update. (2015) *Pharmacological Reports*, 67 (2) 195-203

Reitz, C., Brayne, C., Mayeux, R. (2011) Epidemiology of Alzheimer Disease. *Nature Reviews*, 7 137-152

Roque, A., Ochoa-Zarzosa, A., Torner, L. Maternal separation activates microglial cells and induces an inflammatory response in the hippocampus of male rat pups, independently of hypothalamic and peripheral cytokine levels. *Brain, Behavior and Immunity*. 55 (2016) 39-48

Scheltens, P., Blennow, K,. Breteler, M., Strooper, B., Frisoni, G., Salloway, S., Van der Flier, W. (2016) Alzheimer's Disease. *The Lancet*, 388 (10043) 505-517

Sierksma, A., Vanmierlo, T., De Vry, J., Raijmakers, E., Steinbusch, Harry., Van den Hove, D., Prickaerts, J. Effects of prenatal exposure on soluble Aβ and brain-derived neurotrophic factor signaling in male and female APPswe/PS1dE9 mice. *Neurochemistry International*. 61 (2012) 697-701

Verma, R., Balhara, Y. P., & Gupta, C. S. (2011). Gender differences in stress response: Role of developmental and biological determinants. *Industrial psychiatry journal*, *20*(1), 4–10. doi:10.4103/0972-6748.98407