COMBINED EFFECTS OF REPEATED SOCIAL DEFEAT AND INFLAMMATION ON THE ACCUMULATION OF AMYLOID-β WITHIN THE HIPPOCAMPUS

by

MICAH JACOB EIMERBRINK

Bachelor of Arts, 2006
Texas State University
San Marcos, Texas

Master of Science, 2011
University of Texas at Arlington
Arlington, Texas

Submitted to the Graduate Faculty of the
College of Science and Engineering
Texas Christian University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

May 2017

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Dissertation approved:

(Major Professor)

For The College of Science and Engineering

ACKNOWLEDGEMENTS

The opportunity to pursue this educational goal is one I am deeply thankful for, and I recognize how sincerely blessed I am to have had the support and guidance of so many caring, intelligent, and patient individuals. This achievement would not have been possible without the members of my committee, a countless number of educators, my parents, friends, and beloved wife, and with utmost humility, please know that I cherish of your efforts. Thank you, not just for helping me achieve the degree of Doctor of Philosophy, but for valuing education.

Dr. Gary Boehm, where to begin. I truly don't know how to appropriately thank you for everything you have done for me, as the sentiment of 'thank you' seems too shallow. When you accepted me into your lab, I know you were taking a chance, and I am so thankful that you did. I have learned so much from you and your example, and I am a better person as a result. Thank you for forcing me to discover my own research path through graduate school, and for guiding me through obstacles when your help was needed (which was more often than you probably know). Your mentoring and friendship over the past several years has made all the difference in my life, and I could not have done this without you

Dr. Chumley, I wish I could have gotten to know you better, but I'm thankful for all the time we were able to work together. Your commitment to education and academia exemplify a work ethic that few achieve, and I hope to emulate your example in all my future endeavors.

To the remaining members of my committee, thank you all. You have each individually provided such a positive influence in my life, and I appreciate all the time and effort required to help me complete this achievement. Additionally, I'd like to thank Dr. Cathy Cox. I always

appreciated you smile and wit, even during brief interactions in a hallway, you were never absent of kind words and surprising punchlines.

There are so many other graduate students and lab members to thank. Dinko and Marielle, thank you for welcoming me into the lab. Marielle, working with you was a blast, and was one some of the best years in graduate school. Jordan and Julia, thank you for always being there, any time help was needed. You are both a constant source of support and friendship, and I know you will both do amazing things before you leave. James Taylor, you are an amazing friend with a brilliant intellect, and your friendship is one of the best things to come from my graduate education.

My parents, Donald and Susan Eimerbrink, and Paddy and Lakshmy Padmanabhan, you have all provided such love and support for me throughout this entire process, I can never thank you enough, and this would not have been possible without all of you. Thank you for being a constant source of inspiration and encouragement, and for believing in this goal.

To my wife, Priya, you are the reason for any accomplishment in my life, and your love is an endless source of strength and encouragement. You are a constant inspiration in my life, and I cannot imagine completing this degree without you. Thank you for being the person I always turn to, and for always being there when I do. In addition to wife, you are my best friend, most trusted confidant, and I know how lucky I am to be loved by you. I love you, Priya.

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Chapter 1.

Introduction

The inevitable need of organisms to respond to the external pressures of the world is a commonality that unites all biological entities. The interaction between an organism's biology and environmental circumstance results in an intricate bi-directional relationship capable of modifying all forms of life, a process that has shaped every known species. The importance of understanding this process and how organisms arrived at their modern form is difficult to overstate. However, the discovery that biological processes mediate the ability of organisms to respond and adapt to the needs of their surroundings provided a framework that helped unite psychology and biology. Individual evaluation of a set of stimuli requires several psychological processes, and if the outcome of that evaluation indicate the stimuli represent a threat, a psychologically-induced alteration in biological function can occur to help the organism respond to the identified threat. This interaction between biology and environment has undoubtedly developed to promote survival, but is not without potentially adverse consequences. The purpose of this dissertation is to present a series of studies that demonstrate how the experience of a specific social circumstance can alter an induced immune response to disrupt behavior and trigger the production of biological precursors for Alzheimer's disease.

Introduction to Stress and the Response to Stress

Often regarded as the greatest American physiologist, Walter Cannon ushered a new understanding of stress into the literature with a recognition that much of human biology adaptively fluctuates in response to the needs of a particular circumstance (Cannon, 1914).

This conceptualization of biology implies that physiological states are transitory, and fluctuate in response to periods of intense, as well as diminished activation. Moreover, Cannon observed that organisms undergo a process of physiological balance that maintains biological systems within tolerable activation limits, a process he termed *homeostasis* (Cannon, 1929). Cannon's conceptualization of physiology described an adaptive process that permitted an organism to respond to intermittent threats with increased physiological arousal punctuated with extended periods of activation balance. Homeostasis encompassed this activation balance, and Cannon used the term 'fight or flight response' to describe the periods of increased physiological arousal.

Upon initiation of the 'fight or flight response,' an organism mobilizes muscular activity to respond to threats by means of fleeing or physical engagement (Cannon, 1953). This response can be activated as a result from neural processing within the dorsomedial amygdala or from peripheral somatic information processed within the nucleus of the solitary tract (NTS) (Cunningham, Bohn, & Sawchenko, 1990; Herman et al., 2003; Roldan, Alvarez-Pelaez, & Fernandez de Molina, 1974). Projections from the amygdala facilitate signal transduction to the lateral and posterior hypothalamus (Roldan et al., 1974); from the hypothalamus signals are relayed to the medulla and spinal cord before terminating at the adrenal medulla (Roldan et al., 1974). Within the adrenal medulla, chromaffin cells respond to the incoming stimuli with the production and release of two catecholamines, epinephrine and norepinephrine. Additionally, norepinephrine can be released from pre and post ganglionic sympathetic neurons. The release of catecholamines triggers a generalized increase in excitatory somatic responses within approximately twenty-five seconds (Wenger et al., 1960). Cannon focused much of his research on the somatic response to catecholamines, and prompted the him to articulate the 'fight or flight' response. Today, the biological axis that responds to such stimuli with the release of

catecholamines is described simply as the sympathoadrenomedullary system (SAM). However, Cannon was only studying a small portion of the response to threats with his focus on the SAM.

In the 1950's, Hans Selye extended Cannon's discoveries, and described what he called the "General Adaptation Syndrome[1] (GAS)," characterized by a process of biological recognition and response to any bodily demand (Selye, 1950). He viewed stress as a "non-specific" response in that, regardless of the demand (the stressor), the stress response could be triggered as an effort to respond to the stressor. He divided the GAS into three different phases, alarm, resistance, and exhaustion. The alarm stage of GAS occurs when a threat is recognized. Following recognition, the organisms attempts to adapt and resist the stressor, but if these efforts are not successful, the organism enters the exhaustion phase wherein negative health effects accrue, including death of the organism, in extreme circumstances.

The biology of the GAS is rooted in a separate axis than the SAM identified by Cannon, and attributes the primary response mechanism to stress to the hypothalamic-pituitary-adrenal axis[2] (HPA). Within the hypothalamus, neurons in the medial parvocellular subdivision of the paraventricular nucleus (PVN) synthesize corticotropin-releasing factor (CRF). When an organism encounters a stressor, CRF is released into portal vessels that circulate through the median eminence. When CRF binds to the CRF type 1 receptor in the anterior pituitary, adrenocorticotropic hormone (ACTH) is released into the circulatory system. The primary target of ACTH is the melanocortin type 2 receptor (MC2-R) located on the adrenal cortex. Activation

[1] Selye's work on stress began in the 30's but initially failed to generate differences between his stressed and control animals such that his control animals exhibited the biological indicators of what he aimed to attribute to stress, a result later attributed to his lack of animal husbandry competence.
[2] This may also be referred to as the adrenal cortical axis.

of MC2-R triggers glucocorticoid (GC) production and subsequent release into the circulatory system.

Glucocorticoids are a type of steroid hormone capable of a broad spectrum of peripheral effects that include alterations in behavior and immune function, in addition to physiological effects on blood glucose concentration, metabolism, and the cardiovascular system. The diverse influence of GCs (recall Selye's use of "non-specific") on an organism is mediated through the glucocorticoid receptor (GR). Steroid hormones are lipophilic, a characteristic that allows for movement across the lipid bi-layer of cells into the cytosol where the majority of GRs reside. When GCs bind to the GR, a conformational change occurs that sheds GR regulatory proteins and allows for translocation into the nucleus. Inside the nucleus, GRs have several potential targets. One primary target is the GC response element (GRE) located in the DNA of the cell inside the promotor regions of GR-dependent genes. A second potential target for GRs in the nucleus is nuclear factor-κB (NF-κB), a transcription factor involved in a diverse array of transcription events. This general process is widely applicable to GR activation, whereas the specific effects are dependent on the type of target tissue. Release of GCs into circulation also activates the negative feedback system within the hippocampus, hypothalamus, and pituitary to inhibit further GC release, and return the organism to homeostatic conditions.

Both Cannon and Selye made significant contributions to the modern understanding of stress, but neither individual's understanding or model fits completely with modern standards. Current research recognizes the biological response to stress can vary depending on the type of stressor the organism encounters, as well as the organism's perceived and actual ability to cope with the stressor (Cui et al., 2014; Goldstein, 2000). Research also supports a more inclusive model of stress that extends beyond biological or physiological threats, and includes perceived

threats to homeostasis (Goldstein & McEwen, 2002; McEwen & Stellar, 1993). The inclusion of perception into the determination of stressors incorporates observations that date back to the fifth century philosopher Epictetus, who recognized that individuals are not necessarily disturbed by negative events, but by the way the event is perceived (Epictetus & Gould, 1964). Moreover, an individual's perception of what constitutes a threat is directly influenced by the context in which the event occurred (Lazarus, Opton, Nomikos, & Rankin, 1965), the individual's personality and degree of social support (Kobasa & Puccetti, 1983), learned associations (Edwards, King, & Fray, 1999), and the ability to cope with the potential threat (Lazarus & Folkman, 1984). Such individual variability permits an equally diverse assessment of potential stressors. A particular set of stimuli could be viewed by one individual as threatening, while another could view the same stimuli and conclude that no threat is present.

The perceptual variability in threat assessment also generates a diverse biological response to stress (Smith & Vale, 2006). When peripheral homeostatic alterations are detected within the nucleus of the solitary tract (NTS), the signal is relayed to several other locations within the medulla as well as the PVN of the hypothalamus (Herman et al., 2003; Schwaber, Kapp, Higgins, & Rapp, 1982). Once the signal reaches the PVN from the NTS, the HPA axis can respond to the homeostatic information (Cunningham et al., 1990; Cunningham & Sawchenko, 1988). Simultaneously, the medulla transmits information to limbic forebrain structures, such as the hippocampus, amygdala, and prefrontal cortex (Cunningham et al., 1990; Ulrich-Lai & Herman, 2009). Neural processing of information relating to actual or potential stressors between the NTS in the medulla and forebrain structures allows for the integration of prior associations into the coming hypothalamic response. As the incoming somatic information is integrated with current sensory processing and learned associations, the HPA response can be

modulated to adapt to perceived needs (Herman et al., 2003; Ulrich-Lai & Herman, 2009). The HPA modulation within the PVN is regulated by structures such as the amygdala, hippocampus, bed nucleus of the stria terminalis, and subfornical organ, through innervations of the GABA-ergic neurons in the peri-PVN that provides tonic inhibition to the PVN (Cole & Sawchenko, 2002; J. B. Park, Skalska, Son, & Stern, 2007; Roland & Sawchenko, 1993). Likewise, the autonomic response to evaluated threats is influenced by the dorsomedial hypothalamus (DMH), a structure that can gate the PVN's autonomic influence via inhibitory synaptic projection to the PVN and brainstem (Bailey & Dimicco, 2001; Cullinan, Helmreich, & Watson, 1996; DiMicco, Samuels, Zaretskaia, & Zaretsky, 2002). The principle significance of these overlapping processing pathways is that an organism can cognitively assess a threat and modify the subsequent biological response.

The variability in the stress response can be recognized by discriminating between the neural structures that activate the response and the degree of cognitive evaluation required for activation (Herman & Cullinan, 1997). Structures such as the medial PFC (mPFC), amygdala, and ventral subiculum are associated with 'higher-order' processing of stimuli to modulate the stress response (Ulrich-Lai & Herman, 2009). Predominantly, these structures influence the HPA response, but there is evidence for influence of the SAM response as well. The dorsal mPFC has the ability to decrease CRF expression in response to psychogenic, restraint stress, while the ventral mPFC can enhance the HPA response and simultaneously regulate the autonomic response (Radley, Arias, & Sawchenko, 2006). Within the amygdala, several nuclei have the ability to increase the HPA response to stress, such as the basolateral, medial and central nuclei, through direct synaptic connections with the PVN, but only the central amygdala has excitatory synapses with the NTS that can increase the SAM response (Herman et al., 2003;

Schwaber et al., 1982; van der Kooy, Koda, McGinty, Gerfen, & Bloom, 1984). Finally, the ventral subiculum relays HPA inhibitory signals from the hippocampus to the PVN, though, this pathway appears to be specific to psychological stressors[3] vs systemic threats (Herman, Dolgas, & Carlson, 1998; Herman & Mueller, 2006; Mueller, Dolgas, & Herman, 2004).

Taken together, the stress response represents a ubiquitous component of everyday life and survival that is shrouded in ambiguity with regards to the particular set of stimuli that will activate a potentially variable response. However, within such a highly complex system, there are also consistencies. Activation of the SAM pathway creates downstream alterations in sympathetic and parasympathetic activity, and appears to be an adaptation highly tuned to facilitate success in physical altercations or conflict avoidance. Likewise, the HPA response confers similar benefits to the organism, presumably to increase the probability of success when confronted with a threat, but additionally, the response provides recovery form the initial stressor. However, in addition to the constellation of physiological changes in heart rate and metabolic function, the response to stress can also influence an organism's immune response, specifically the inflammatory response.

Inflammation and Stress

Inflammation is an adaptive innate immune response activated by the detection of a pathogen or tissue damage in order to facilitate a return to homeostasis (Medzhitov, 2008). With regard to pathogen recognition, the process usually begins when a neutrophil, monocyte, or macrophage is activated in response to a microorganism. Such activation results in a number of processes that promote an inflammatory response, and include the secretion of proinflammatory

[3] Authors used open field and elevated plus as psychological stressors and hypoxia as a systemic threat with CRF and corticosterone as dependent variables.

cytokines. In general, cytokines are small molecular weight signaling proteins used to help coordinate an immune response, and are typically characterized as either pro or anti-inflammatory. Proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are involved in numerous components of the inflammatory response that include activation of other immunocompetent cells to phagocytose pathogens (Netea et al., 2010), release of reactive oxygen and nitrogen species (Netea et al., 2010), stimulation of the production of additional proinflamamtory cytokines (Chung et al., 2009), and help facilitate the transition from innate to adaptive immunity (Chung et al., 2009). While the function of the inflammatory response remains evolutionally advantageous, there are several links between inflammation and diseases progression that include conditions such as cancer (Pesic & Greten, 2016), irritable bowel syndrome (Martin-Vinas & Quigley, 2016), arteriosclerosis (Franklin, Mangan, & Latz, 2016), and Alzheimer's disease (Van Eldik et al., 2016), to name only a few. Therefore, as numerous diseases are linked to the inflammatory response, any factor capable of influencing inflammation could indirectly alter disease genesis or progression. The stress response is a prime example of a physiological process that can influence the overall immune function of an organism, but more specifically, stress can directly modulate the extent and severity of the inflammatory response.

A highly salient connection between stress and immune function involves sympathetic nerve fibers that extend from the spinal cord to the primary and secondary lymphoid tissues, such as the thymus and spleen, respectively (Felten & Felten, 1994). Additionally, individual immune cells have receptors for both catecholamines and glucocorticoids, and activation of these receptors can produce a variety of outcomes within the cell, that include gene expression, suppression of intercellular signaling, and altered cell trafficking (Dhabhar, Malarkey, Neri, &

McEwen, 2012; Dhabhar, Miller, McEwen, & Spencer, 1996; Padgett & Glaser, 2003). When an interaction occurs between stress related factors and inflammation, the outcome is either proinflammatory or anti-inflammatory. However, each outcome can be achieved through a variety of pathways, for example, an interaction between stress and immune function that reduces the inflammatory response can be achieved by enhancing the anti-inflammatory response, or by inhibiting the proinflammatory response.

As with many phenomena in science, *the precise mechanisms* that mediate the connection between stress and inflammation remain unknown. Early research conducted by Selye identified a strong connection between stress-related hormones and anti-inflammatory outcomes, such that the anti-inflammatory effects of GCs became a well-accepted characteristic and function of GCs[4] (Selye, 1950). In 1956, Selye extended and refined his model to a broader interpretation that concluded that stress itself was immunosuppressive (Selye, 1956). However, conducting research is never so simple, as the scientific process appears to be fond of presenting a preponderance of evidence for a particular phenomenon, only to later present evidence in complete contradiction to previous results. Fortunately, the occurrence of apparent contradiction is also where research and the scientific method truly shine. The lack of consistent results within the field generated an abundance of literature on stress, so much so that there is an overwhelming wealth of publications, even when refined to the interaction of stress and immune function. Therefore, for the remainder of this document, the focus will shift directly to the interaction between psychological stress and pro-inflammatory outcomes.

[4] Selye did describe the possibility for GC enhancement of inflammation in his 1954 editorial (Selye, 1954), but the bulk of his work supported the anti-inflammatory model.

Herbert and Cohen (1993) were some of the first researchers to reconcile seemingly contradictory accounts of both pro and anti-inflammatory effects of stress after conducting a meta-analysis on available literature to identify specific factors that influence the pro or anti-inflammatory outcome of stress-related immune interactions. They identified that the *duration* of the stressor was a key determinant when predicting the influence on immune function. For example, a differential outcome was identified in circulating suppressor/cytotoxic T cells, such that acute laboratory stressors (characterized as less than thirty minutes in duration) increased the peripheral abundance within blood samples, in contrast to long-term stressors (characterized as lasting longer than one month) that produced a decrease in abundance (Herbert & Cohen, 1993). These results directly relate to the inflammatory response as cytotoxic T cells can release pronflammatory cytokines such as interferon-γ and TNF-α, in addition to the proinflammatory cytokine increase that results from the lysis of cells targeted by T cells.

Within the same year, research conducted by Barber et al. (1993) assessed the temporal influence of glucocorticoid administration on the proinflammatory response to lipopolysaccharide (LPS), an isolated portion of cell-wall from Gram-negative bacteria, and a potent proinflammatory stimulus. Five different groups of human participants were studied. One group did not receive any cortisol prior to LPS challenge, one received cortisol six hours before and concomitant with the LPS challenge, and the final three received cortisol either 6, 12, or 144 hours prior to LPS challenge. Following LPS challenge, the proinflammatory immune response and the hormonal stress response was monitored for six hours. Surprisingly, and accidently, the results demonstrated that administration of cortisol at either 12 or 144 hours prior to an LPS challenge significantly increased the proinflammatory response, as measured by interleukin-6 (IL-6) and TNF-α, relative to control participants that were only administered LPS.

Additionally, participants treated 12 or 144 hours prior to the immune challenge also had significantly elevated levels of C-reactive protein, a protein associated with inflammation, relative to participants only treated with LPS[5].

Additional evidence for stress-induced increases in peripheral inflammation soon followed, despite the initial hypotheses to the contrary. Cell culture studies demonstrated that glucocorticoid-treated splenic lymphocytes exhibited an increased mitogen-induced proliferation response, an effect blocked by the glucocorticoid agonist, RU486 (Wiegers et al., 1995). Following exposure to dexamethasone (DEX), a potent synthetic glucocorticoid agonist, and subsequent stimulation with a chemical probe, PMA (to induce cellular differentiation and gene expression), THP-1 cells released the more interleukin-1β (IL-1β) relative to cells treated only with DEX, those treated only with PMA, or controls, indicating that GC agonists can enhance the inflammatory response (Wang, Zhang, Dai, Lei, & Pike, 1997). Similarly, a study of T lymphocytes and endogenous stress hormones (cortisol, epinephrine, and norepinephrine) demonstrated that elevated concentrations of serum stress-related hormones triggered increased expression of genes related to both cellular proliferation and apoptosis in T cells, evidence supporting the hypothesis that stress can either prime immune cells to proliferate in response to a pathogen, or undergo apoptosis (Flint et al., 2005).

Data from animal models also supported the emerging hypothesis that GCs have complicated effects within organisms, beyond the well-documented anti-inflammatory properties. For example, a series of experiments conducted in Maier's lab (Johnson, O'Connor, Deak, Spencer, et al., 2002; Johnson, O'Connor, Deak, Stark, et al., 2002; Johnson, O'Connor,

[5] Although the data were not included in the publication, researchers replicated the increased inflammatory response to LPS at both 36 and 72 hours following cortisol administration.

Hansen, Watkins, & Maier, 2003) identified that an extreme acute stressor, such as repeated inescapable tail shocks[6] (IS), increased the proinflammatory response to LPS up to 24 hours after the stressor was administered as indicated by peripheral measures of IL-1β, IL-6, and TNF-α, and an exacerbated central IL-1β response to LPS was identified up to 4 days after the stressor (Johnson, O'Connor, Deak, Stark, et al., 2002). After replicating the proinflammatory effects of IS, the results were extended to include a time of day (0900 or 2200) as a variable for data collection to determine whether the proinflammatory effects were maximized when measured during the AM collection time, a time when cort production in response to LPS was significantly elevated in stressed animals relative to control animals (Johnson et al., 2003). Restated, the proinflammatory effects of stress where increased when measured in the morning, a period of time when cort levels were elevated due to IS. The increased proinflammatory effect during a period of increased GC expression supported the hypothesis of glucocorticoid resistance whereby the exacerbated inflammatory response resulted from a resistance to the normally immune-suppressive effects of GCs (O'Connor, Johnson, Hammack, et al., 2003). An alternative hypothesis was presented that attributed the stress-induced increase to a "priming" mechanism used to prepare an organism for a potential immunological challenge (Flint et al., 2005; Johnson, O'Connor, Deak, Spencer, et al., 2002; Johnson, O'Connor, Deak, Stark, et al., 2002; Johnson et al., 2003; Johnson, O'Connor, Watkins, & Maier, 2004).

In a review by Sorrells and Sapolsky (2007), one of the assertions put forth was that the literature favored a hypothesis of GC priming, based on evidence from *in vitro* and *in vivo* studies that showed the increased inflammatory response to be independent of the level or

[6] Study was conducted with rats and the stress protocol consisted of 100 5-s, 1.6mA shocks with an average ITI of 60s (Johnson, O'Connor, Deak, Stark, et al., 2002).

presence of GCs at the time of the immune challenge or throughout the duration of the immune challenge (Barber et al., 1993; Johnson, O'Connor, Deak, Spencer, et al., 2002; Johnson, O'Connor, Deak, Stark, et al., 2002; Johnson et al., 2003; Johnson et al., 2004; O'Connor, Johnson, Hansen, et al., 2003). Additionally, the authors suggested seven modifications to the traditional perception of GCs as strictly anti-inflammatory, and while all seven are important points, the most relevant to this discussion is the fourth suggestion stating that exposure to GCs or stress prior to an immune challenge can prime the immune system for an inflammatory response (Sorrells & Sapolsky, 2007). Indeed, this suggestion is in line with a hypothesis put forth a decade earlier (but refined in 2012) that provided an alternative explanation for GC-induced depletion of circulating leukocytes, noting that the apparent depletion could be metaphorically attributed to a reorganization of immune soldiers toward the 'battlefields' where the potential for surface tissue damage is maximized[7] (Dhabhar et al., 2012; Dhabhar et al., 1996; McEwen et al., 1997). The migration of immune cells toward the periphery, areas with potential tissue damage, are consistent with the concept of GC priming of the immune response. Taken together, the priming effect would begin with GC-induced migration of immune competent cells toward potential wound sites, and would later be observed as an increased inflammatory response to immune challenge at a particular site given the increased presence of immune responders.

A similar conclusion was reached by Frank, Watkins, and Maier (2013), and their hypothesis that GCs have a differential function as an organism begins to experience and

[7] This metaphor is best articulated by the original author, Dhabhar (2012):

> "… we propose a model explaining how stress hormones represent a 'call to arms' and induce the body's 'soldiers' (immune cells) to leave their 'barracks' (marginated pool, spleen, bone marrow), travel through the 'boulevards' (blood) and take up positions at ongoing or potential 'battlefields' (e.g. skin) during or following stress."

ultimately reconciles a fight or flight response. Accordingly, the classic anti-inflammatory processes exist to help the organism during the experience of the stressor, but as the event or stressor is reconciled, the GCs facilitate a secondary function in the form of a warning signal that prepares the organism to respond to any potential unreconciled danger. Therefore, GC sensitization can be understood as a means of potentiating the pro-inflammatory response induced by a post-stress immune challenge, maximizing the potential for the immune system to respond to any acquired challenge (Frank et al., 2013). If this hypothesis is extended to the CNS, GC-induced immune sensitization would increase the probability that an immune challenge would result in an exacerbated neural-immune response.

Immune Function

Expression of Sickness Behaviors

Sickness behaviors represent an immune-induced motivational[8] shift toward adaptive behaviors that reduce the probability of spreading the infectious agent while simultaneously emphasizing behaviors that promote elimination of the pathogen (Dantzer, 2001). Therefore, sickness behaviors provide an example of the immune system's ability to influence the central nervous system. This highly conserved behavioral pattern is characterized by a constellation of nonspecific symptoms that include fatigue, anorexia, irritability, shivering, fever, and malaise, and are reliably expressed following immune activation or proinflammatory cytokine[9] administration (Dantzer, 2001; Dantzer & Kelley, 1989; Kent, Bluthe, Kelley, & Dantzer, 1992).

[8] The motivational aspect of sickness behaviors was elegantly demonstrated in rats that were trained to lever press in order to rest during a period of forced running. If sickness behaviors were purely depressive of behavior then animals would decrease lever pressing following administration of endotoxin, however, lever pressing increased to gain additional periods of rest (Miller, 1964).

[9] Cytokines are small molecule immune messengers that help coordinate an immune response, the designation of "pro-inflammatory" in this instance indicates that these messengers coordinate and help initiate an inflammatory response.

Proinflammatory cytokines are released in response to numerous signals, such as macrophage[10] pathogen recognition, and an immune challenge reliably utilized to initiate a pro-inflammatory response is peripheral administration of lipopolysaccharide (LPS).

LPS is a bacterial mimetic derived from Gram-negative bacteria that consists of three distinct regions, a core oligosaccharide, an O side chain, and the lipophilic region, lipid A (Raetz & Whitfield, 2002). Within an organism, LPS-binding protein recognizes and attaches to the lipid A region before trafficking LPS to CD14[11], a common marker for monocytes and macrophages (Galanos et al., 1985; Wright, Tobias, Ulevitch, & Ramos, 1989). Once bound to CD14, LPS is recognized by the MD-2-Toll-like receptor-4 (TLR4) receptor complex, and initiates two intercellular signaling cascades, one pathway is MyD88-dependent and the other is TRIF-dependent (Lu, Yeh, & Ohashi, 2008; Wright et al., 1989; B. Yu & Wright, 1996).

Activation of the MyD88-dependent pathway stimulates a rapid production of certain proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, through both NF-κB dependent (Lu et al., 2008) and independent (Yamamoto et al., 2004) signaling. Activation of the TRIF-dependent pathway is slower than the MyD88-dependent pathway and increases production of Type I interferons, again through both NF-κB dependent (Chuang & Ulevitch, 2004), and independent (Oganesyan et al., 2006) signaling. Although each pathway accounts for a small portion of the total inflammatory response, they function synergistically in order to achieve the full inflammatory response (Hoebe et al., 2003). Restated, peripheral macrophage recognition of LPS initiates an immune response that results in production of proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6.

[10] Tissue dwelling phagocyte derived from blood monocytes and contribute to the innate immune response.
[11] However, LPS can bind directly to CD14 without LPS-binding protein.

Ordinarily, cytokines are restricted from passing through the blood brain barrier (BBB), however, multiple mechanisms exist to facilitate peripherally-stimulated central inflammation and/or entry of proinflammatory mediators into the CNS. Sub-diaphragmatic vagal afferents contain proinflammatory cytokine receptors capable of transducing peripheral immune signaling and inducing central inflammation and the corresponding sickness behaviors (Goehler et al., 1997; Laye et al., 1995). Cytokine signaling through the CNS via circumventricular organs (CVOs) is possible through interactions with neural projections connecting CVOs with central autonomic structures (Marvel, Chen, Badr, Gaykema, & Goehler, 2004; Wei et al., 2013), although there is little evidence that cytokines diffuse across the CVOs (Banks, 2005; Peruzzo et al., 2000). There is also a complex interaction between LPS, cytokines, and the BBB, such that LPS can compromise the tight junctions of the BBB which allows for larger molecules to pass through (Jaeger et al., 2009), while simultaneously stimulating the release of additional cytokines from epithelial cells of the BBB (Verma, Nakaoke, Dohgu, & Banks, 2006). However, regardless of the entrance modality, cytokine entry into the CNS initiates a central inflammatory response through interaction with neurons, microglia, and astrocytes, all of which are able to express cytokine receptors, while microglia and astrocytes are also able to produce and release cytokines (Rothwell, Luheshi, & Toulmond, 1996).

Microglia

Microglia are critical immunocompetent cells in the brain parenchyma that are often characterized as the resident macrophages of the CNS (Gehrmann, Matsumoto, & Kreutzberg, 1995). Under normal physiological conditions, microglia can be described[12] as "resting," a state

[12] The literature has yet to come to consensus on how microglia should be discussed and classified, and the terminology presented here represents one of the potential ways to discuss microglia. I personally chose the "activated" and "resting" terminology as I feel this communicated the level of specificity necessary for the

characterized by rapid extension and retraction of cellular processes (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Such dynamic morphological transformation allows the cell to sample the local environment, effectively screening for any changes that require an immune response (Nimmerjahn et al., 2005; van Rossum & Hanisch, 2004). Recognition of an environmental change, such as an increase in pro-inflammatory cytokine concentration (Hanisch, 2002), can trigger a change in state from "resting" to "activated" (Nimmerjahn et al., 2005; Raivich et al., 1999).

Activated microglia can be further classified into two functional phenotypes, alternative activation, and classical activation (Gordon, 2003; Mills, Kincaid, Alt, Heilman, & Hill, 2000; Varin & Gordon, 2009). The alternative activation phenotype is characterized by the ability protect and repair tissue following a bout of inflammation (Varin & Gordon, 2009), and is often identified by the increased expression of arginase I (Munder et al., 1999). The classical activation phenotype is functionally associated with the elimination of pathogens, the expression of pro-inflammatory cytokines (Skeen, Miller, Shinnick, & Ziegler, 1996), and can be identified by the expression of Iba1 and/or iNOS (Cherry, Olschowka, & O'Banion, 2014; Frank et al., 2013). Restated, microglia survey the brain's parenchyma from a resting state and transition into an activated state to respond to various environmental stimuli.

Activation stimuli for both the central and peripheral immunity can be broadly classified with three terms: alarmin, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs) (Bianchi, 2007). Although the literature has yet to come to consensus as to the operational definitions for these terms, this document will adhere to the

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document; however, this terminology does simplify the highly complex task of classifying the different activation states of microglia while accounting for all the subtle distinctions of each state.

recommendations that the term *alarmin* refer broadly to both DAMPs and PAMPs (Chan et al., 2012; Clark & Vissel, 2015). PAMPs are exogenous molecular motifs that elicit an innate immune response, such as LPS (Bianchi, 2007). Similarly, DAMPs are endogenous early inflammatory signals that are primarily found in the nucleus or cytosol of a cell, but can be released as a result of tissue damage, necrosis, or environmental challenge (Lotze et al., 2007), but are distinguished from cytokines based on receptor affinity (Carta, Lavieri, & Rubartelli, 2013).

While the function of DAMPs closely resembles that of some pro-inflammatory cytokines, DAMPs and their respective receptors, appear earlier in evolution than cytokine receptors, leading to the hypothesis that DAMPs are among the first evolutionary mediators of inflammation. The later evolution of cytokines and cytokine receptors provided receptor activation specificity that increased the probability of positive inflammatory outcomes (Carta et al., 2013). As a result, pattern recognition receptors for DAMPs are less sensitive to stimulus specificity and can therefore be activated by a broad range of alarmins. In contrast, cytokine receptors are more discriminative, and activate in response to more specific ligands. The increased selectivity of cytokine receptors may have contributed to the evolution of cytokine diversity, in addition to conserving inflammatory responses that optimized the probability of survival. Returning to the previous example of peripheral administration of LPS, LPS serves as a PAMP that elicits a peripheral immune response. Part of the peripheral response to LPS is the production of proinflammatory cytokines that trigger a central neuroinflammatory response through a variety of pathways. Moreover, activation of central inflammation initiates a cascade that releases two DAMPs of interest, high mobility group box 1 (HMGB1) (Carta et al., 2013) and amyloid-beta (Aβ) (Kahn et al., 2012; Lee et al., 2008).

High Mobility Group Box 1

The proteins in the high mobility group were first reported in 1973 and the "high-mobility" descriptor in the name was selected from the protein's ability to migrate efficiently through a gel during electrophoresis (Goodwin & Johns, 1973; Goodwin, Sanders, & Johns, 1973). Almost 30 years later, the proteins in the 'high mobility group' were further categorized into three distinct families, HMGB was one of the families created (Bustin, 2001). The addition of the "B" for "box" into the name represents the motif that defines inclusion in the family, further, proteins within that Box family were given sequential numeric tags for identification, thus HMGB1 (Bustin, 2001). The genetic coding region for HMGB1 is one of the oldest evolutionary mammalian genes, conserved for over 525 million years (Sharman, Hay-Schmidt, & Holland, 1997), and is composed of two separate domains, box-A and box-B (Yang & Tracey, 2005). Moreover, the similarity between expression of HMGB1 mRNA is 99% homologous between human and rodent (Gariboldi et al., 1995; Wen, Huang, Johnson, & Reeck, 1989). The functional role of HMGB1 is similarly conserved.

HMGB1 is able to shuttle between the cytoplasm and the nucleus of a cell, with the majority of HMGB1 accumulating in the nucleus where it can bind to the minor groove of chromatin to facilitate gene transcription and regulate glucocorticoid receptor expression (Isackson, Bidney, Reeck, Neihart, & Bustin, 1980; Sapojnikova et al., 2005). This functional role is critical to the survival of an organism as HMGB1 -/- mice die after birth from an inability to regulate glucocorticoid receptor expression, and therefore have difficulty accessing the energy reserves in the liver (Calogero et al., 1999). Moreover, this example helps illustrate the connection between HMGB1 and the stress response, a connection that helps to bridge the understanding of the how stress can influence immunity as HMGB1 has been characterized as

the “master regulator” of the innate immune response (Castiglioni, Canti, Rovere-Querini, & Manfredi, 2011).

In order to exert influence on the innate immune response, HMGB1 must first exit the cell and enter the extracellular space where HMGB1 can interact with various receptors such as TLR2, TLR4, and RAGE (Matzinger, 2002). Trafficking of HMGB1 from the cell occurs through two primary methods of release that can be classified as either passive or active (Frank, Weber, Watkins, & Maier, 2015). Passive release is achieved when HMGB1 spills from damaged or necrotic cells (Hamana & Kawada, 1989), but importantly, during apoptosis HMGB1 remains bound to chromatin and is not released into the extracellular space after the cell self-terminates (Scaffidi, Misteli, & Bianchi, 2002). This indicates that the extracellular effects of HMGB1 can be modulated though the mechanism of cellular demise. When cellular death is an “intentional” process, such as apoptosis, HMGB1 is not released into the surrounding environment, but necrotic, “unintentional” death, floods the surrounding environment with HMGB1, allowing the protein to function as a pro-inflammatory mediator (Scaffidi et al., 2002). Moreover, during active release, immunocompetent cells secrete HMGB1 from secretory vesicles in response to distress or immune challenges to promote an inflammatory response (Zimmermann et al., 2004). However, the precise influence HMGB1 exerts depends on the redox (oxidation) state of the molecule.

HMGB1 exists in three distinct redox states. When fully oxidized, HMGB1 is effectively inert and retains no apparent endogenous function or biological value (Weber, Frank, Tracey, Watkins, & Maier, 2015). In contrast, the fully reduced (frHMGB1) form performs the functions needed during basal conditions (regulations of GR expression), and is primarily located in the cytosol and nucleus (Yang, Antoine, Andersson, & Tracey, 2013). Inflammation triggers the

release of frHMGB1 that functions as a chemoattractant in the extracellular space to increase the immune cell presence in the area (Schiraldi et al., 2012). As activated microglia begin to migrate to frHMGB1 signals, the mitochondria within begin to produce reactive oxygen species (ROS[13]) in response to inflammatory challenges, and glucocorticoids can further increase ROS abundance (J. Park et al., 2015; You et al., 2009). Interactions between ROSs and the frHMGB1 can result in a change in HMGB1 oxidation state from a fully reduced form to a disulfide (dHMGB1) form (Kazama et al., 2008).

Disulfide HMGB1 (dHMGB1) binds with MD-2 and initiates a TLR4 signaling cascade that produces pro-inflammatory cytokines (Yang et al., 2015). Moreover, dHMGB1 can bind with a diverse array of inflammatory signaling molecules, including IL-1β and LPS, to increase the downstream immune response that follows when the complex binds to the target receptor (Bianchi, 2009). Finally, HMGB1-dependent activation of RAGE initiates signaling that phosphorylates and degrades IκB, a process that facilitates NF-κB-mediated gene activation (Huttunen & Rauvala, 2004; Taguchi et al., 2000). This process masterfully facilitates an inflammatory response whereby frHMGB1 attracts immunocompetent cells to an area and the response of those cells to the initial stimulus produces ROSs that shift frHMGB1 to the proinflammatory dHMGB1.

HMGB1-Primed Inflammation

The lack of specificity that permits secretion of HMGB1 in response to a diverse assortment of stimuli, is the same property that facilitates the exaggerated inflammatory response. For example, cellular distress in the form of ROS production is sufficient to trigger the

[13] Not to be confused with ROUSs (This one's for you, Gary).

release of HMGB1 within the CNS (Y. Yu, Tang, & Kang, 2015), and as previously stated, GCs are capable of generating ROSs within a cell (You et al., 2009). The secretion of HMGB1 then attracts and activates microglia (Gao et al., 2011), a process that increases localized ROS production, and shifts the HMGB1 oxidation state to favor dHMGB1 from frHMGB1, resulting in activation of TLR4 and TLR2 (Weber, Frank, Sobesky, Watkins, & Maier, 2013). In microglia, the initial activation of TLR4 and TLR2 facilitates the synthesis and cytosol accumulation of pro-IL-1β and NLRP3 (Babelova et al., 2009). Once the accumulation of NLRP3 in the cytosol surpasses a threshold, the NLRP3 inflammasome assembles (Tschopp & Schroder, 2010).

After the inflammasome is assembled, the cell is primed to respond to any subsequent activation through an interaction between ASC and the NLRP3 inflammasome that sequesters and cleaves pro-caspase-1 into caspase-1, which in turn cleaves pro-IL-1β into IL-1β (Cassel, Joly, & Sutterwala, 2009). This pathway provides a probable explanation for how stress can prime microglia and exaggerate an immune response. Stress triggers the release of frHMGB1 that is oxidized into dHMGB1, an oxidation state that can activate TLR4. Activation of TLR4 facilitates the formation of the NLRP3 inflammasome, priming the cell to produce proinflammatory cytokines as the assembly of the inflammasome is already complete. Once primed, interactions with any immunological threat, such as LPS, will result in an exaggerated immune response (Frank et al., 2015).

Additionally, extracellular HMGB1 can bind to its high-affinity receptor, RAGE, to trigger a cascade that degrades IκB, an NF-κB inhibitor (Huttunen & Rauvala, 2004; Taguchi et al., 2000). Once free of IκB, NFκB can translocate to the nucleus and initiate transcription of NF-κB-dependent transcripts, such as pro-inflammatory cytokines and RAGE (Chuang &

Ulevitch, 2004; Lu et al., 2008; Yan et al., 1994). The promoter region for RAGE contains an NF-κB binding site, therefore, RAGE-dependent activation of NF-κB signaling initiates a positive feedback mechanism that increases the cell's RAGE receptor density and heightens the sensitivity of the cell to localized HMGB1 (Yan et al., 1994). Therefore, HMGB1 can prime the inflammatory response of immunocompetent cells by facilitating the production and assembly of the NLRP3 inflammasome, and increasing cellular sensitivity to proinflammatory HMGB1 signaling by upregulating RAGE expression.

Such priming has reliably been shown using the paradigm of repeated social defeat (RSD) (Wohleb et al., 2011). Briefly, this paradigm involves introducing a retired male CD1 breeder into a home cage of three smaller, typically C57BL/6, mice for two hours each day, for six consecutive days (Avitsur, Stark, & Sheridan, 2001). Following the introduction of the CD1 intruder, animals are then monitored for approximately ten minutes to ensure the intruder usurps dominance within the cage, as indicated by submissive behaviors from the C57 mice such as upright posture, fleeing and crouching (Avitsur et al., 2001; Stark et al., 2001). If the CD1 fails to elicit submissive behaviors from the C57s, the intruder is replaced with a different CD1. In order to prevent habituation to the CD1, on each consecutive day of RSD, a novel CD1 intruder is introduced to the C57 home cage. After completion of the two-hour session, the intruder is removed from the cage, and all animals undergo a health inspection. As a control, a comparable group of animals remain undisturbed in their home cage.

Using a similar social stress paradigm, microglia extracted from socially defeated animals were hyper-responsive to *ex vivo* application of LPS (Wohleb et al., 2011), and had a heightened susceptibility to endotoxic shock (Quan et al., 2001). Social defeat also increases the expression of IL-1β, IL-6, and TNF-α in the prefrontal cortex in response to an LPS challenge (Audet,

Jacobson-Pick, Wann, & Anisman, 2011). The exaggerated immune response has been attributed to primed CD11b+ cells (microglia and macrophages), as these cells are key regulators of the neuroinflammatory response, and the socially defeated animals also expressed an elevated level of IL-1β mRNA (Wohleb et al., 2011). In addition, animals in the social defeat paradigm had a significant increase in the number of microglia within the hippocampus, amygdala, and prefrontal cortex (Wohleb et al., 2012).

Related paradigms have shown similar stress-related elevations in the neuroinflammatory response to LPS following inescapable tailshock that include an elevated fever response and exaggerated neuroinflammatory response (Johnson, O'Connor, Deak, Stark, et al., 2002; Johnson et al., 2003). As a follow-up to these studies, *ex vivo* studies of microglia isolated from rat hippocampi 24h after completion of the stress protocol identified that stress increases the number of activated microglia present in addition to an elevated pro-inflammatory response (Frank, Baratta, Sprunger, Watkins, & Maier, 2007; Frank, Miguel, Watkins, & Maier, 2010). Importantly, the *ex vivo* studies demonstrate that the priming effect occurred prior to exposure to LPS and was not dependent on any physiological difference at the time of injection as all isolated cells were maintained under uniform culture conditions (Frank et al., 2013). The exaggerated inflammatory response to LPS could also be prevented by administering RU486 prior to the stress protocol to block stress-induced GC-signaling (Frank et al., 2013).

The link between stress and HMGB1 was identified with the initial recognition that repeated tail-shocks triggered the active release of HMGB1 within the hippocampus (Weber et al., 2015). The release of HMGB1 was deemed active, as opposed to the passive release from damaged or necrotic cells, after the hippocampi of stressed and control animals were compared but identified no difference in cell viability. If the release was passive, one would expect to see a

decrease in cell viability in the stressed condition. Further, to show that HMGB1 was responsible for the microglial priming effects seen in various stress studies, a group of animals completed the stress protocol after being treated with the HMGB1 antagonist, box-A. Microglia from the box-A treated animals failed to produce the exaggerated inflammatory response to LPS which lead the authors to conclude that HMGB1 accounted for the stress-induced priming of microglia, an effect that was further refined to the dHMGB1 redox state (Weber et al., 2015). Moreover, stress prompts the active release of dHMGB1 within the hippocampus that attracts and primes immunocompetent cells and exaggerates the immune response. This response clearly affords adaptive advantages, however, given the relationship between inflammation and Alzheimer's disease (AD), such an exaggerated immune response may result in an increased risk for the production of markers of AD pathology, such as amyloid-β peptides (Aβ).

AD, Inflammation, and Amyloid- β

Alzheimer's disease is a fatal neurodegenerative illness that gradually degrades the cognitive ability of afflicted individuals, depriving them of memory, critical thinking, and the ability to care for themselves (Arshavsky, 2010; Fischer et al., 2008; Hoyer, 1994). There is currently no cure for AD, and despite an abundance of research, the pathophysiology of the disease remains incomplete. However, the link between AD and Aβ remains the leading hypothesis for the disease pathogenesis (Van Eldik et al., 2016), and Aβ plaques are one of the two hallmarks for AD pathology[14]. The production of Aβ begins when β-amyloid precursor protein (APP) is cleaved by β-secretase[15] (BACE1), and is subsequently cleaved by γ-secretase

[14] Although there is a select focus on Aβ within this document, there is an abundance of evidence to show that Aβ is but one of several factors that contribute to AD and the associated complications.

[15] In this instance, APP cleavage by BACE1 appears to be the critical step for Aβ production as substitution of BACE1 for α-secretase results in the production of p3, not Aβ, following cleavage by γ-secretase (Vardy, Catto, & Hooper, 2005).

(Salomone, Caraci, Leggio, Fedotova, & Drago, 2012; Shi et al., 2003). The result from this two-step protein cleavage is an Aβ monomer of either 40 or 42 residues in length, the Aβ42 variant being more neurotoxic and likely to aggregate (Heppner, Ransohoff, & Becher, 2015). Aggregation occurs when the production of Aβ monomers exceeds the clearance rate, thereby allowing the monomers to accumulate and spontaneously combine (Hardy, 2009). Monomer aggregation into plaques is a reversible[16] process that begins with monomers forming oligomers, and is followed with the formation of protofibrils, Aβ fibrils, and finally the aggregation of Aβ fibrils into plaques (Hardy, 2009; Heppner et al., 2015). However, this process is not uniform in progression as the Aβ coding domain of APP partially determines which aggregates will form from different monomers (Haass, Kaether, Thinakaran, & Sisodia, 2012; Mucke & Selkoe, 2012).

Of particular interest is the ability of immune activation to increase the production of Aβ, and more specifically, the ability of peripherally-induced neuroinflammation to elicit an increased production within the hippocampus (Kahn et al., 2012; Lee et al., 2008). One of the first studies to examine the influence of inflammation on Aβ found that a single injection of LPS was sufficient to disrupt cognition, induce Aβ production, decrease α-secretase abundance while increasing β-secretase, and that these effects could be mitigated with an anti-inflammatory (Lee et al., 2008). This research was later extended to a regimen of seven injections of LPS, delivered once per day, that resulted in an increased expression of hippocampal Aβ and cognitive deficits (Kahn et al., 2012). When this model was paired with the γ-secretase inhibitor, imatinib methanesulfonate, LPS administration failed to increase hippocampal Aβ and no cognitive

[16] Of important note, while the process of aggregation from monomers to plaques is technically reversible, the process biochemically favors aggregation.

deficits were identified among treated animals (Weintraub et al., 2013). This research is congruent with the role of γ-secretase in Aβ production, and supports the hypothesis that inflammation-induced elevations in hippocampal Aβ, independent of plaque formation, are capable of disrupting learning and memory.

Despite an incomplete understanding of all the processes that contribute to inflammation-induced Aβ elevations, a connection between inflammation to BACE1[17] provides one possible pathway. Transgenic models of AD demonstrate that cognitive deficits associated with AD pathology occur concomitant with increased neuroinflammation, and elevated expression of both RAGE and BACE1 (Barroso et al., 2013). Activation of RAGE also initiates a signaling pathway that increases the expression of BACE1 following NF-κB-induced transcription (Guglielmotto et al., 2012). This is consistent with the increase in BACE1 expression a single injection of LPS (Lee et al., 2008). Inflammation also increases that activity of the kinase PKR, an enzyme involved in generating the inflammatory response, synapse degradation, cognitive deficits, and apoptosis (De Felice & Ferreira, 2014). Inflammation-induced Aβ production is also dependent, in some way, on PKR, as $PKR^{-/-}$ mice have a reduced neuroinflammatory response and produce less Aβ relative to wildtype controls following LPS treatment (Carret-Rebillat et al., 2015).

Unpublished Research Related to Repeated Social Defeat, HMGB1 and Amyloid-β

Three relevant studies were conducted in preparation for the current research to elucidate the relationship between RSD-induced stress, LPS-induced inflammation, and Aβ production. The goal of the first experiment was to replicate an effect within the literature that cited stress-

[17] Recall that BACE1 is the enzyme that cleaves APP so that subsequent cleavage by γ-secretase produces Aβ monomers.

induced exacerbation of the inflammatory response within the hippocampus. This effect was indicated by elevated expression of IL-1β mRNA and HMGB1 protein expression, however, we expanded the investigation to include a measure of HMGB1 mRNA. The second study examined the effect of RSD on sickness behaviors by examining the effect of RSD on LPS-induced burrowing deficits. Finally, the third study directly examined the relationship between RSD, LPS-induced inflammation, Aβ production, and cognition. The combined results of these studies supported the experimental progression toward the current research.

The initial study in our investigation of the relationship between RSD and inflammation hypothesized that animals subjected to six consecutive days of RSD would demonstrate an exacerbated central immune response to LPS (at doses of 125 μg/kg and 250 μg/kg) as indicated by mRNA expression of IL-1β and HMGB1. Results from the mRNA analysis demonstrated a dose dependent significant increase in IL-1β production for RSD animals with a similar, but reduced, effect within home cage control (HCC) animals (Figure 1). The principal finding from the IL-1β mRNA data was that, at both doses of LPS, animals in the RSD condition demonstrated an exacerbated immune response relative to HCC animals (Figure 1). Analysis of the HMGB1 mRNA data revealed that saline-treated HCC animals expressed the least HMGB1 mRNA, and this expression was significantly less than all other groups (Figure 2). HMGB1 protein expression was quantified using western blots, and identified that RSD animals expressed significantly more HMGB1 protein at each level of treatment, in addition to a dose dependent increase in HMGB1 expression (Figure 3). Importantly, this research demonstrated the RSD could increase expression of HMGB1 without increasing IL-1β mRNA (seen in the RSD/saline group), therefore, the significantly elevated expression of IL-1β in RSD animals treated with

LPS could not be attributed to any preexisting differences, indicating that a RSD-induced effect was responsible for the exaggerated immune response.

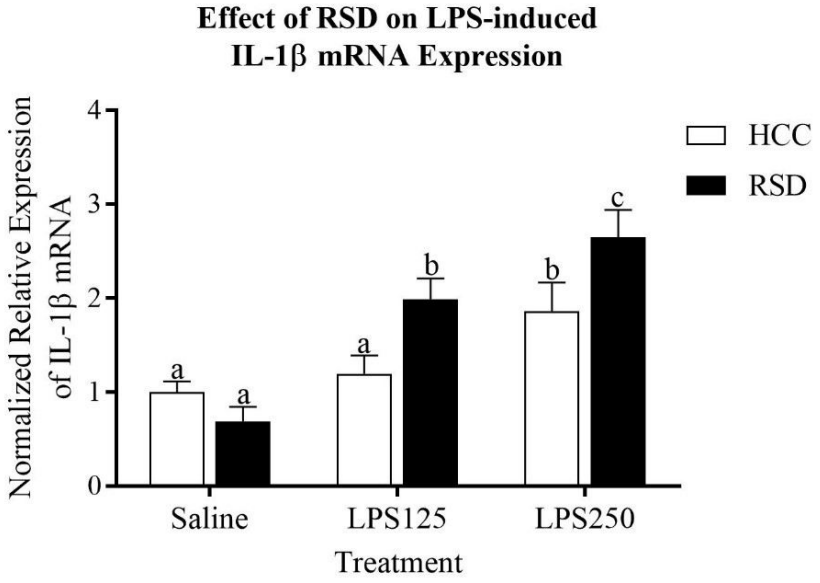

Figure 1. Effect of RSD on LPS-induced IL-1β mRNA Expression. Results show that the combination of RSD and LPS significantly increase the inflammatory response to LPS. Different characters represent significant ($p < .05$) difference, error bars equal standard error of the mean (SEM) ($n = 8$).

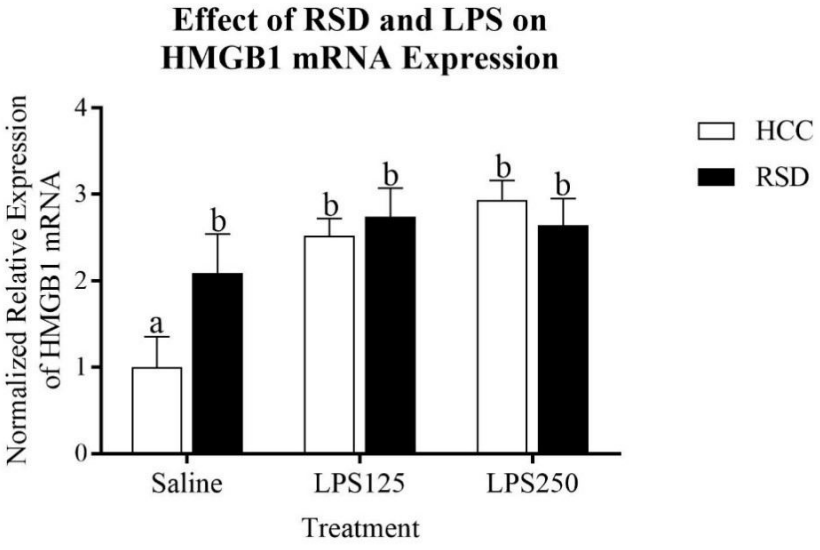

Figure 2. Effect of RSD and LPS on HMGB1 mRNA Expression. RSD and LPS increase HMGB1 mRNA expression in the dorsal hippocampus. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 8$).

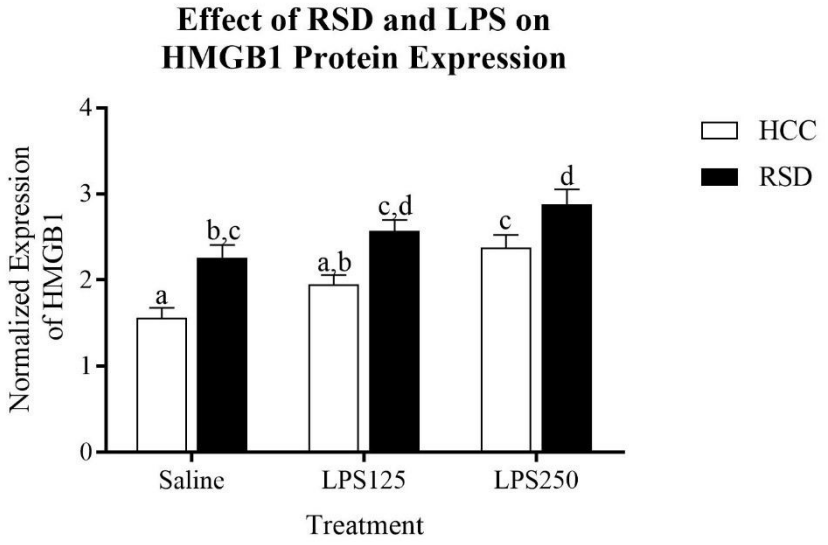

Figure 3. Effect of RSD and LPS on HMGB1 Protein Expression. RSD and LPS increase HMGB1 protein expression. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 8$).

The second experiment examined the influence of RSD on LPS-induced "*sickness behaviors*," behaviors linked to immune activation that include a diverse constellation of nonspecific symptoms such as fatigue, diminished foraging, fever, and general malaise (Dantzer, 2001; Kent et al., 1992). These behaviors are common across multiple species and can be activated by either an immune challenge or cytokine administration, a connection that indicates sickness behaviors are linked to elevations in proinflammatory cytokines (Dantzer & Kelley, 1989). Therefore, activation of the immune system increases the expression of proinflammatory cytokines. As expression of proinflammatory cytokines increases, the motivational state of an organism is adaptively reorganized to favor a behavioral profile consistent with pathogen elimination (Dantzer, 2001; McCusker & Kelley, 2013). In mice, such behavioral alterations can reliably be assessed through changes in burrowing behavior, an innate behavior highly sensitive to elevations in proinflammatory cytokines (Deacon, 2006). Therefore, the purpose of the

second experiment was to assess the influence of RSD on LPS-induced alterations in burrowing behavior.

Animals were simultaneously trained in the burrowing procedure while undergoing RSD. The morning after the final RSD session, animals received a dose of either saline or LPS (5 μg/kg, i.p.). Two hours after the injection, animals were moved into a private burrowing chamber with free access to their burrowing tube. Eight hours after the injection, LPS-treated RSD animals maintained a burrowing deficit relative to all other groups (Figure 4). As burrowing behavior is susceptible to elevations in pro-inflammatory cytokines, and RSD exaggerates the inflammatory response to LPS, results from this study support the hypothesis that the RSD can potentiate LPS-induced sickness behaviors.

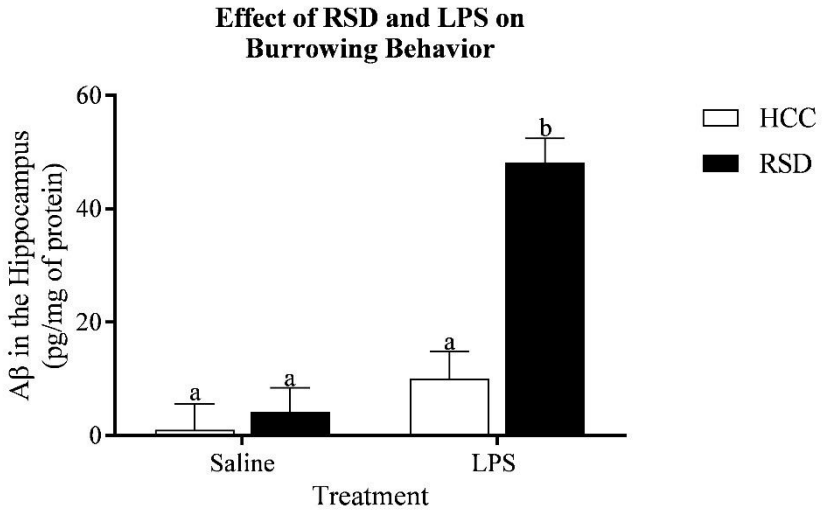

Figure 4. Effect of RSD and LPS on Burrowing Behavior. RSD potentiates LPS-induced burrowing deficits. Figure shows that LPS-treated RSD animals continue to display a burrowing deficit eight hours after injection. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 7-9$).

The third study examined the effects of repeated bouts of inflammation on cognition and accumulation of Aβ within the hippocampus. Our lab has repeatedly shown that consecutive daily bouts of LPS-induced peripheral inflammation result in a significant, LPS-induced,

elevation in Aβ, the peptide building blocks for senile plaques associated with AD (Kahn et al., 2012; Weintraub et al., 2013). This model of AD-like pathology uses seven injections of LPS (250 μg/kg) administered once per day for seven consecutive days to elevate hippocampal Aβ. The morning after the seventh injection, animals are trained in contextual fear conditioning. Twenty-four hours after training, animals are returned to the conditioning chamber for testing (Kahn et al., 2012; Weintraub et al., 2013). This paradigm is ideal for testing the influence of hippocampal Aβ on cognitive performance as the task primarily depends on the integrity of the hippocampus to form an association between the context and aversive stimulus (Barrientos et al., 2002; Celerier, Pierard, & Beracochea, 2004). Importantly, the dorsal portion of the hippocampus is required to form the contextual association as ablation of the dorsal hippocampus, despite an intact ventral hippocampus, prevents the formation of an association between the context and the aversive stimulus (Celerier et al., 2004).

Results from CFC testing showed that both groups of LPS-treated animals displayed a significant reduction in freezing behavior during testing (Figure 5). However, analysis of hippocampal Aβ revealed that while both groups of LPS-treated animals had significantly more Aβ when compared to saline-treated animals, LPS-treated RSD animals also had significantly more Aβ than LPS-treated HCC animals (Figure 6). Together, these data suggested that although RSD can exaggerate LPS-induced Aβ production, the elevation did not induce further behavioral deficits in freezing behavior. According to previous research, accumulation of Aβ in the hippocampus accounts for approximately one third of deficits in freezing behavior during CFC testing (Weintraub et al., 2014). Therefore, one possible alternative explanation for the behavioral data would be that LPS-treated RSD animals had an insufficient accumulation of Aβ relative to LPS-treated HCC animals to demonstrate an exaggerated deficit in freezing behavior.

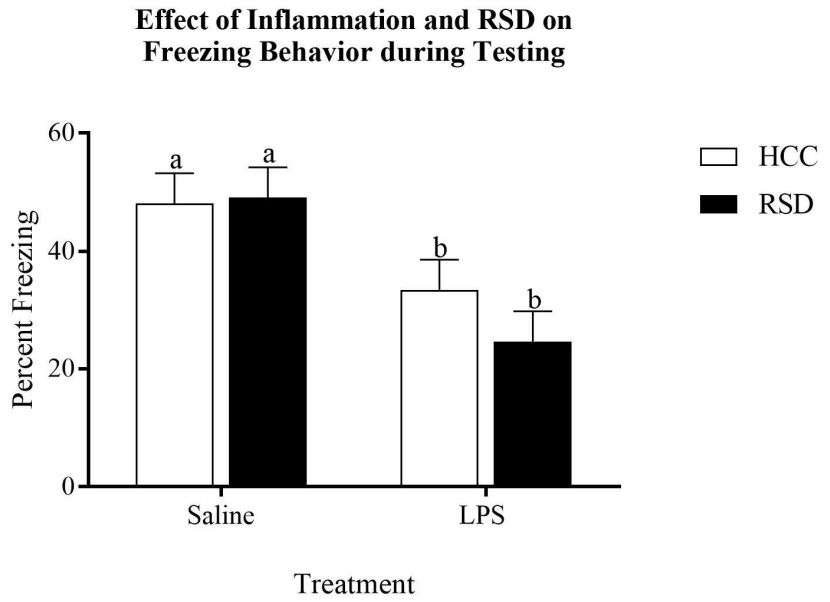

Figure 5. Effect of Inflammation and RSD on Freezing Behavior during Testing. RSD potentiates LPS-induced burrowing deficits. Figure shows that LPS-treated RSD animals continue to display a burrowing deficit eight hours after injection. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 7-9$).

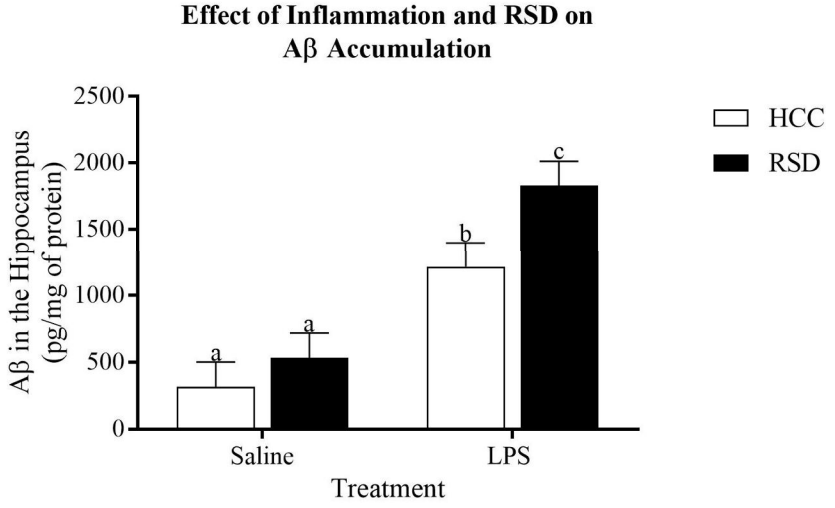

Figure 6. Effect of Inflammation and RSD on Aβ Accumulation. RSD and LPS interact to exacerbate LPS induced Aβ accumulation in the hippocampus. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 7-9$).

Alternatively, deficits in freezing behavior could have reached a floor, and that the accumulation of Aβ in LPS-treated animals, regardless of condition, has maximumly disrupted freezing

behavior. However, there is likely some combination of these, and other unknown effects, contributing to the lack of behavioral difference.

Summary

The survival of most organisms is directly related to their ability to observe and respond to threats within their environment. When confronted with a circumstance or stimuli that constitutes a perceived threat to the organism, a stress response is initiated that involves both the SAM and HPA axes. Following repeated activation of the stress response, the immune system of an organism can become *primed* such that an interaction with an immune challenge results in an exacerbated response. This sequence of events is biologically adaptive, however, there may be an unintended cost associated. When the immune system responds to challenges with a systemic inflammatory response, the neural-inflammation that results generates Aβ, the building blocks for senile plaques associated with AD.

Results from our unpublished research support the hypothesis that elevations of HMGB1 can prime the immune system and exaggerate the inflammatory response to an immune stimulus. The primed immune response was shown to potentiate sickness behaviors, and exaggerate Aβ production. However, the additional production of Aβ in LPS-treated RSD animals failed to confer cognitive deficits beyond those of LPS-treated HCC animals. To address this result, the dose of LPS was reduced by 50% before retesting the hypothesis that RSD-induced immune alterations would increase cognitive deficits beyond those observed by the HCC and result in significantly elevated accumulation of hippocampal Aβ. Additionally, this study tested the hypothesis that inhibiting HMGB1 during RSD constitutes a sufficient intervention to prevent the elevated production of Aβ. As a final measure, regional expression (dorsal and ventral) of

HMGB1 and IL-1β mRNA within the hippocampus was evaluated to test the hypothesis RSD and LPS differentially effect functionally distinct regions of the hippocampus.

Chapter 2.

Experiment 1

REPEATED SOCIAL DEFEAT EXACERBATES LPS-INDUCED COGNITIVE DEFICITS AND AMYLOID-β PRODUCTION, AN EFFECT MITIGATED BY TREATEMENT WITH GLYCYRRHIZIN

The present study tested two hypotheses. The first hypothesis predicted that RSD would elevate LPS-induced Aβ production resulting in exaggerated cognitive deficits. Additionally, this study examined the role of HMGB1 in the exacerbated accumulation of LPS-induced Aβ within hippocampus of RSD animals by administering the HMGB1 inhibitor glycyrrhizin to test the hypothesis that inhibiting HMGB1 could prevent the RSD-induced deficits.

Glycyrrhizin (GL) is an anti-inflammatory derived from licorice root extract, and is capable of inhibiting LPS-induced inflammation by disrupting the interaction between LPS and the MD-2/TLR4 receptor complex (Honda et al., 2012). The anti-inflammatory properties of GL are also attributed to the compound's ability to inhibit the phosphorylation and release of HMGB1 (S.-W. Kim et al., 2012; Xiong et al., 2016). Inhibiting the phosphorylation and release of HMGB1 provides a compound approach to reducing HMGB1 activity as less dHMGB1 is produced due to the lack of phosphorylation and the activity of dHMGB1 that is produced is inhibited by the reduction in overall release.

This mechanism is consistent with the results of Honda et al. (2012) as dHMGB1 can interact with MD-2 to promote inflammation thought TLR4 (Yang et al., 2015). In addition to HMGB1 inhibition, GL was also found to have anti-oxidative effects in neurons (S.-W. Kim et al., 2012), results that further support the use of GL to inhibit HMGB1. However, the ability of GL to prevent RSD-induced alterations in the inflammatory response has not yet been tested.

Therefore, the current research builds on the previous research by testing two hypotheses. The first hypothesis predicted that RSD would exaggerate both LPS-induced Aβ accumulation and Aβ-induced cognitive deficits. The second hypothesis predicted that GL treatment would prevent any RSD-induced alterations in Aβ production and cognition.

Experiment 1: Methods

Experimental Progression

To assess the influence of RSD on inflammation-induced Aβ production, animals were first assigned to one of two Conditions, RSD or HCC, and to one of two Block treatments, glycyrrhizin (GL) or saline. After completing of the RSD protocol (described below), each of the four groups was split into two additional groups based on the Treatment, LPS or saline. Therefore, this study utilized a 2 (Condition: HCC or RSD) x (Treatment: LPS or saline) x 2 (Block: glycyrrhizin or saline) design to create a total of eight groups.

The series of immune injections began on the morning of experimental day eight to ensure clearance of glycyrrhizin metabolites, as there is a peak in secondary metabolites approximately 12 hours after dosage, however, all metabolites should have dissipated by 24 hours post dosage (Takeda et al., 1996). Animals were injected with LPS (125 μg/kg) or saline between the hours of 0900 and 1000, once per day for seven consecutive days. Twenty-four hours after the final injection, animals were trained in contextual fear conditioning (described below), and the freezing behavior was evaluated the following day. After CFC testing was complete, animals were sacrificed and their hippocampi removed for use in an Aβ ELISA.

Experimental Subjects

Animals for this experiment were experimentally naive 4−6 month old C57BL6/J mice bred and housed in the Texas Christian University vivarium from stock acquired from Jackson Laboratory (Bar Harbor, ME). Animals aged in Allentown (Allentown, NJ) NexGen cages (40 x 19 x 18 cm). Prior to beginning experimentation, animals were moved and housed in a separate testing room in standard polycarbonate cages (30 x 20 x 16 cm) with three animals per cage. Food and water was available *ad libitum*, and lights were automatically maintained at a light-dark cycle of 0700 to 1900 respectively. All animal care was in compliance with the *Guide for the Care and Use of Laboratory Animals*, and all experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Texas Christian University.

Treatment Conditions

Animals were randomly assigned by home cage to either RSD or HCC. Following Condition assignment, animals were semi-randomly assigned to each of the subsequent treatment groups (Block: GL or saline; Treatment: LPS or saline). To determine if GL can inhibit RSD-induced elevations in hippocampal Aβ, animals were administered an i.p injection of GL (4 mg/kg) or saline 15 minutes prior to the beginning of RSD, and immediately after completion of the RSD session. Injections of LPS (125 µg/kg) or saline were administered once per day for seven consecutive days beginning on the morning of experimental day eight.

Repeated Social Defeat

The protocol used in this experiment was similar to the protocol described in the literature as both *repeated social defeat (*RSD) and *social disruption stress* (RSD is a

modification of the social disruption stress paradigm) (Reader et al., 2015; Wohleb et al., 2012). Animals housed in groups of three were first randomly divided by home cage into either the RSD or home cage control (HCC) condition. Animals in the HCC condition were left undisturbed throughout the RSD protocol, while animals in the RSD condition experienced six consecutive days of social defeat. During social defeat, a male CD-1 intruder (retired breeder of 10 to 12 months of age) was introduced into the home cage of a cohort of three C57BL6/J mice between the hours of 1600 and 1800 for six consecutive nights. Within the first 10 to 15 minutes, if the intruder did not initiate an attack, or was unable to assert dominance over the cohort, the intruder was replaced with an alternate. During this same timeframe, C57BL6/J mice were monitored for displays of submission such as upright posture, retreat, and crouching to verify that the intruder mouse usurped the dominant position within the cage (Avitsur et al., 2001). Monitoring continued throughout the entire RSD session to ensure the health and safety of all animals. At the end of the session, the intruder mouse was returned to his respective cage, and all animals were weighted and given a health inspection. Any animal that was moribund or displayed signs of physical injury were excluded from further experimentation.

Contextual Fear Conditioning

The protocol for CFC closely replicated the methodology used in previous studies designed to test the effect of inflammation-induced elevations in Aβ (Kahn et al., 2012; Weintraub et al., 2013). Briefly, animals were trained in CFC on the morning of 15th day of experimentation. The conditioning protocol consisted of a 120s acclimation period followed by a 2s mild foot-shock (0.5mA). Following the foot-shock, there was a final 60s interval before the animals were removed from the conditioning chamber (Coulbourn Insturments, Whitehall, PA). The chamber walls were accented with a polka dot pattern, and a peppermint olfactory cue

was added to facilitate the association between the context and the aversive stimulus. After the protocol was complete, animals were returned to their home cage, and the chamber was thoroughly cleaned before the next animal began training. The following morning, animals were returned to the chamber for testing, and freezing behavior was monitored using FreezeFrame™ software for 120s. The percentage of time the animal spent freezing during testing was used as the dependent variable to infer the strength of the association formed between the context and the aversive stimulus.

Tissue Collection

To determine the abundance of Aβ within the hippocampus, animals were sacrificed after the completion of CFC testing and their hippocampi were collected. Collected tissue was homogenized in a protein stabilizer (PRO-PREP, Boca Scientific, Boca Raton, FL) before being flash frozen on dry ice and stored at -80°C until total sample protein levels were quantified using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Samples were prepped for the protein assay by first being centrifuged at 16,000g for 20 minutes to pellet tissue debris, and the remaining lysate was removed for the assay and then diluted to uniform concentration for the ELISA.

Aβ ELISA

Our lab has had repeated success with the Legend Max™ $A\beta_{x\text{-}42}$ ELISA (BioLegend, San Diego, CA, Previously Covance # SID-38956), conducted per manufacturer specifications. Samples were diluted to a uniform concentration with an incubation buffer that contains HRP-labeled detection antibody. Samples were then processed in duplicate, and the plate was

incubated overnight at 2−8°C. The following day, the plate was washed and incubated with TMB substrate before being read at 620nm (BMG LabTech FLOUstar Omega; Cary, NC).

Experiment 1: Results

Glycyrrhizin prevents cognitive deficits in LPS-treated RSD animals

To determine the effect of glycyrrhizin on RSD-induced manipulation of the inflammatory response to LPS, a 2 x 2 x 2[18] factorial ANOVA was conducted on CFC testing data. Results identified a significant effect for Treatment, $F(1,75) = 12.43$, ($p < 0.05$), Condition, $F(1,75) = 6.15$, ($p < 0.05$), and Block, $F(1,75) = 8.41$, ($p < 0.05$). Analysis of significant effects identified that, overall, animals treated with saline froze significantly more (M = 51.59) during testing compared to LPS-treated animals (M = 40.91). Additionally, HCC animals froze significantly more (M = 50.01) than RSD animals (M = 42.5), and animals treated with GL froze significantly more (M = 50.65) than animals treated with saline (M = 41.87). Analyses continued with investigation of all pairwise comparisons, using a Bonferroni correction, to probe for the predicted between groups effects.

Results from all pairwise comparisons revealed that RSD animals treated with LPS and saline froze significantly[19] less ($p < .05$) than all other groups, and no other significant differences were identified. Within this context, the three main effects can be understood more clearly as RSD animals injected with saline and LPS appear to drive all main effects (Figure 7). Taken together, results support the hypothesis that administration of GL can prevent the effect of RSD on LPS-induced disruptions of cognitive performance during CFC.

[18] Condition: RSD or HCC x Treatment: saline or LPS x Block: saline or GL
[19] SPSS data output back calculates the *p*-value so interpretation of the data after a correction is applied can still be identified as less than .05.

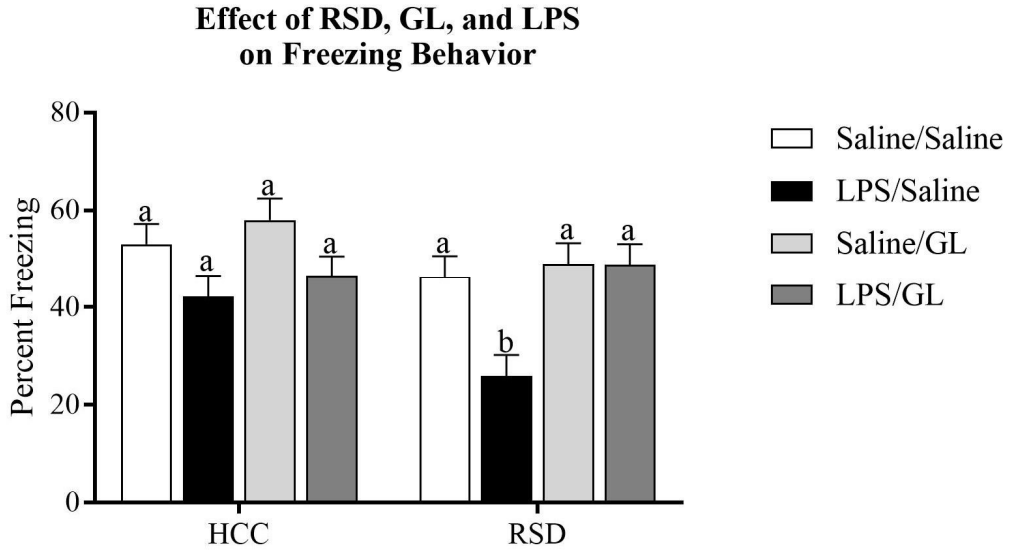

Figure 7. Effect of RSD, GL, and LPS on Freezing Behavior. Data shows that RSD combined with repeated LPS injections induces cognitive deficits, however, administration of glycyrrhizin throughout RSD is protective. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 10-11$).

Mixed effects of glycyrrhizin on hippocampal Aβ accumulation

As before, a factorial ANOVA was conducted on the data from the Aβ ELISA[20]. Results identified a significant effect for Treatment, $F(1,73) = 32.32$, ($p < 0.05$), such that LPS-treated animals expressed significantly more Aβ (M = 3927.58 pg/mg) compared to saline-treated animals (M = 2180.99 pg/mg). To further investigate the predicted between-groups effects, all pairwise comparisons were analyzed using a Bonferroni correction. Results identified that animals in the RSD/LPS/saline group had the highest overall accumulation of Aβ (M = 4839.64 pg/mg); however, this accumulation was not significantly higher than animals in the HCC/LPS/saline group (M = 4015.89 pg/ml), despite being significantly higher ($p < .05$) than the remaining six groups (Figure 8). Additionally, there was no significant difference identified between animals in the HCC/LPS/saline group, and animals in the HCC/LPS/GL group (M =

[20] Two samples were removed from the analysis as the total protein within the sample was deemed too low for inclusion in the ELISA.

3671.01 pg/ml) or animals in the RSD/LPS/GL group (M = 31.83.80pg/mg), although these three groups were significantly higher ($p < .05$) than the remaining four groups (Figure 8). No other significant differences were identified between groups. After analyzing all between-group effects, results support the hypothesis that glycyrrhizin protects against RSD-induced exaggerations of LPS-induced inflammation (Figure 8).

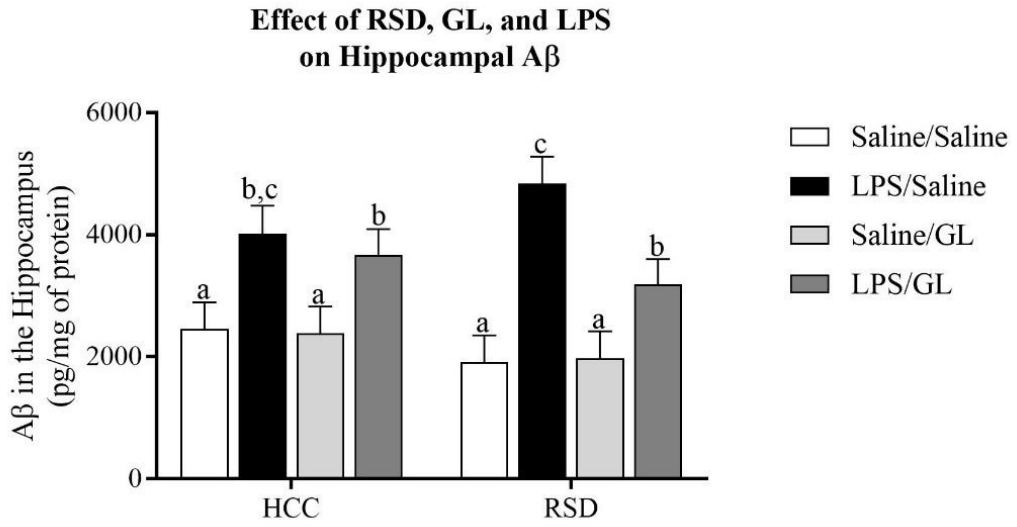

Figure 8. Effect of RSD, GL, and LPS on Hippocampal Aβ. Data shows that RSD animals treated with LPS and saline have the highest levels of Aβ accumulation, but are not significantly elevated compared to HCC animals of comparable treatment. Glycyrrhizin administration throughout the RSD protocol significantly reduces RSD-induced exaggeration of LPS-induced Aβ accumulation in the hippocampus. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 10-11$).

Experiment 1: Brief Discussion

The purpose of the presented study was to test two hypotheses, 1) that RSD would increase LPS-induced accumulation of Aβ within the hippocampus and create significant cognitive deficits, and 2) that administration of glycyrrhizin would protect against RSD-induced exaggerations of LPS-induced cognitive deficits, and prevent RSD-induced elevations of LPS-induced Aβ accumulation within the hippocampus. Overall, results from this experiment support the hypothesis that RSD increases LPS-induced cognitive deficits, and that glycyrrhizin can

protect against RSD-induced exaggerations of LPS-induced cognitive deficits. However, RSD did not significantly elevate LPS-induced Aβ accumulation, despite an overall higher expression of Aβ in RSD/LPS/saline animals relative to the comparable HCC group. Analysis of CFC testing data identified that animals in the RSD/LPS/saline group froze significantly less during testing compared to all other groups, but no other between-group differences were identified. Further, analysis of Aβ accumulation within the hippocampus revealed that animals in the RSD/LPS/saline group expressed the highest levels of Aβ accumulation, and were significantly elevated relative to all other groups except the HCC/LPS/saline group. However, animals in the HCC/LPS/saline group also expressed a comparable Aβ load relative to animals in the HCC/LPS/GL group and the RSD/LPS/GL group. These results support the use of GL as a prophylactic measure against RSD-induced exaggerations of LPS-induced hippocampal Aβ accumulation.

Previously, a similar study was conducted in our lab whereby animals completed six days of RSD and then received seven days of daily injections of LPS (250 μg/kg). Results from that study failed to detect any behavioral difference between HCC and RSD animals treated with LPS (Figure 5), and prompted the reduced dosage of LPS (125 μg/kg) used in the present study. The repeated injections paradigm relies on the LPS-induced inflammatory response to generate Aβ, and the accumulation of Aβ within the hippocampus accounts for a significant portion of cognitive deficits displayed by LPS-treated animals during CFC testing. Therefore, by reducing the dosage of LPS, the present research relied on the RSD-induced exaggerations of the immune response to LPS (Figure 1) to generate Aβ beyond that of HCC animals and disrupt cognition during CFC testing.

Additionally, results from previous literature, and data from our lab (Figure 2 and 3), support the hypothesis that HMGB1 accounts for the RSD-induced exaggerations of the inflammatory response (Reader et al., 2015; Wohleb et al., 2012; Wohleb et al., 2011). Glycyrrhizin inhibits the phosphorylation and release of HMGB1, and is therefore capable of preventing the activity of dHMGB1 (S.-W. Kim et al., 2012). Data from the present research supports the use of GL to prevent RSD-induced exaggerations of the inflammatory response as indicated by the comparable accumulation of Aβ between the RSD/LPS/GL, HCC/LPS/GL and HCC/LPS/saline groups. Had the exaggerated inflammatory effect of RSD occurred independent of HMGB1 activity, then Aβ accumulation in RSD/LPS/GL animals would be expected to approximate Aβ accumulation identified in RSD/LPS/saline animals. Therefore, results from this study support the hypothesis that a recurrent social stressor followed by repeated bouts of inflammation negatively impact cognitive performance, and contribute to exaggerated accumulation of hippocampal Aβ. However, administration of an HGMB1 inhibitor, such as glycyrrhizin, throughout periods of stress can prevent inflammation-induced deficits. Moreover, experiencing a repeated social stressor prior to several bouts of inflammation enhances the production of Aβ, an effect also mitigated though administration of glycyrrhizin during periods of stress.

Chapter 3.

Experiment 2

REPEATED SOCIAL DEFEAT ALTERS EXPRESSION OF HMGB1 mRNA IN THE DORSAL, BUT NOT VENTRAL, HIPPOCAMPUS, AND EXAGERATES PRODUCTION OF INTERLUKIN 1-β mRNA

The purpose this study was to test the hypothesis that regionally distinct areas of the hippocampus are associated with a differential response to RSD and LPS. The initial analysis of mRNA data (Figures 1 & 2) examined the effect of RSD on LPS-induced exaggerations of the inflammatory response in the *dorsal* hippocampus. While the dorsal hippocampus is highly associated with contextual fear conditioning (Celerier et al., 2004), the ventral hippocampus partially regulates emotional experiences (Fanselow & Dong, 2010) and the stress response (Henke, 1990). The pro-inflammatory effect of repeated social defeat was hypothesized to originate from stress-induced elevations in HMGB1 production that prime the immune system to respond to an immune challenge. This effect was demonstrated in the dorsal hippocampus by data presented in Figures 1 and 2, however, there were no previous reports in the literature of a direct comparison between the dorsal and ventral response to RSD and LPS. In response to this absence in the literature, the present study tested the hypothesis that LPS-treated RSD animals would increase expression of HMGB1 in the ventral hippocampus. Additionally, given the well documented influence of proinflammatory cytokines on contextual conditioning, and that contextual conditioning is dependent on the dorsal portion of the hippocampus, this study tested the hypothesis that RSD-induced exaggerations of the inflammatory response to LPS would predominantly be found in the dorsal hippocampus.

Experiment 2: Methods

Experimental subjects and Repeated Social Defeat Protocol

The description of experimental subjects for this study was identical to those previously discussed in experiment one. Additionally, the RSD protocol was conducted as described in experiment one except for the administration of GL before and after each RSD session. The morning after the sixth RSD session, animals were given an injection of LPS (250 μg/kg, i.p) or saline and tissue was collected four hours after the injection.

RT-qPCR Procedure

Tissue samples stored at -20°C were processed according to Maxwell® 16 Tissue Purification Kit (Promega, Madison, WI) and total RNA yield was quantified (NanoDrop, ThermoFisher Scientific, Wilmington, DE) prior to being diluted to a uniform concentration for RT-qPCR analysis. The RT portion of the procedure was conducted with a 7500 RealTime PCR Thermal Cycling System (Applied Biosystems, Foster City, CA) using iScript™ reverse transcription supermix (BioRad, Hercules, CA), and the qPCR portion was conducted using the CFX Connect™ System (BioRad, Hercules, CA). All probes and primers were obtained from BioRad (Hercules, CA), and were amplified in accordance with the manufacture's recommended protocols. Regression quantification cycle determination was used to evaluate amplification data before target gene expression was normalized relative to β-actin, and analyzed using the CFX Manager Gene Study software (BioRad), and all statistics were performed with Prism GraphPad (Version 6.07).

Statistical approach to mRNA expression analysis

The CFX Manager Gene Study software was used to normalize target gene expression to our selected reference gene, β-actin. Once the gene was normalized, the software provides information about each group within the study, such as the mean normalized expression and the standard error of the mean. However, for this particular study, each subject contributed two regionally distinct areas of the hippocampus for analysis, the dorsal and ventral regions. While our preferred method of analysis would include a within-subjects analysis of dorsal and ventral regions, the absence of information for each individual data point prevented this approach. Therefore, in order to analyze the data for all between-group effects, the data set for each target was first split by Treatment (LPS or saline), and a subsequent 2x2 ANOVA was conducted to identify the influence of Condition (RSD or HCC) and Region (dorsal or ventral hippocampus) on target mRNA expression.

Experiment 2: Results

RSD-induced expression of HMGB1 mRNA occurs in the dorsal, but not ventral hippocampus in saline-treated animals

The first set of analyses examined HMGB1 mRNA expression in saline-treated animals within the dorsal and ventral hippocampus. Results from the 2x2 ANOVA identified a significant interaction between Condition (RSD or HCC) and Region (dorsal or ventral), $F(1,24) = 5.07$, $p < .05$, as well as a main effect for both Condition, $F(1,24) = 7.15$, $p < .05$, and Region, $F(1,24) = 5.07$, $p < .05$. Results for all between-group comparisons identified that RSD animals expressed significantly more HMGB1 mRNA within the dorsal hippocampus relative to all other groups, and no other significant differences were identified. While these results do not support

the predicted expression within the ventral hippocampus, the data does replicate previous results (Figure 2) for expression within the dorsal hippocampus (Figure 9).

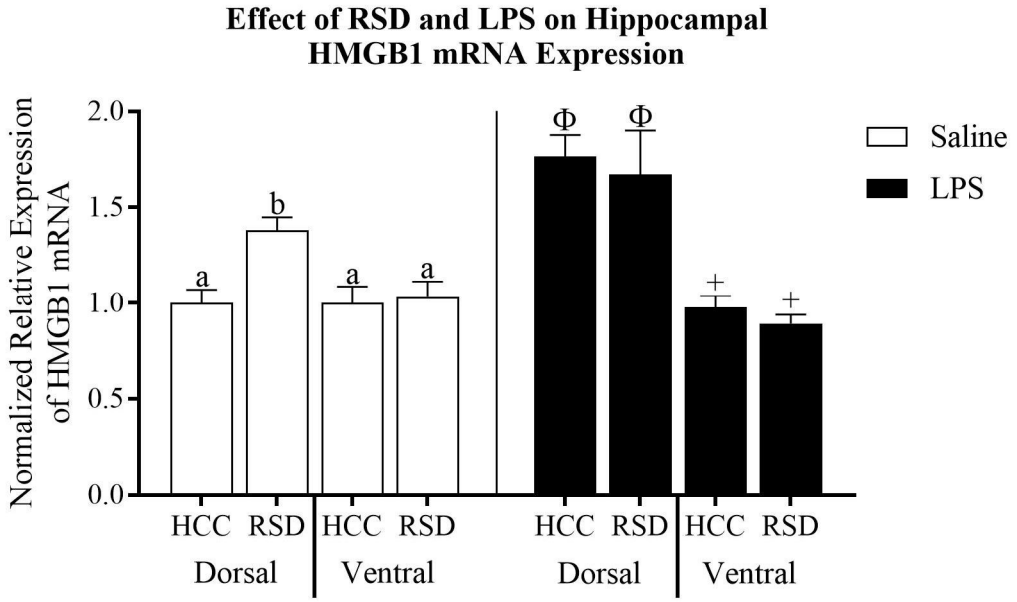

Figure 9. Effect of RSD and LPS on Hippocampal HMGB1 mRNA Expression. RSD exaggerates HMGB1 mRNA expression in the dorsal, but not ventral hippocampus, of saline-treated animals. Likewise, administration of LPS also increases HMGB1 mRNA expression in the dorsal, but not ventral hippocampus. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 7$).

LPS administration facilitates expression of HMGB1 mRNA in the dorsal, but not ventral hippocampus

A second 2x2 ANOVA was conducted to determine the influence of LPS on HMGB1 mRNA expression. Results failed to support the stated hypothesis, identifying a main effect for Region, $F(1,24) = 5.07$, $p < .05$. Analysis of all between group effects revealed both groups of LPS-treated animals had a comparable expression of HMGB1 within the dorsal hippocampus that was significantly elevated ($p < .05$) relative to the ventral hippocampus. When these results are viewed in conjunction with data from saline-treated animals, the data supports a hypothesis

predicting the majority of HMGB1 mRNA production within the hippocampus would be localized toward the dorsal, as opposed to the ventral, hippocampus (Figure 9).

RSD reduces expression of IL-1β mRNA within the dorsal but not ventral hippocampus in saline-treated animals

Contrary to any predicted effect, analysis of data for IL-1β mRNA expression in saline-treated animals identified a main effect for Condition, $F(1,24) = 5.07$, $p < .05$, such that, overall, HCC animals expressed significantly more hippocampal IL-1β mRNA compared to RSD animals. Complete analysis of all between-group effects identified that RSD animals expressed significantly less ($p < .05$) IL-1β mRNA in the dorsal hippocampus when compared to either region of HCC animals, but the ventral region of the RSD animals was not significantly different from any of the other three regions (Figure 10).

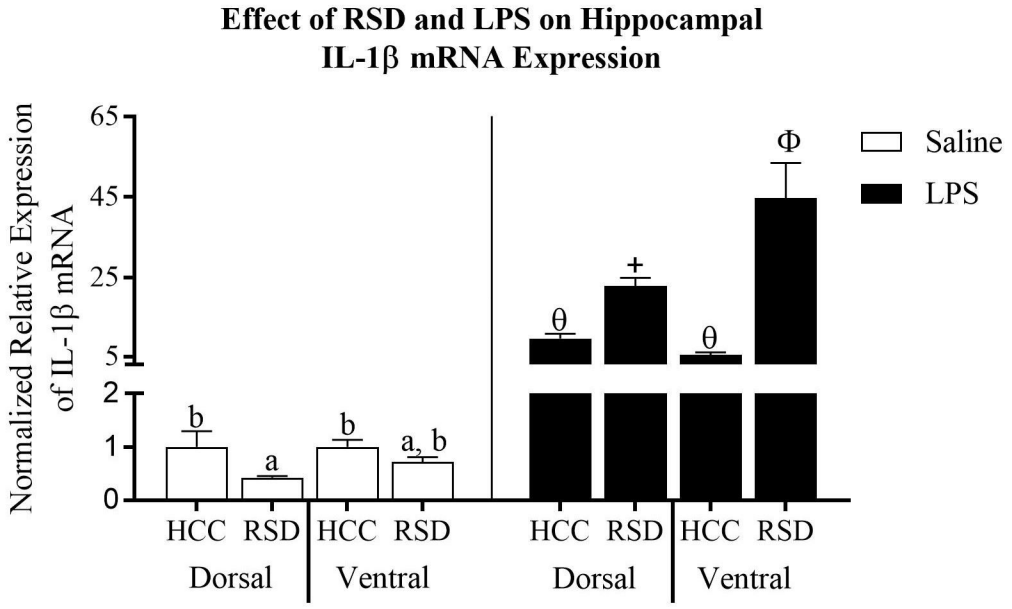

Figure 10. Effect of RSD and LPS on Hippocampal IL-1β mRNA Expression. Differential effects of RSD on IL-1β mRNA expression. Independent of LPS, RSD appears to reduce IL-1β mRNA in the dorsal hippocampus. However, the combination of RSD and LPS exaggerate IL-1β mRNA expression in the ventral and dorsal hippocampus relative to HCC animals. Different characters represent significant ($p < .05$) difference, error bars equal SEM ($n = 7$).

RSD interacts with LPS to enhances production of IL-1β mRNA within the dorsal and ventral hippocampus

Data from IL-1β mRNA analysis continued to deviate from predicted results, revealing a significant interaction between Condition and Region, $F(1,24) = 8.11$, $p < .05$, and a main effect for Condition, $F(1,24) = 33.86$, $p < .05$. Between-groups analysis identified that LPS-treated HCC animals did not express significantly different levels of IL-1β mRNA between the dorsal and ventral hippocampus. Additionally, LPS-treated RSD animals expressed significantly more ($p < .05$) IL-1β mRNA in the dorsal hippocampus when compared to either HCC region, but surprisingly, the ventral hippocampus of LPS-treated RSD animals expressed significantly more ($p < .05$) IL-1β mRNA than all other groups (Figure 10). When these results are considered with regard to saline-treated animals, the data supports the hypothesis that RSD diminishes expression of IL-1β mRNA in the dorsal hippocampus, but also interacts with LPS to enhance expression beyond HCC animals in both the dorsal and ventral hippocampus.

Experiment 2: Brief Discussion

The purpose of this experiment was to identify the influence of RSD and LPS on the expression of HMGB1 and Il-1β mRNA in the dorsal and ventral hippocampus. These regionally distinct areas of the hippocampus are also associated with functionally distinct activation, such that the dorsal hippocampus is associated more with cognition and encoding of fear related memory, while the ventral hippocampus controls behaviors related to anxiety and the response to stress, in addition to numerous other subtle contribution (Bannerman et al., 2004; Fanselow & Dong, 2010; Kheirbek et al., 2013; Risold & Swanson, 1996). The association of the ventral hippocampus with the response to stress prompted the hypothesis that the ventral hippocampus would express HMGB1 mRNA in a greater abundance relative to the dorsal

hippocampus, as the protein HMGB1 can be upregulated in response to stress. Not only did we fail to find support for that hypothesis, our data suggest the dorsal hippocampus as the primary region of hippocampal HMGB1 mRNA expression. Fortunately, data from the dorsal hippocampus closely replicated data previously found regarding the significant increase in HMGB1 mRNA expression in the dorsal hippocampus of saline-treated RSD animals (Figure 2), and the uniform increase in HMGB1 mRNA expression in response to LPS administration.

Data from IL-1β mRNA expression also revealed some interesting and curious effects. Despite our incorrect hypothesis regarding elevated dorsal production of IL-1β mRNA relative to ventral production, analysis of the data from saline-treated animals identified a significant difference in IL-1β mRNA expression between both HCC areas and the dorsal region of the RSD animals. Given the data reported in Figure 1 that shows a similar, but non-significant decline in IL-1β mRNA in saline-treated RSD animals, this data may represent a subtle decline in available IL-1β mRNA in this group. Further research should attempt to replicate this effect, in addition to coupling the mRNA expression with a measure of cytokine expression, as the decline in mRNA within the dorsal hippocampus may be a product of RSD-induced immune priming and the translation of mRNA into the components for IL-1β assembly.

Analysis of IL-1β mRNA expression in LPS-treated animals was also surprising, as the ventral hippocampus of RSD animals exhibited the most robust response to an inflammatory challenge as indicated by the highest increase in expression compared to all other groups. This effect was surprising for at least two reasons. First, based on the data from HMGB1, administration of LPS increases expression of HMGB1 regardless of Condition. HMGB1 is highly related to the inflammatory response, therefore, elevations in HMGB1 mRNA expression would be expected to coincide with elevations in IL-1β. However, that is not the pattern of

expression we identified. Second, analysis of the HCC animals treated with LPS showed a decline in the expression of IL-1β mRNA between the dorsal (M = 9.47) and ventral (M = 5.44) regions of almost fifty percent, an effect reversed in RSD animals. When RSD animals were dosed with LPS, expression of IL-1β mRNA in the ventral region of the hippocampus was approximately double that identified in the dorsal region. The most probable explanation for this effect depends on stress-induced migration of bone-marrow derived cells into the hippocampus (Brevet et al., 2010). Once these cells reach the hippocampus, they differentiate into microglia and migrate toward the ventral region of the hippocampus (Brevet et al., 2010). This stress-induced migration of bone-marrow derived microglia toward the ventral hippocampus was reported to occur over a period of five days, a timeline comparable with our RSD protocol. If the ventral hippocampus of RSD animals was inundated with newly differentiated microglia, a subsequent LPS immune challenge could result in the data pattern we observed in our study.

Chapter 4.

Concluding Discussion

The two experiments presented in this document are best understood when viewed in conjunction with the three preceding experiments conducted by our lab regarding repeated social defeat. The first of our studies simply aimed at replicating an effect present in the literature whereby repeated social defeat was used to upregulate protein expression of HMGB1 and exacerbate the inflammatory response to LPS. We were successful in this pursuit as results found that saline-treated RSD animal expressed significantly more protein and mRNA for HMGB1 in the dorsal hippocampus. Additionally, we found that LPS-treated RSD animals expressed significantly more IL-1β mRNA when compared to LPS-treated HCC animals. Results from this study supported the hypothesis that RSD is a manipulation capable of initiating a biological process that upregulates expression of HMGB1 and primes the immune system to respond to challenges with an exaggerated response.

Additionally, evidence within the literature characterize several RSD-induced alterations in peripheral and central biology, as well as several behavioral changes consistent with altered immune function. Initiation of these processes beings with activation of the stress response when animals in the social defeat paradigm are confronted with a physically superior home cage intruder. Within a laboratory setting, murine social hierarchies are generally accepted to consist of an alpha and subordinates, although instances of shared dominance exist (Schein, 1975). The introduction of an intruder prompts a reorganization of the social hierarchy as residents attempt, and fail, to defend the resources of the cage from the intruder (Tamashiro, Nguyen, & Sakai, 2005). Over the duration of the six consecutive days of social defeat, the first RSD-induced behavioral alterations emerged as the frequency of submissive behaviors increases (Figure 11).

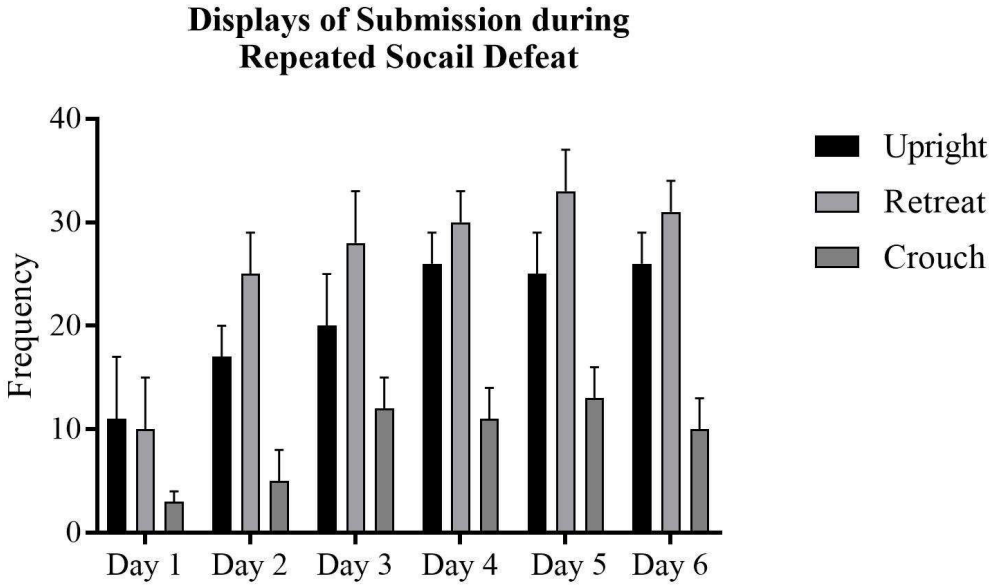

Figure 11. Displays of Submission during Repeated Social Defeat. Data was collected during three separate RSD experiments. Results were obtained by monitoring a single cage of animals for the first 15 minutes of each RSD session. Overall, the data shows that the frequency of submissive behaviors generally increases as a function of time (error bars = SEM).

After completion of the RSD, the primary behavioral alteration of defeated mice includes an increased expression of anxiety behaviors. Kinsey, Bailey, Sheridan, Padgett, & Avitsur (2007) reported that during the light/dark preference test, RSD animals spent significantly more time in dark box and had an increased latency to emerge from the box, behaviors associated with increased anxiety. In the same study, open field data showed that RSD animals also made less entries into and spent less time in the center of the arena despite having comparable locomotor activity (Kinsey et al., 2007). Additionally, these effects persisted for up to 14 days, however, other reports of identical behaviors only persisted for approximately eight days (Kinsey et al., 2007; Wohleb, Powell, Godbout, & Sheridan, 2013). The contribution of Wohleb et al. (2013) extended the analysis of RSD behavioral alterations to include measures of central immunological changes that paralleled the increase in anxiety behaviors such that central recruitment of bone-marrow derived monocytes coincided with expression of anxiety behaviors.

Taken together, these studies linked RSD to alterations in immune function with concomitant elevations of anxiety behaviors.

Separately, evidence emerged supporting the hypothesis that RSD-induced alterations in the inflammatory response were mediated through stress-induced production of the protein HMGB1 (Weber et al., 2015). In this context, RSD can be viewed as a paradigm that temporarily transforms an organism into a state of anxious immunological hyper-responsiveness. However, other than reports of RSD failing to induce alterations in measures of depression (Kinsey et al., 2007), few other behavioral tests were investigated. Moreover, the opportunity to investigate how RSD-induced immune alterations could interact with LPS-induced behavioral changes was a unique opportunity. After replicating the effect of RSD-induced expression of HMGB1 protein and mRNA, and exaggeration of the inflammatory response to LPS, our lab had the opportunity to incorporate this manipulation into behavioral models that measured sickness behaviors, as well as, LPS-induced AD-like pathology.

To evaluate sickness behaviors, animals were first subjected to six consecutive days of RSD while simultaneously undergoing training for the burrowing task. The morning after the final RSD session, animals were given a dose of either saline or LPS (5 μg/kg) and were allowed access to individual burrowing tubes two hours after the injection. Results from this study demonstrated that eight hours after the injection, HCC animals treated with LPS no longer exhibited a burrowing deficit, however, LPS-treated RSD animals continued to display evidence of LPS-induced sickness behaviors. Results from this experiment demonstrated that although no apparent behavioral deficit existed between RSD and HCC animals, as demonstrated by a comparable burrowing behavior between saline-treated RSD and HCC groups, RSD animals were predisposed to immunological threats such that administration of LPS caused a potentiation

of sickness behaviors. In this regard, RSD facilitated alterations in the central nervous system that were behaviorally hidden with regard to the specific task, however, given the appropriate trigger, RSD-induced immunological priming significantly potentiated performance deficits.

Within the context of sickness behaviors, there is little room to evaluate these changes as positive or negative, as the benefit or hindrance of exaggerated sickness behaviors would be dependent upon the context of the situation. However, as an American, there is a certain obligation to render some form of condemning judgement on situationally dependent phenomena; therefore, the following experiments examined the influence of RSD-induced exaggerations of the immune response in the context of AD-like pathology. Our lab has had repeated success elevating hippocampal Aβ following repeated bouts of acute inflammation that impairs performance during cognitive tasks, such as contextual fear conditioning (Kahn et al., 2012; Weintraub et al., 2013; Weintraub et al., 2014). However, an important caveat to consider when interpreting research using the repeated injections of LPS is that while this research aims to model an extended inflammatory state, the use of LPS induces an acute inflammatory response per injection. Therefore, the research more closely models repeated bouts of acute inflammation rather than chronic inflammation.

Two experiments were conducted to examine the physiological and behavioral effects of RSD-induced exaggerations of the immune response to LPS, and the subsequent accumulation of LPS-induced Aβ within the hippocampus. The first iteration of this experiment failed to identify behavioral differences between LPS-treated RSD and HCC animals, however, LPS-treated RSD animals had significantly elevated accumulation of hippocampal Aβ. The second iteration of the study reduced the dosage of LPS, but also evaluated the efficacy of the HMGB1 inhibitor, glycyrrhizin, at preventing RSD-induced alterations. Results found that animals in the

RSD/LPS/saline group displayed significant cognitive deficits during CFC testing, but no other significant between group effects were identified. These results are significant for at least two reasons, 1) this data represent a second instance of behavioral disruptions that emerge as a result from RSD-induced alterations in the immune response to LPS, and 2) that administration of GL prior to and after a session of RSD confers a protective effect against RSD-induced immune priming.

There are two known components of glycyrrhizin's mechanism of action that contribute to the inhibition of HMGB1 activity. Intracellular inhibition occurs as glycyrrhizin prevents phosphorylation of HMGB1, a necessary step for the secretion of HMGB1 into the extracellular space where HMGB1 exerts both chemotactic and proinflammatory influences (S.-W. Kim et al., 2012; Y. H. Kim et al., 2016; Y. M. Kim, Kim, & Chang, 2015). Extracellular inhibition of HMGB1 occurs as glycyrrhizin directly binds with the protein to prevent mitogenic, chemotactic, and receptor mediated proinflammatory activity (Mollica et al., 2007; Musumeci, Roviello, & Montesarchio, 2014; Wu et al., 2015). Although the use of glycyrrhizin to inhibit the influence of HMGB1 on inflammation is well represented in the literature, this is the first known use of glycyrrhizin to inhibit HMGB1-induced immune priming.

The protective effect conferred by glycyrrhizin during RSD is apparent in the data from the Aβ ELISA. Animals in the RSD/LPS/saline group expressed the highest mean accumulation of Aβ, and were significantly higher than all other groups except the HCC/LPS/saline. Moreover, there were no significant difference between the HCC/LPS/saline group and either of the groups treated with GL, indicating that administration of GL prevented RSD-induced HMGB1 effects on inflammation, and did so without any perceived effects on the HCC animals.

However, the use of glycyrrhizin is debated with regard to potential side effects as dosage and routes of administration elicit different effects on bioavailability.

The principal cause for concern over unrestricted use of glycyrrhizin pertained to reports that excess consumption disrupts mineralocorticoid and cortisol activity, and induces hypokalemia (low blood potassium) and muscle weakness (Omar et al., 2012). However, emergence of these side-effects depends largely on the method of administration, as oral consumption is subject to intestinal modification that converts glycyrrhizin into the primary metabolite, glycyrrhetic acid, an extremely effective inhibitor of 11-β-hydoxysteroid dehydrogenase, the enzyme responsible for converting cortisol to cortisone (Chan et al., 2012). Therefore, oral ingestion of high doses of glycyrrhizin can reduce blood potassium concentration, elevate mineralocorticoid activity, and prevent the reversible reaction that converts cortisol to cortisone (Hattori et al., 1985). Currently, the most widely accepted human maximal dose is 2 mg/kg/day (Omar et al., 2012), however, oral dose limitations are easily circumnavigated through the use of an injectable delivery for glycyrrhizin that is not associated with the side effects oral administration. Such practice is common in Japan, as intravenously delivered glycyrrhizin as a common treatment for Hepatitis B, a treatment also known to completely reverse the viral infection (Sato et al., 1996; Takahara, Watanabe, & Shiraki, 1994).

The final experiment presented returned to the methodology of the initial experiment to test the hypothesis that there is a differential response to RSD and LPS between the dorsal and ventral hippocampus. For this study, animals completed the RSD protocol and were subsequently injected with LPS or saline the morning after the final RSD session. Tissue was collected four hours post injection and samples from the dorsal and ventral hippocampus were taken from each animal. Analysis of HMGB1 mRNA yielded similar results to the first study

such that in saline-treated RSD animals, the dorsal portion of the hippocampus expressed significantly more HMGB1 mRNA when compared to all other saline-treated animals, however, no other significant differences were identified between any saline-treated animals. These data reinforce the hypothesis that RSD induces elevated expression of HMGB1 mRNA in the dorsal hippocampus, independent of any immune stimulus. Analysis of data from the LPS-treated animals found that treatment with LPS, regardless of condition, results in a comparable expression of HMGB1 mRNA in the dorsal hippocampus. Finally, data from this study indicate that the ventral hippocampus is not as susceptible as the dorsal to manipulation of HMGB1 mRNA expression.

Analysis of data from the IL-1β gene study suggests that RSD reduces expression of Il-1β mRNA in the dorsal hippocampus of saline-treated animals. However, analysis of IL-1β protein expression would be a prudent follow up analysis as the reduction of mRNA may be a result of increased IL-1β protein expression resulting from stress-induced priming of the NLRP3 inflammasome. The final results obtained from this study are some of the most interesting,[21] as they clearly demonstrate a novel RSD-induced interaction between LPS and regional expression of an inflammatory marker. Regarding LPS-treated HCC animals, no significant difference was identified between regions, however, the dorsal region had a higher mean expression relative to the ventral. Further analysis of between group effects revealed that the dorsal hippocampus of RSD animals expressed significantly more IL-1β mRNA relative to either region of HCC animals, but the ventral region of LPS-treated RSD animals expressed significantly more than all other LPS-treated groups[22]. This effect is also significant in that the increase in IL-1β mRNA in

[21] And credit for the investigation of regional variability in gene expression is entirely credited to the committee's request.
[22] Surprising effects like this are easily one of my favorite things about science.

the ventral hippocampus occurs independently of evidence for increased expression of HMGB1 mRNA expression within the same region.

We hypothesize that the exaggerated response to LPS in the ventral hippocampus of RSD animals was driven by stress-induced migration of bone marrow-derived monocytes toward the hippocampus. Multiple reports of such stress-induced migration are present within the literature. Wohleb et al. (2013) used GFP^+ bone marrow chimeric mice to show that RSD increased the percentage of circulating monocytes and macrophages within the perivascular space of the brain, in addition to significantly increasing recruitment of peripherally circulating monocytes to the brain. Additional evidence for a similar effect was presented by Brevet et al. (2010) who used six days of repeated foot-shock to induce stress and potentiate recruitment of bone marrow-derived cells. This research tracked the migration of bone marrow-derived cells to identify that as cells entered the hippocampus they would adopt microglia characteristics, and migrate toward the ventral hippocampus. This cell migration toward the ventral hippocampus, the region of the hippocampus associated with anxiety behaviors, is also consistent with RSD-induced alterations in anxiety behavior reported by Wohleb et al. (2013), providing further evidence for an immunological link between anxiety and inflammation. Therefore, the elevated production of IL-1β mRNA observed within the ventral hippocampus of LPS-treated RSD mice could be attributed to stress-induced migration bone marrow-cells.

Final Thoughts

The last paragraph of document is always important, as the author has one final chance to remind the audience *why* the information they just read *was* or *is* important. This would be a perfect opportunity to remind everyone that stress, inflammation, and Alzheimer's are, by and large, negative aspects of life (despite the possibility of acquiring some existential meaning from

a forced confrontation with the myriad of possible negative experiences any of these three factors can deliver into your life). Of course, there is also the opportunity for some obvious fear-mongering, of which we are all very familiar with, so here is an obligatory warning, "*don't get stressed and sick or you'll get Alzheimer's*!" But what attracted me to this research, and to graduate school, was idea that cognitive experiences can influence one's physical health, and my education extended that interest an additional step to include how our physical health can shape our cognitive experiences.

The *big picture* of this research illustrates an example of how the reciprocal relationship between an organism and their environment results in a continuous exchange of bidirectional influence. Within the context of the present research, a forced stress response set a biological stage for an environmental trigger to alter cognitive experiences. As an aspiring psychologist, my interests are rooted in understanding cognition and behavior, both of which are inextricably linked to biology. In some instances, biology clearly exerts a dominate influence on the determinants of behavior, such as functional deficits that arise from neural damage. In others, cognition can influence biological function to alter behavior, such as the release of hormones in response to a thought. The evolution of this connection was driven without purpose, but cognitive awareness of the connection allows for opportunity, for choice – the choice to view our circumstances through the lens of our own preference. And, because humans have the ability to choose, choose to not get stressed, otherwise, you'll get sick and end up with Alzheimer's.

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VITA

Personal Background	Micah J. Eimerbrink Keller, TX Son of Donald and Susan Eimerbrink Married Priya A. Iyer, August 23, 2017
Education	Dipolma, Keller High School, 1999 Bachelor of Arts, Psychology Texas State University, 2006 Master of Science, Experimental Psychology University of Texas at Arlington, 2011 Doctor of Philosophy, Experimental Psychology Texas Christian University, 2017
Experience	Community Health Specialist, Department of State Health Services, 2006 - 2009 Adjunct Instructor Columbia College, 2013 - 2015 Teaching Assistant Texas Christian University, 2011- 2016
Professional Memberships	Society for Neuroscience PsychoNeuroImmunology Research Society
Awards	“Dannon Yogurt and Probiotic Fellowship” Funding Amount: $25,000 Dannon Company Inc., 2016 Global Outlook Ambassadorship Texas Christian University, 2016

ABSTRACT

COMBINED EFFECTS OF REPEATED SOCIAL DEFEAT AND INFLAMMATION ON THE ACCUMULATION OF AMYLOID-β WITHIN THE HIPPOCAMPUS

By Micah Jacob Eimerbrink
Department of Psychology
Texas Christian University

Dissertation Advisor: Dr. Gary W. Boehm, Professor of Psychology

All organisms are united by the need to respond to environmental pressures, or stress. The response to stress initiates a biological cascade that attempts to facilitate a reconciliation of the stressor, either through escape or confrontation. This response occurs in numerous forms, but in instances of a repeated psychosocial stressor, there is a specific set of psychological and physiological changes can arise. However, recent evidence suggests that in addition to reconciliation, the response to a repeated stressor also facilitates biological changes that can prime the immune system to respond to immunological threats with an exacerbated response. While adaptive, activation of the immune system's inflammatory response has also been linked to the genesis and progression of numerous diseases. Specifically, research within our lab has demonstrated a link between repeated bouts of immune activation and the production of amyloid-β (Aβ), the protein associated with plaques of Alzheimer's disease. The following data presents support for the hypothesis that repeated stress followed by repeated activation of the immune system elevate hippocampal Aβ and induce significant cognitive deficits. We also demonstrate that administration of glycyrrhizin prior to and immediately after the experience of stress can prevent the stress-induced alterations in the immune response. Additionally, we show that the inflammatory response within the dorsal and ventral hippocampus interacts with repeated

stress, such that stress alters the regional expression of IL-1β and HMGB1 mRNA. Cumulatively, previous data with our lab, combined with the present experiments, demonstrate that repeated stress can exaggerate an induced immune response, potentiate sickness behaviors, elevate the accumulation of inflammatory induced Aβ, and create regionally distinct patterns of immune activation within the hippocampus.