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Neurochemistry, Physiology, And Behavior In A Model Of Gulf War Illness

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DEDICATION

I would like to dedicate this work to all the veterans with Gulf War Illness – I hope that this work provides a platform for better treatment strategies to improve your quality of life. I would also like to dedicate this work to all the animals whose lives made this progress possible.
ACKNOWLEDGEMENTS

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ABSTRACT

Upon returning from the first Gulf War, soldiers cited a plethora of unexplained physical and cognitive deficits which have since been termed Gulf War Illness (GWI). Presentation of GWI is positively correlated with pyridostigmine bromide (PB) use, which was prophylactically administered to soldiers in response to threats of chemical warfare. To test the overarching hypothesis that PB interacted with stress of deployment to alter neural, endocrine, and immune systems, the following studies used a 2×2 rodent model with 14 days of drug treatment (vehicle; PB) and 10 days of repeated restraint stress (stressed) or non-stressed-control conditions. Results indicate that PB decreases cholinesterase activity acutely but sensitizes it by three months post-treatment selectively in rats also subjected to stress. Similarly, only rats in the PB-stressed condition exhibit elevations in corticosterone three months post-treatment. These results suggest that PB and stress interact to progressively disrupt homeostasis in peripheral measures. To test whether PB and stress also interact to influence central neurotransmitter function, an in vivo microdialysis study was performed in a separate GWI cohort where rats were subjected to an immune challenge (30 µg/kg lipopolysaccharide; LPS) followed 48 hours later by an acute restraint stress challenge. Acetylcholine and glutamate levels were assessed in both the prefrontal cortex (PFC) and hippocampus. Results indicate that a history of restraint stress attenuates the cholinergic and glutamatergic responses to both LPS and restraint stress challenges. PB
in the absence of stress decreases the cholinergic response to restraint stress challenge in both the PFC and hippocampus. In contrast, PB in combination with a history of restraint stress interact to preferentially increase the glutamatergic response in the hippocampus. These results indicate that PB preferentially disrupts cholinergic systems whereas PB and stress interact synergistically to disrupt glutamatergic systems, providing insight into possible mechanisms underlying cognitive impairments observed in soldiers with GWI.
PREFACE

The 1990-1991 Gulf War was brief, but the repercussions of that encounter can still be seen today in the 42% of Gulf War soldiers deployed in active combat zones who now report chronic multi-symptom illness (L. Steele, 2000). Although the federal government originally denied any association with Gulf War deployment and ontogeny of symptomology, this stance was revised as collective reports from veterans emerged and were collectively presented to Congress in 1997 (Burton, 1997). The excerpt which follows is from Sergeant Chris Kornkven’s testimony on his experiences with GWI to the U.S. House Committee on Government Reform and Oversight (Burton, 1997). The symptoms reported by Sergeant Kornkven are consistent with those reported by other soldiers with GWI:

“While still in the Gulf I began experiencing symptoms that continue to this day. I had difficulty remembering significant events that happened days earlier... my knees and shoulders were especially painful... and fatigue stayed with me constantly... I reported blinding headaches with only offers of aspirin. I reported memory loss... dismissed as stress. I reported skin problems... and was told ‘it’s not cancer yet... come back as needed.’ I reported intestinal problems... and rectal bleeding... dismissed and no follow-up. I reported joint pain... diagnosed as fibromyalgia... no treatment other than Motrin. I reported chest pains... and
racing heart beats... and was told it was due to an abnormal heart valve... which was hereditary.”

Since this Congressional presentation, the Department of Defense and Veterans Affairs have funded numerous epidemiological, clinical, and preclinical studies to determine the cause, pathology, and treatment strategies for GWI. In the decade since this initiative was launched, we have gained much insight into potential causal factors. However, the specific underlying mechanisms driving the continued progression of GWI or successful treatment regimens remain to be elucidated. The following studies aim to advance our understanding of the pathophysiology of GWI as a precursor to the development of more targeted and successful pharmacological interventions in this population.
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LIST OF ABBREVIATIONS

AChE ........................................................................................................... Acetylcholinesterase
ACTH ........................................................................................................... Adrenocorticotropic hormone
AP ............................................................................................................... Anterior-Posterior
BChE ......................................................................................................... Butyrylcholinesterase
DV ............................................................................................................... Dorsoventral
GR ........................................................................................................... Glucocorticoid Receptor
GWI ............................................................................................................... Gulf War Illness
HPA ........................................................................................................... Hypothalamic Pituitary Adrenal
IL ............................................................................................................... Interleukin
EPI ............................................................................................................... Epinephrine
KMO ........................................................................................................ kynurenine 3-monooxygenase
L ................................................................................................................. Lateral
LPS .......................................................................................................... Lipopolysaccharide
LTD ........................................................................................................ Long-Term Depression
LTP ........................................................................................................ Long-Term Potentiation
MR ........................................................................................................ Minerocorticoid Receptor
NE ........................................................................................................ Norepinephrine
NF-κB ........................................ Nuclear Factor Kappa-light chain-enhancer of activated B cells
NSC ........................................................................................................ Non-Stressed Control
NTS ........................................................................................................ Nucleus Tractus Solitarius
PB ...................................................................................................................... Pyridostigmine Bromide
PFC .................................................................................................................. Prefrontal Cortex
PTSD ............................................................................................................... Post-Traumatic Stress Disorder
CHAPTER 1
A HISTORY OF GULF WAR ILLNESS

On August 2, 1990, the United States along with a coalition of 35 nations invaded Iraq in response to its annexation of Kuwait. In total, this conflict lasted only one hundred hours, and this swift victory was paralleled by the lowest incidence of combat deaths in any war in U.S. history. Originally hailed as a military success, the latent costs of this victory emerged as returning veterans began citing a plethora of unexplained and unprecedented medical symptoms. These varied symptoms have been clustered into three broad classifications: impaired cognition, confusion-ataxia, and arthro-myo-neuropathy (Haley, Kurt, & Hom, 1997). Impaired cognition includes memory loss, attention deficits, and decreased working memory capacity; confusion-ataxia refers to spatial disorientation, confusion, and balance disturbances; arthro-myo-neuropathy refers to muscle weakness and fatigue. These symptoms are differentiable from post-traumatic stress disorder (PTSD) and symptoms reported by veterans of other wars, suggesting that unknown factor(s) in the Gulf War caused the onset of these symptoms (Haley, Kurt, & Hom, 1997). Furthermore, these same symptoms are reported by veterans of the Gulf War from other countries at similar rates to U.S. soldiers, making GWI a global phenomenon (Ikin et al., 2004; Unwin et al., 1999). In response to emerging concerns, the Department of Defense and Veteran Affairs began to
1.1 PYRIDOSTIGMINE BROMIDE AND THE RESPONSE TO CHEMICAL WARFARE

One of the factors unique to the Gulf War which gained much attention was the direct threat of the use of G-series nerve gas agents by Saddam Hussein. In a famous broadcast by Saddam Hussein on April 2, 1990, he said: “By God, spare us your evil. Pick
up your goods and leave. We do not need an atomic bomb. We have the dual chemical. Let them take note of this. We have the dual chemical. It exists in Iraq” (Comprehensive Report of the Special Advisor to the DCI on Iraq’s WMD With Addendums, 2004). The dual chemical refers to a method for storing precursors for chemical warfare agents which become activated upon firing with munitions. This use of a binary form in chemical warfare enabled stockpiling large quantities G-series agents such as Sarin and Soman and posed a serious threat to the safety of the coalition of soldiers preparing for an invasion.

These G-series nerve gas agents, so-named due to their original synthesis by German scientists in World War II, are the most toxic chemical warfare agents known (Sidell & Borak, 1992). They produce toxicity by irreversibly inhibiting cholinesterase (ChE) enzymes: acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Of the two, AChE is the primary enzyme which metabolizes acetylcholine. Cholinesterases hydrolize the neurotransmitter acetylcholine, resulting in its rapid breakdown into acetate and choline. Tight enzymatic control of cholinergic function is critical for the central and peripheral nervous systems, and disruption of cholinergic transmission has profound consequences on functioning.

In the central nervous system, cholinergic projections are particularly dense in the neocortex and hippocampus where they modulate excitation of local circuitry to influence attention, arousal, and learning and memory (Fadel, 2011). While moderate hyperstimulation of central cholinergic function can disrupt these cognitive functions, cholinergic toxicity induced by G-series agents results in seizures and convulsions.
Irreversible inhibition of cholinesterase in the periphery has even more detrimental results. Acetylcholine is the critical neurotransmitter at neuromuscular junctions, secretory glands, and smooth muscles. As such, peripheral cholinergic hyperstimulation following irreversible cholinesterase inhibition results in vomiting, paralysis and ultimately death by asphyxiation (Pope, Karanth, & Liu, 2005; Tokuda, Kikuchi, Takahashi, & Stein, Gerald, 2006). Collectively, these symptoms following cholinergic overload are termed a “cholinergic crisis.”

Because G-series nerve gas agents are aerosolized and rapidly soluble through all tissues, including the blood-brain-barrier, the threat of the use of G-series agents was a primary concern for the U.S. government. In response, the U.S. government found hope in preclinical literature which suggested that prophylactic administration of PB was protective against death in the event of exposure to G-series chemicals when combined with post-exposure doses of atropine and oximes (Dirnhuber, French, Green, Leadbeater, & Stratton, 1979; Von Bredow, Adams, Groff, & Vlck, 1991). PB is a reversible cholinesterase (ChE) inhibitor which binds competitively to the same site on cholinesterase as Sarin and Soman, thus protecting a portion of cholinesterases from Sarin and Soman’s irreversible inhibition (Aquilonius & Hartvig, 1986). Because repeated administration of this dose of PB in primates did not result in any overt behavioral changes, it was concluded that PB could be safely administered chronically to all soldiers deployed in active combat-zones where nerve gas exposure was a potential threat (Keeler, Hurst, & Dunn, 1991; Philippens et al., 1998; Von Bredow et al., 1991). The
result was that an estimated quarter-million soldiers in high-risk combat zones were authorized to take PB on a daily basis (Golomb, 2008).

This authorization of the use of a pharmacological agent in humans in the absence of clinical trials or informed consent regarding that use was an historic event. The decision was based off the consideration that conductance of human clinical trials on lethality is inherently unethical. Eleven days prior to Operation Desert Storm, following requests from the Department of Defense, the Food and Drug Administration (FDA) created an interim rule to approve the use of PB on soldiers as an “investigational new drug” which enabled the experimental administration of PB in potentially lethal military situations in the absence of informed consent (Aebersold, 2012). PB remains formally approved as a prophylactic treatment for chemical warfare agents under what is colloquially termed “The Animal Rule” which was passed by the FDA in 2002 (Snoy, 2010). The Animal Rule states that if traditional human clinical efficacy studies are not feasible or ethical, a drug or biological product that targets lethal or severely toxic conditions may be approved for human use following sufficient animal testing and reasonable expectation that the drug should be both safe and effective in humans. PB was the first of nine drugs ever approved under this legislation (CDER Drug and Biologic Animal Rule Approvals, 2017).

The use of PB in soldiers was not expected to cause any adverse effects. This assessment was partially based off the fact that its use had already been approved for the treatment of myasthenia gravis, an autoimmune disorder which results in neuromuscular weakness. In addition, the dose necessary for protection of soldiers (30
mg, administered orally every 8 hours) is 10× lower than the dose prescribed for myasthenia gravis. These facts, in addition to large bodies of animal studies suggesting its efficacy as a nerve-gas pre-treatment and low-likelihood of crossing the blood-brain barrier due to a quaternary ammonium group (Ray et al., 1991) led confidence to the fact that its use should not negatively impact soldiers. Despite this confidence, mounting epidemiological evidence suggested that out of all of the various exposures to soldiers during the Gulf War, PB has the most consistently reported to have a positive correlation with the development of GWI with some evidence even supporting a positive dose-response relationship (Golomb, 2008; L Steele, Sastre, Gerkovitch, & Cook, 2012).

The mechanism by which PB may have caused some of these effects remains controversial. For example, PB was not anticipated to cause any cognitive side effects as it was thought that PB does not cross the blood-brain barrier due to the presence of a charged quaternary ammonium group (Ray et al., 1991). However, soldiers emerging from the Gulf War reported a plethora of cognitive symptoms such as deficits in attention, concentration, learning, visuospatial functioning, depression, anxiety, sensorimotor abilities, and working and long-term memory (Robert W. Haley et al., 1997; Lange et al., 2001). These clinical observations left researchers to determine how a peripherally acting drug could cause such drastic central nervous system effects. One theory which has garnered much support at preclinical levels, is that PB interacted with stress to produce unexpected and chronic physiological changes, contributing to the
development of GWI symptomology in both central and peripheral systems (Nisenbaum, Barrett, Reyes, & Reeves, 2000; L Steele et al., 2012).

1.2 ESTABLISHING A MODEL OF GULF WAR ILLNESS

Rodents have long been used to model human physiology and in health and disease because of their similarities in drug metabolism, cellular structure, and organization of nervous, cardiovascular, and immune systems. They provide an advantage over epidemiological studies due to their ability to control confounding variables and establish causal relationships between different variables. Over the last few decades, several rodent models of GWI have emerged to support this theory of an interaction between stress and PB on cognitive abilities. For example, acutely, PB exaggerates the behavioral response to acoustic startle in rats (Servatius et al., 2000). More chronic studies have demonstrated similar interactions between stress and cholinesterase inhibitors. Five minutes of daily restraint stress over the course of four weeks exacerbates the adverse effects of a cocktail of PB, permethrin, and DEET on memory formation and retrieval in the Morris Water Maze (Parihar, Hattiangady, Shuai, & Shetty, 2013) as well as performance in novel object and novel place recognition tasks (Hattiangady et al., 2014). These studies suggest that PB and stress cause deficits in learning and working memory. While these exacerbated cognitive deficits by combinations of stress and PB are well-replicated across preclinical literature, what remains unclear is 1) the specific role of PB in these cholinesterase-stress interactions, and 2) the mechanism by which stress and PB interact to induce these deficits.
Therefore, the current study used a model of repeated administration of PB by gavage in combination with repeated restraint stress. The following sections will address the validity in examining PB and modeling stress in a rodent model of GWI.

**Pyridostigmine Bromide in GWI**

In GWI, several different rodent models have emerged with varying advantages and limitations. Most notably, the majority of rodent models of GWI use a cocktail of chemicals to mimic the vast array of exposures soldiers may have experienced. The chemicals included in this cocktail vary across studies and include combinations of low levels of nerve gas agents (e.g. diisopropyl fluorophosphate, Sarin, Soman), PB, DEET, chlorpyrifos oxon, and permethrin (Abdel-Rahman, Abou-Donia, El-Masry, Shetty, & Abou-Donia, 2004; Abdel-Rahman, Shetty, & Abou-Donia, 2002; Abou-Donia et al., 2004; Locker et al., 2017). While this chemical cocktail can arguably mimic the range of conditions soldiers experienced during the Gulf War, the exact contribution PB has towards cognitive and physiological outcome measures remains unclear. As such, the use of PB in combat conditions remains controversial. The following studies will therefore selectively evaluate the contribution of PB in the absence of other chemical cocktails to the pathophysiology of GWI.

**Pharmacokinetics and Pharmacodynamics of Pyridostigmine Bromide**

PB was used during the Gulf War due to its effect on the enzymes which metabolize acetylcholine - AChE and BChE. PB is hydrolyzed by cholinesterases to reversibly inhibit these enzymes in both humans and rats (Jiří, Kuča, & Jun, 2004). By binding to the same target on cholinesterases as Sarin and Soman, PB can dramatically
increase survival rates in the event of exposure to these irreversible cholinesterase inhibitors. The efficacy of PB in this role has been established across a variety of species, including rats, mice, guinea pigs, rabbits, and primates (Gordon, Leadbeater, & Maidment, 1978; Maxwell, Brecht, Lenz, & O’Neill, 1988; Von Bredow et al., 1991). Human dosing regimens were ultimately based on primate studies which determined that inhibition of 40-50% of peripheral cholinesterase was sufficient to produce significantly greater rates of survival in the event of exposure to Sarin or Soman and that increasing the dosage beyond this point did not significantly increase survival (Von Bredow et al., 1991).

Pyridostigmine is a carbamate compound which is stabilized by the addition of a bromide salt, resulting in pyridostigmine bromide (PB). PB is water-soluble and distributes evenly across a variety of tissues with maximal bioavailability at approximately 2 hours after oral administration in humans and 30 minutes in rats (Barber, Bourne, Calvey, & Muir, 1975; Marino et al., 1998). The bioavailability of PB when administered orally is estimated to be as low as 12% (Marino et al., 1998), which is suspected to be due to either poor gastrointestinal absorption or rapid first-pass metabolism by the liver.

Metabolism of PB proceeds via first-order kinetics (Kornfeld, Samuels, Wolf, & Osserman, 1970). PB is metabolized by microsomal enzymes (e.g. cytochrome p450) in the liver into 3-hydroxy-N-methylpyridinium and excreted in the urine as both the metabolite and unaltered pyridostigmine (Yakatan & Tien, 1979). This results in a relatively short half-life of PB across species. As such, soldiers were recommended to
take 30 mg tablets PB orally every 8 hours for up to 21 days (Madsen, Hurst, MacIntosh, & Romano, 2003). Oral administration in humans can be mimicked successfully by gavage in rats. Studies have found that a daily dose of 1.3 mg/kg PB in rats reduces plasma cholinesterase activity by approximately 50% which matches the target levels in soldiers (Abou-Donia et al., 2004).

**Modeling Stress in GWI**

Soldiers in theater experienced a variety of deployment related stressors including uncertainty of tour length, crowded living, harsh climates, sleep deprivation, boredom, long work hours, isolation from home culture and comforts, threats from biological and chemical warfare, and fear of injury or death, in addition to post-deployment stressors associated with difficulties readjusting to civilian life (Gifford, Ursano, Stuart, & Engel, 2006; Nisenbaum et al., 2000). The stressors from deployment are hypothesized to be significant contributing factors to the development of GWI symptoms in a way that is unique from stress-contributions to the development of other war-related disorders such as PTSD (Robert W. Haley, 1997; Nisenbaum et al., 2000). However, variations in soldiers’ experiences, poor reliability of self-report methods, and poor record-keeping during war-time makes disentangling the unique contributing effects of stress with other factors soldiers experienced during deployment difficult. As such, to understand the casual roles and mechanisms by which stress contributes to the pathophysiology of GWI, research has turned to preclinical models.

Although no model can recapitulate the stress of deployment into active combat zones, rodent models of GWI have used a variety of stressors in their paradigms. For
example, some studies have mimicked stress by adding corticosterone into the animals’ drinking water (Locker et al., 2017). Corticosterone is a hormone released by the adrenal glands following stress-induced activation of the hypothalamic-pituitary adrenal (HPA) axis, and as such, is an important hormone in the stress-response. However, stress also activates the sympathetic nervous system and instigates cognitive processing of the stressful event. As such, corticosterone alone cannot accurately mimic the entirety of the stress response. Other models have subjected rodents directly to stressful stimuli. These stimuli vary from forced swim to restraint, to cold stress, to heat stress, to avoidance-escape foot-shock stress (Grauer, Alkalai, Kapon, Cohen, & Raveh, 2000; Kant et al., 2001; Lallement et al., 1998; Servatius et al., 2000; Sinton, Fitch, Petty, & Haley, 2000). The effects of stress on physiology are heterogeneous with results contingent upon both the prior history, the type of stressful stimuli, and the conditions under which stressful stimuli are presented, and the brain region or system being examined (M. A. Wilson, Grillo, Fadel, & Reagan, 2015). As such, there could be variability between different stress models in rodent models of GWI.

The following studies used six hours a day of repeated restraint stress for the course of ten days. This stress paradigm was selected because 1) of all these models of stress, psychological stress due to repeated restraint has the most robust and consistent literature on behavioral and physiological effects of this type of stress (S. Cook & Wellman, 2004; Macht & Reagan, 2017; B.S. McEwen, 2001; Jason J. Radley et al., 2008; Watanabe, Gould, & McEwen, 1992; Wellman, 2001), and 2) the morphological and behavioral effects of repeated restraint for this duration are reversible following
removal of the stressor or pharmacological intervention (Bloss et al., 2010; Conrad et al., 1999; Luine et al., 1996, 1994; Magarinos and McEwen, 1995; Magarinos et al., 1999; Radley et al., 2005). The reliability of the effects of restraint stress on outcome measures make it an ideal stressor by which to assess the synergistic effects of PB and stress over time. This way, synergistic effects between PB and stress will not be attributable to inconsistent variations in the stress paradigm on outcome measures.

The nervous system is extremely sensitive to the effects of restraint stress, and while acute stress can promote neuroplasticity and facilitate cognitive performance, chronic stress has been implicated in a host of cognitive disorders including anxiety, major depression, Alzheimer’s, and PTSD. Therefore, it is possible that the chronic stress from deployment contributed to the pathophysiology of some of the cognitive symptoms evidenced in GWI. For example, the prefrontal cortex (PFC) and hippocampus are sensitive to the effects of chronic stress and produce reproducible effects on behavior in addition to morphology and spine density of glutamatergic. These brain regions are also critical for a variety of cognitive functions, including attentional processing, working memory, and long-term memory – all of which are impaired in GWI. However, the morphological and behavioral effects of repeated restraint are reversible following removal of the stressor (Bloss et al., 2010; Conrad et al., 1999; Luine et al., 1996, 1994; J.J. Radley et al., 2005). This suggests that stress alone is unlikely to have caused the long term and progressive symptoms in GWI.

**Interactions between Pyridostigmine Bromide and Stress on the Blood-Brain Barrier**

Stress is not thought to impact the metabolism or distribution of PB in peripheral
tissues (Francesconi, Hubbard, & Mager, 1983). However, an early hypothesis that garnered much interest was that stress affects the integrity of the blood-brain barrier, creating a “leaking effect” which allows PB to cross into the central nervous system and directly alter central cholinesterase activity and consequently, cognitive function. In 1996, Friedman et al. determined that mice subjected to forced swim stress increased the sensitivity of brain AChE messenger ribonucleic acid (mRNA) to PB, such that less than 1% of the original dose was required to alter brain AChE activity levels under these conditions. This was paralleled by increased excitability of synaptic circuits in the CA1 region of the hippocampus relative to controls. Other studies suggested that twenty-eight days of a chemical cocktail of PB, DEET, and permethrin in combination with five minutes per day of restraint stress exhibited significant increases in blood-brain barrier permeability in a brain-region specific manner with the cingulate cortex, dentate gyrus, thalamus and hypothalamus being particularly sensitive, relative to water-treated non-stressed controls (Abdel-Rahman et al., 2002). Using this same “toxic soup” paradigm, Abdel-Rahman et al. (2004) found that the combination of mild restraint stress and these toxic chemicals altered brain AChE levels in the midbrain, brainstem, and cerebellum. Unfortunately, other studies have failed to replicate these early findings of stress-induced blood-brain barrier permeability to PB (Amourette et al., 2009; Grauer et al., 2000; Kant et al., 2001; Song, Tian, Bressler, Pruett, & Pope, 2002; Tian, Song, Bressler, Pruett, & Pope, 2002). As such, it remains unclear whether stress disrupts the blood-brain barrier to allow PB to directly impact central neurotransmitter function.
The Immune System as the Crossroads between Pyridostigmine Bromide and Stress

Even if PB does not cross the blood-brain barrier, clinical (Golier, Schmeidler, Legge, & Yehuda, 2007; Nisenbaum et al., 2000; L Steele et al., 2012; Sullivan et al., 2003) and preclinical (Amourette et al., 2009; Hattiangady et al., 2014) studies have supported indirect synergistic effects of PB and stress on cognitive function, suggesting that PB may interact with stress through peripheral systems to alter central neurological function. Exactly which systems PB and stress interact on and how this feeds back to impact the brain and behavior is currently unknown; hence, characterizing the interactions between stress and PB on peripheral systems and determining how this correlates with behavior is an important precursor to determining the effects of stress and PB on the central nervous system.

The following set of studies were based on the hypothesis that the immune system provides the mechanistic link by which peripheral effects of PB interact with chronic stress to produce changes in neural function in the brain. This section will discuss four primary pieces of evidence which led to this hypothesis: 1) stress and PB both independently impact immune function, 2) veterans with GWI exhibit changes in the HPA axis and immunological markers that are consistent with those seen in preclinical chronic stress paradigms, 3) preclinical models using PB have found both central and peripheral immune changes, and 4) the cholinergic anti-inflammatory pathway may provide the immunological link by which PB could interact to impact central nervous system function without crossing the blood-brain barrier.
One classic study linking stress to the immune system emerged in 1987 with the finding that medical students exhibited immunosuppression and increased sickness behaviors during their examination period (Glaser et al., 1987). In the decades since, multiple studies have worked to elucidate the bi-directional interactions between stress and the immune system, resulting in a multifold increase in neuro-immune publications over the last two decades (Quan & Banks, 2007). There are two major pathways by which stress directly affects immune function: the HPA axis and the catecholamine-driven sympathetic nervous system (Besedovsky, Rey, Sorkin, Da Prada, & Keller, 1979). Both the HPA axis and the sympathetic nervous system are activated during the stress response, and chronic activation of these systems results in chronic production of both glucocorticoids and catecholamines (Padget & Glaser, 2003). A variety of immune cells express receptors for both glucocorticoids and catecholamines, and the sympathetic nervous system directly innervates lymph nodes, suggesting that the HPA axis and sympathetic nervous system have multiple regulatory points on the immune response (Besedovsky et al., 1979; del Rey, Besedovsky, Sorkin, da Prada, & Arrenbrecht, 1981; Gametchu, Watson, & Wu, 1993).

Similarly, the immune system also influences neural function in the CNS, highlighting that this stress-immune dynamic is bidirectional (Miller, Galpern, Dunlap, Dinarello, & Turner, 1991; Plata-Salamán & Ffrench-Mullen, 1992). The central effects of cytokines on neurotransmitter systems were first directly recorded in amino acid neurotransmitter systems, GABA and glutamate (Miller et al., 1991; Plata-Salamán & Ffrench-Mullen, 1992). While these cytokine-mediated effects on amino acid
neurotransmitter systems were originally thought to be driven by cytokines released by immunocompetent cells in the central nervous system – microglia and astrocytes – research suggests that peripheral cytokines can may also impact central neurotransmitter system function by crossing the blood-brain barrier though a saturable transport system (Banks, Kastin, & Broadwell, 1995; Habif, Lipton, & Cantell, 1975). This suggests that peripheral and central cytokine signaling cues can both potentially influence neurotransmitter function in the central nervous system.

A third and critical point of interaction between the periphery and central nervous system regulation of the immune response resides in the vagal nerve anti-inflammatory reflex (V. Pavlov, Wang, Czura, Friedman, & Tracey, 2003). This reflex was originally discovered after it was reported that stimulation of the vagus (“wandering”) nerve inhibits the macrophage production of the cytokine TNF-α following administration the endotoxin lipopolysaccharide (LPS) (Borovikova et al., 2000). The vagus nerve is a critical point of communication between the central and peripheral nervous systems. Vagal efferents directly regulate the peripheral immune response while vagal afferents relay information to the central nervous system on the state of inflammation in the periphery (Rosas-Ballina & Tracey, 2009). In GWI, the effects of chronic stress may be compounded by PB interactions at the cholinergic anti-inflammatory pathway of the vagus nerve (Bauer, Perks, Lightman, & Shanks, 2001). Vagus nerve efferents release acetylcholine which binds to nicotinic α7 receptors on peripheral macrophages to decrease the production of a variety of pro-inflammatory cytokines, particularly TNF-α (Tracey, 2002). Since PB inhibits ChE, chronic
administration of PB may have altered the immune system by increasing the effects of acetylcholine at nicotinic α7 receptors on both macrophages and the adrenal glands, thereby altering both peripheral immune signals and feedback cues to the central nervous system via vagal afferents.

Collectively, these studies suggest that the interplay between the central and peripheral nervous system, immune system, and endocrine system is complex with the function of each system depending on input from the others. This has important implications for clinical pathologies as differences in the dysregulation of each neuro-immune and endocrine system can be associated with different clinical states.

**Endocrine and Immune Dysfunction in GWI**

Veterans with GWI exhibit alterations in both endocrine and immune systems that are distinct from other war-related (PTSD) and non-war related (chronic fatigue syndrome) disorders, suggesting that despite overlapping symptomology, GWI symptoms are driven by unique mechanisms. For example, Golier et al., (2007) determined that GWI but not PTSD was associated with low levels of adrenocorticotropic hormone (ACTH) and consequently a higher cortisol: ACTH ratio. This unique endocrine profile in veterans with GWI was also positively correlated with the presentation of both PB treatment and cognitive symptoms. In addition, Whistler et al., (2009) determined that the cortisol response to an exercise challenge was dampened in individuals with GWI relative to the control group - sedentary veterans from the National Guard. Collectively, these studies suggest that GWI is characterized at least in part by dysregulation of the HPA axis.
GWI is also repeatedly associated with shifts in the immune system, although specific cytokine shifts are often inconsistent between studies. For example, unlike in chronic fatigue syndrome, fatigue symptoms in GWI have not been associated with increased IL-1β, a pro-inflammatory cytokine classically associated with fatigue in sickness (Parkitny, Middleton, Baker, & Younger, 2015). Veterans with GWI do exhibit decreases in natural killer cell number and sensitivity to cytotoxicity in addition to elevations in the cytokine interleukin (IL)-5 compared to sedentary control veterans during an exercise challenge (Whistler et al., 2009). Preclinical studies have confirmed that PB decreases natural killer cell activity and T-cell dependent antibody responsiveness, suggesting that these cytokine shifts in clinical populations may have been driven by PB (Peden-Adams et al., 2004). In contrast, Broderick et al. (2011) found a heightened IL-2, IL-10, IFN-γ, and TNF-α response to an exercise challenge in GWI relative to healthy controls but not non-veteran subjects with chronic fatigue syndrome. While part of these differences may be attributable to differences in control populations or other experimental parameters, other evidence suggests that the immune system in veterans with GWI is extremely variable from day to day relative to healthy veterans who served in the Gulf War (Parkitny et al., 2015). As such, while shifts in immune parameters in GWI are undoubtedly a critical component of this illness, exactly what those shifts are, under what experimental conditions, and how those shifts contribute to the presentation of symptoms in GWI remain to be elucidated. Animal models of GWI which examine shifts in endocrine and immune systems under basal conditions and in response to stressful stimuli could help clarify these findings.
These alterations to the peripheral immune system by both stress and PB could have altered feedback to the central nervous system, potentially resulting in synergistic effects of stress and PB on both immune function and neural networks. In support of this idea, a preclinical model of GWI using a combination of stress and variety of Gulf War chemicals (i.e. DEET, permethrin, PB) suggested that the combination of Gulf War chemicals and stress resulted in greater density of microglia and astrocyte hypertrophy in the hippocampus than Gulf War chemicals alone. This provides evidence that stress and Gulf War chemicals exhibit synergistic effects on the immune system that are evident in the central nervous system (Parihar et al., 2013). While this study provides insight into possible central nervous system effects of Gulf War conditions, the combination of multiple chemicals makes isolation of the effects of PB impossible to untangle.

Based on these studies, the following experiments examined interactions between stress and PB on the immune system and on neural function. In light of the clinical studies suggesting that immune effects in GWI are exacerbated when challenged, the following experiments used a preclinical model of GWI to examine physiological and behavioral effects under both basal conditions and following a challenge (e.g. LPS, restraint stress).

**Overarching Experimental Hypotheses**

The following research aims to assess the mechanism by which PB and stress could interact to cause GWI symptomology in a rodent model. The overarching general hypotheses for the following experiments are that 1) chronic stress interacts with PB to
adversely impact homeostasis of peripheral endocrine, cholinergic, and immune systems in a progressive manner, and 2) chronic stress interacts with PB to adversely impact central neurotransmitter responses to future stressful stimuli. To address these hypotheses, the following work is divided into three separate experiments which are addressed in the following chapters. The first set of experiments (Chapter 2) test the progression of peripheral and behavioral consequences to repeated psychological (restraint) stress and PB. The second and third sets of experiments (Chapters 3 and 4, respectively) test neurochemical changes to an LPS and acute restraint stress challenge using the GWI paradigm. Specific hypotheses for each experiment will be addressed in each subsequent chapter.

1.3 EXPERIMENTAL DESIGN

The following experimental design encompasses the general animal model of GWI which provided the basis for all subsequent studies. More specific methods, relating to each individual set of experiments, will be provided in the appropriate corresponding chapters.

Animal Housing

All studies used young adult male Sprague Dawley rats which were individually housed at the University of South Carolina School of Medicine’s animal facility in a temperature-controlled facility (22°C) with 12/12 h light-dark cycle with lights on at 7:00 AM and ad libitum access to food and water. Although both males and females have reported GWI, this study used males since the majority of soldiers and hence incidences
of GWI were in men (Nettleman, 2015). For all studies, rats habituated to the animal facility for one week prior to the start of the experiments. After a week of acclimation to the animal facility, each rat was randomly assigned to a treatment condition for subsequent studies (Figure 1.1). Experiments ranged from approximately one to three months in duration. All housing procedures for these experiments are in accord to all guidelines and regulations by the University of South Carolina’s Animal Care and Use Committee.

**GWI Paradigm**

Because preclinical studies determined that PB is only effective when administered chronically prior to the onset of nerve gas agent exposure (Gordon et al., 1978; Von Bredow et al., 1991), soldiers were authorized to take PB before deployment if they were being sent to high risk zones (Madsen et al., 2003). Animal models assessing the contribution of PB to GWI must therefore recapitulate this repeated dosing paradigm with PB treatment preceding stress. The current study therefore used a paradigm of 4 days of 1.3 mg/kg/day PB treatment by oral gavage followed by a combination of PB with 10 days of restraint stress. Rats were weighed every other day at 9:00 AM to ensure accurate dosing. PB was prepared fresh daily in sterile water with a final concentration of 0.13 mg/mL. Rats were gavaged daily with either water or PB between 9:30-10:00 AM.

On day 5 of treatment, rats were randomly assigned to either repeated restraint stress (stress) or non-stressed conditions (NSC). Stressed rats were housed separately from non-stressed controls and placed in wire mesh restrainers from 10:00 AM to 4:00
PM on treatment days 5-14. Restraint lasted six hours as this duration increases hippocampal vulnerability in a manner not observed with shorter daily stress durations (McLaughlin, Gomez, Baran, & Conrad, 2007; M. A. Wilson et al., 2015). Restrainers had painted rubberized edges for protection. Once the rats were placed in the restrainers, the ends were secured with clips to keep the rat in from escaping while permitting small movements (as described in Reznikov et al., (2008) and Reagan et al., (2004)). Tail bleeds were performed on day 14 of treatment for all rats, approximately 30 minutes after the onset of restraint stress. At the end of treatment, rats were assigned to either early or delayed evaluation time points (Figure 1.2). As such, the rats comprising the early or delayed time point are separate cohorts of animals. For euthanasia, rats were lightly anesthetized with isoflurane, after which trunk blood was collected for all experiments at early (day 24) and delayed (day 104) time points for further analyses.
Figure 1.1. Experimental Groups. This experiment had a 2×2 design with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (NSC, stressed). PB is prepared fresh daily in sterile water with a final concentration of 0.13 mg/mL.
Figure 1.2 Timeline for Gulf War Illness Paradigm. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control, repeated restraint stress). PB treatment preceded the beginning of the stress paradigm by 4 days. Between 9:30-10:00 AM, rats were gavaged with either vehicle or PB. On the 5th day of treatment, rats undergoing restraint stress were moved to a separate room for restraint stress. Rats were placed in wire mesh restrainers at approximately 10:00 AM, just after gavage. Restraint stress lasted 6 hours per day for a total of 10 days. The entirety of the treatment paradigm lasted 14 days.
CHAPTER 2

EXPERIMENT ONE: GULF WAR ILLNESS FROM PHYSIOLOGY TO BEHAVIOR

Although GWI is considered unique to the Gulf War, symptomology of GWI parallels symptoms of other conditions in civilian populations, including chronic fatigue syndrome, major depression, PTSD, and fibromyalgia. However, epidemiological studies have consistently demonstrated that the underlying physiology driving these symptoms is unique in GWI. For example, although both chronic fatigue syndrome and GWI exhibit altered immune functions, the cytokine profiles in both populations are distinct (G. J. Johnson, Slater, Leis, Rector, & Bach, 2016; Khaiboullina et al., 2015; Parkitny et al., 2015; Smylie et al., 2013).

Endocrine profiles are also altered in veterans with GWI. Veterans with GWI exhibit exaggerated cortisol suppression in response to a low dose of dexamethasone relative to non-deployed veterans when PTSD, smoking, weight, and depression are taken into account (Golier, Schmeidler, Legge, & Yehuda, 2006). Veterans with GWI also exhibit a significant elevation in their cortisol to ACTH ratios, further emphasizing dysregulation of endocrine systems in this population (Golier et al., 2007). Predictive computational models of GWI have suggested that 1) GWI in males is characterized by disruption of homeostatic states which consist of hypercortisolism and a shift towards a pro-inflammatory immune profile, and 2) persistence of clinical symptoms across the
decades is perpetuated by continued disruption of these homeostatic systems (Craddock et al., 2014). One of the primary hypotheses of the computational model of GWI is that under normal physiological conditions where a challenge is presented (e.g. stress, drug exposure, etc.), systems respond to the event and then return to prior basal levels. However, when a disruption is of significant duration and magnitude, the system assumes a new resting state. In some cases, this new resting state can be maladaptive. In the case of GWI, a shift in the resting state of physiological parameters may underlie a variety of clinical symptoms evidenced in this population.

**Hypotheses**

The aim of the current study was to assess whether the pathogenesis of PB and stress-induced changes in physiological markers – immune, endocrine, cholinergic – paralleled changes in behavior. Specifically, on the last day of treatment, we hypothesized that cholinesterase activity would be reduced in PB treated rats, that corticosterone would be increased in rats undergoing restraint stress, and that PB would attenuate the inflammatory response to stress in PB-stressed rats. In addition, because veterans with GWI exhibit progressive symptom severity, we hypothesized that the combined effects of PB and stress on these parameters would become exacerbated over time. We therefore examined the effect of PB and stress on each of these measures at three separate time points: on the last day of treatment, ten days following the cessation of treatment, and three months following the cessation of treatment. PB and stress effects on peripheral cytokines were hypothesized to be the most sensitive to the
interactive effects of PB and stress due to the abundant evidence of immune
dysfunction in veterans with GWI. Thus, this study expands upon data from
epidemiological and computational models by providing causal evidence for the role of
PB and stress in the pathophysiology of GWI. In addition, we examined the effects of PB
and stress on fear conditioning to determine whether shifts in these physiological
parameters paralleled shifts in cognitive function. Because veterans with GWI exhibit
significant memory deficits in a manner that is independent from PTSD symptom
presentation, we hypothesized that fear conditioning will be impaired in rats which
received a combination of PB and repeated restraint stress and that these deficits will
become exacerbated over time.

2.1 METHODS AND MATERIALS

The first experiment examined the progression of GWI from a variety of
peripheral measures: cholinesterase activity, corticosterone, and cytokines. These
measures were assessed at three different time points: day 14 of treatment (acute),
one-week post-treatment (early), and three months post treatment (delayed) (Figure
2.1). Early and delayed time points consisted of separate cohorts of animals with N =
106 for the entire study.

Plasma Collection

Tail bleeds from the acute time point were taken 30 minutes following
administration of drug treatment and onset of stress. Trunk blood was also collected on
ice in EDTA-treated tubes during euthanasia for both the early (Day 24) and delayed
Figure 2.1 Timeline for Experiment One. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control, repeated restraint stress). Thirty minutes following gavage on day 14, all rats underwent a tail bleed to assess plasma levels of AChE activity, corticosterone, and levels of 12 different cytokines. Following, rats were further subdivided into either early or delayed conditions. Rats in the early condition underwent conditioned freezing on Days 20-21 and were euthanized followed by perfusion on Day 24 (n = 13 per group). Rats in the delayed condition went through conditioned freezing on days 100-101 and euthanized followed by perfusion on day 104 (n = 13-14 per group). Plasma from the trunk blood was then assessed for each of these peripheral measures at these time points.
(Day 104) time points. Importantly, all plasma measures from early and delayed time points were conducted on animals which had undergone fear conditioning. However, a substantial recovery period was allocated between fear conditioning and euthanasia. For euthanasia, 6-7 rats per group which had gone through fear conditioning were lightly anesthetized and then transcardially perfused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The remaining rats in each group were rapidly decapitated for a separate study. All blood was collected on ice and then spun down for 15 minutes at 13,000 x g. Plasma supernatant was removed from each vial and frozen at -80°C.

**Contextually-Conditioned Freezing**

The conditioned freezing paradigm was performed as described previously (Sharko, Fadel, Kaigler, & Wilson, 2017). For rats in the early cohort, behavioral testing was performed from days 17-20. For rats in the separate, delayed cohort, behavioral testing was performed from days 97-100. During the light phase on each test day, rats were transported to the behavioral testing suite where they were placed in a 46 x 24 x 22 cm acoustically isolated shock box. For the acquisition phase, rats were exposed to the testing box for 3 min to assess unconditioned freezing in the novel context. Rats were then given three 10 sec tones (2 KHz, 80 dB) that co-terminated with a 1 second, 1 mA shock at 1 minute inter-stimulus intervals. Twenty-four hours following acquisition of the fear response, rats were returned to the testing box in the absence of tones or shocks for a total of 8 minutes. The chamber was wiped clean with 5% ammonium hydroxide in between each rat’s test period during both test days. All tests were
recorded on video tapes using FreezeScan (Clever Systems, Inc). Freezing was defined as the absence of movement excluding respiration. For both acquisition and contextual freezing, percent freezing behavior was automatically assessed in 1 min bins using FreezeScan software. Accuracy of automated software was verified by a blind observer.

**Cholinesterase Assay**

A cholinesterase activity assay measuring AChE and BChE activity was performed on plasma from tail bleeds (day 14) and on trunk blood at early (day 24) and delayed (day 104) time points using the Abcam acetylcholinesterase assay kit (#ab138871). All components of the acetylcholinesterase assay kit were thawed to room temperature prior to use. Standards were 300, 100, 30, 10, 3, 1, and 0 mU/mL. 50 µL of standards and samples were added to the 96-well plate in duplicates. Then 50 µL of acetylthiocholine reaction mixture was added to each well, following which the plate incubated at room temperature for 20 minutes, protected from light. The plate was read using a microplate reader at 410 nm absorbance settings. Cholinesterase activity from samples was interpolated from the linear standard curve. Due to assay to assay variability, data at each time point were expressed as a percentage of the mean of their respective vehicle-NSC groups in order to allow clear interpretations of the data, where the mean cholinesterase activity across all assays for vehicle-NSC rats was 43.03 ± 2.30 mU/mL.

**Corticosterone Assay**

Plasma corticosterone was assessed using a corticosterone ELISA kit from Enzo-Life Sciences (#ADI-900-097) on plasma from tail bleeds (day 14) and on trunk blood at early (day 24) and delayed (day 104) time points. All components of the assay kit were
brought to room temperature prior to use. Samples were diluted 1:40 with steroid displacement reagent and kept on ice. Standards were 20,000, 4,000, 800, 160, and 32 pg/mL corticosterone and prepared using standard diluent. 100 µL of either the samples or standards were then added in duplicate to each well. Standard diluent was pipetted into the non-standard blank (NSB) and B₀ wells. Subsequently, 50 µL of alkaline phosphatase conjugated with corticosterone was added to each well except total activity and blank wells, after which 50 µL of polyclonal sheep anti-corticosterone antibody was pipetted into each well except total activity, NSB and B₀ wells. The plate was then sealed and placed on an orbital shaker for 2 hours at 500 rpm. Plate contents were then washed 3X3 times with wash buffer. 5 µL of alkaline phosphatase conjugated with corticosterone was added to the total activity wells, after which 200 µL pNpp substrate solution was added to every well. The plate was then incubated a second time for 1 hour without shaking. 50 µL of stop solution was added to each well to stop the reaction, after which the plate was immediately read using a microplate reader at 590 nm absorbance. Corticosterone activity was interpolated from standards using a 4-parameter logistic curve.

**Cytokine Assay**

A rat cytokine assay kit from Bio-Plex (#171k1002M) was used to quantify immune molecules in plasma from tail bleeds (day 14) and trunk blood at early (day 24) and delayed (day 104) time points. The Bio-Plex assay uses premixed coupled magnetic beads and detection antibodies to detect 12 different rat cytokines. Solutions for the assay were brought to room temperature 30 minutes prior to initiation of assay
procedures. Eight four-fold dilution standards were prepared using the provided diluent and reconstituted standard, and samples were thawed and diluted 1:4 with diluent. 288 µL of 20x beads were vortexed for 30 seconds and then diluted with 5472 µL of assay buffer for a total volume of 5760 µL. 50 µL of the diluted beads were then added to be bottom of a 96-well plate, followed by 50 µL of standards and samples. The assay plate incubated on an orbital shaker plate for 1 hour at room temperature. After 3 washes with wash buffer, 25 µL of detection antibody was added to each well, followed by a 30-minute incubation at room temperature on an orbital shaker. The detection antibody was washed from the plate with 3 washes using wash buffer. 50 µL of SA-PE was added to each well and the plate was incubated on an orbital shaker at room temperature for 10 minutes. After the incubation, the plate was washed 3 times with wash buffer and then the beads were re-suspended in 125 µL assay buffer. After 30 seconds, the plate was read on a Luminex Bio-Plex Multiplex plate reader using high photomultiplier voltage. Results were interpreted from a logarithmic standard curve. Due to assay to assay variability, data at each time point were expressed as a percentage of the mean of their respective vehicle-NSC groups in order to allow clear interpretations of the data.

**Statistical Analyses**

All data unless otherwise specified was analyzed using a 2 X 2 between-groups analysis of variance (ANOVA) with significance set at α = 0.05. The between groups factors were drug treatment (i.e. vehicle, PB) and stress (i.e. non-stressed control, repeated restraint stress). Early and delayed groups were analyzed separately as determined *a priori*. Sample size was determined by power analysis for behavioral
studies and suggested that 13 animals per group were sufficient to achieve statistical significance given an effect size of $f = 0.6$ and $\alpha = 0.05$. As behavioral measures exhibit the most variability, N-sizes for plasma measures were the same as those of behavior.

Cytokines were analyzed as a multivariate ANOVA with univariate ANOVA as a post-hoc follow-up when appropriate. Contextually conditioned freezing behavior was analyzed using a repeated measures ANOVA with the addition of the factor time in conjunction to the factors of treatment and stress. Following significant interactions, simple effects tests were performed with Bonferroni post-hoc corrections for family-wise error.

2.2 RESULTS

*Contextually-Conditioned Freezing*

Contextually-conditioned freezing was first assessed at the early time point, approximately one-week after the cessation of drug and stress exposure. During the first day (acquisition) of the contextually-conditioned freezing paradigm, freezing increased with the three tone-shock pairings, as expected, $F(5, 240)=490.0, p < 0.001$. This suggests that all rats appropriately acquisitioned the fear response. Twenty-four hours following acquisition of the fear response, rats were returned to the testing box in the absence of tones or shocks for a total of eight minutes. Contextual freezing was impacted by time, $F(7, 336)=25.79, p < 0.001$. Freezing behavior increased incrementally over the first three minutes, ($p < 0.001$ and 0.001, respectively). There was also a main effect of stress such that stress decreased context-associated freezing, $F(1, 48)=7.34, p =$
Follow-up analyses determined that this effect is driven by the group which received PB in addition to stress where PB-stress rats froze less than NSC counterparts during the first 4 time points ($p = 0.004, 0.004, 0.004, \text{and} 0.036$, respectively). This suggests that while there is a main effect of stress on conditioned freezing, this deficit in context-associated fear conditioning is primarily driven by rats which were stressed and received PB (Figure 2.2).

At the delayed time point, freezing increased with the three tone-shock pairings, $F(5, 240)=641.7, p < 0.001$ (Figure 2.3). These data indicate that all rats appropriately acquired the fear-response. Twenty-four hours later, rats were returned to the testing box in the absence of tones or shocks for a total of eight minutes. Results indicate that time significantly affected contextual freezing within all groups, $F(7, 366)=15.42, p < 0.001$. Specifically, freezing increased incrementally from minutes 1-3, ($p < 0.001$ and 0.001, respectively). There was also a trend for a stress × time interaction, $F(7, 350)=1.81, p = 0.08$, and a stress × drug interaction, $F(1, 50)=3.82, p=0.056$. Specifically, prior history of stress increased freezing during the first three minutes in vehicle-treated but not PB-treated rats relative to vehicle-NSC rats ($p = 0.001, 0.007, \text{and} 0.049$, respectively). These results suggest that a prior history of restraint stress in the absence of PB treatment sensitizes the contextual conditioned fear response.

**Cholinesterase Assay**

PB decreased plasma cholinesterase activity by approximately 50% relative to vehicle-treated controls on the last day of drug treatment (day 14), $F(1, 23)=69.40, p < 0.001$ (Figure 2.4). This reduction in cholinesterase activity following PB administration
Figure 2.2. Conditioned Freezing One Week Post-Treatment. Panel A. There was no effect of any factor on acquisition of freezing behavior in response to three shocks. This suggests that neither stress nor PB impacts learning of fear memory at this time point. Panel B. Stressed rats froze less in the context where the shocks had occurred relative to NSC rats. Further analysis indicates that this effect is driven by the condition in which rats received PB and stress, suggesting that PB may exacerbate the effects of stress on consolidation or recall of fear memories. Results are expressed as group mean percent freezing ± SEM at each time point.

A: *: significant effect of time on freezing behavior relative to previous time point, $p < 0.05$; ↓: 1 second shock paired with a 10 second tone
B: *: significant effect of stress in PB-treated rats, $p < 0.05$. 
Figure 2.3. Conditioned Freezing Three Months Post-Treatment. Panel A. There was no effect of any factor on acquisition of freezing behavior in response to three shocks. This suggests that neither stress nor PB impacts learning of fear memory at this time point. Panel B. Rats with a prior stress history without PB treatment froze more in the context where the shocks occurred, suggesting that a prior stress history sensitizes the fear response. Results are expressed as group mean percent freezing ± SEM at each time point.

A: *: significant effect of time on freezing behavior relative to previous time point, $p < 0.05$; ↓: 1 second shock paired with a 10 second tone

B: *: significant effect of stress in vehicle-treated rats, $p < 0.05$. 
aligns with the targeted cholinesterase activity in soldiers whom were administered PB, suggesting that the dose of PB administered here accurately reflects that received by the target population. At the time of euthanasia for the early cohort (10 days following the cessation of treatment), rats treated with PB continued to exhibit a significant, 15% decrease in plasma cholinesterase activity relative to vehicle-treated controls, \( F(1, 32) = 5.82, p = 0.02 \). Conversely, at the delayed time point, approximately 3 months following the cessation of PB treatment, there was a significant effect of PB on cholinesterase activity, \( F(1, 32) = 4.88, p = 0.04 \). Specifically, PB-stressed rats exhibited a 30% increase in plasma cholinesterase activity relative to vehicle-treated controls, \( p = 0.031 \). These data suggest that stress and PB interact over time to influence peripheral levels of cholinesterase activity.

**Corticosterone Assay**

Corticosterone was significantly elevated 30 minutes into restraint on the last day of treatment (day 14) in both vehicle and PB treated rats undergoing restraint stress relative to non-stressed controls, \( F(1, 33) = 12.38, p = 0.001 \) (Figure 2.5). Interestingly, corticosterone remained above baseline in rats exposed to restraint stress regardless of drug treatment ten days following the cessation of the stress paradigm (early group), \( F(1, 30) = 16.21, p < 0.001 \). Three months following the cessation of treatment, there was an interaction between stress and drug treatment on plasma corticosterone levels, \( F(1, 29) = 7.165, p = 0.01 \). Corticosterone was significantly elevated in rats with prior
Figure 2.4. Plasma Cholinesterase Activity. **Panel A.** PB decreased plasma acetylcholinesterase activity by approximately 50%, which is proportional to pharmacological target values of soldiers. This plasma was taken on the last day of PB treatment. **Panel B.** PB treatment continued to decrease plasma acetylcholinesterase activity by approximately 15% ten days following the cessation of treatment. This suggests that while PB has a persistent effect, acetylcholinesterase activity following PB-treatment has begun to normalize. **Panel C.** Three months following the cessation of treatment, acetylcholinesterase activity is elevated selectively in rats which were exposed to restraint stress in conjunction with PB. This suggests that PB and stress interact to produce long-term disruption in the metabolism of peripheral acetylcholine. All data are expressed as a percentage of vehicle-NSC rats. Values are expressed as mean±SEM at each time point.

*: significant difference between designated groups, \( p < 0.05 \).
Figure 2.5. Plasma Corticosterone. **Panel A.** During the first thirty minutes of restraint stress on the last day of the restraint stress paradigm, stressed rats have significantly higher levels of corticosterone than non-stressed rats. **Panel B.** Rats with a prior stress history have elevated corticosterone. This is ten days after the end of the restraint stress paradigm and 72 hours following context-retrieval of conditioned freezing. **Panel C.** Three months following the cessation of treatment, corticosterone is elevated selectively in rats which were exposed to restraint stress in conjunction with PB relative to non-stressed counterparts. This is also 72 hours following context-retrieval of conditioned freezing. Collectively, these data suggest that the effects of PB and stress on corticosterone change over time. Values are expressed as mean± SEM at each time point.

*: significant difference between designated groups, $p < 0.05$. 
history of PB treatment in conjunction to chronic restraint stress relative to PB-NSC rats, 
$p = 0.029$. While prior exposure to the fear conditioning paradigm could be partially
attributable to these increases in corticosterone, these data suggest that stress and PB
interact to influence plasma corticosterone differently over time, even in the absence of
continuation of chronic restraint stress and drug treatment.

**Cytokine Assay**

On day 14, approximately 30 minutes into the restraint stress session, there was
a significant interaction between PB and stress on levels of **IL-1α** [$F(1, 30) = 5.51, p =
0.03$], **IL-1β** [$F(1, 30) = 6.92, p = 0.01$], **IL-2** [$F(1, 30) = 4.37, p = 0.04$], **IL-5** [$F(1, 30) = 6.23,
p = 0.02$], **IL-6** [$F(1, 30) = 4.92, p = 0.03$], **IL-10** [$F(1, 30) = 5.75, p = 0.02$], **IL-12** [$F(1, 30) =
5.41, p = 0.03$], **IL-13** [$F(1, 30) = 6.19, p = 0.02$], **GM-CSF** [$F(1, 30) = 6.57, p = 0.02$], **IFN-γ**
[$F(1, 30) = 4.69, p = 0.04$], and **TNF-α** [$F(1, 30) = 5.33, p = 0.03$] (**Table 2.1**). The only
cytokine not impacted by either stress or drug treatment was **IL-4**, $p = 0.74$. Follow-up
ANOVARs revealed that relative to vehicle-NSC rats, both PB-NSC and vehicle-stressed
rats exhibit increased plasma levels of **IL-1α** ($p = 0.002, 0.007$), **IL-1β** ($p = 0.002, 0.015$),
**IL-2** ($p = 0.027, 0.046$), **IL-5** ($p = 0.001, 0.003$), **IL-10** ($p = 0.003, 0.049$), **IL-12** ($p = 0.008,
0.029$), **IL-13** ($p = 0.001, 0.016$), **GM-CSF** ($p = 0.004, 0.017$), and **IFN-γ** ($p = 0.002, 0.041$).
However, only PB-NSC rats exhibited significant elevations in **IL-6** ($p = 0.003$) and **TNF-α**
($p = 0.017$). These results suggest that in NSC conditions, PB can mimic the effects of
stress on many plasma cytokines. In addition, these results suggest that PB exhibits a
selective pro-inflammatory effect on **IL-6** and **TNF-α** with PB producing the largest effect
on levels of **IL-6**. Interestingly, PB treatment in combination with stress blocked these
increases in plasma cytokines, suggesting that PB produces anti-inflammatory effects only in the context of another pro-inflammatory stimulus.

Ten days following the cessation of treatment, there was no effect of either PB or stress on any plasma cytokine ($p = 0.715$ and 0.692, respectively; Table 2.2). This suggests that shortly after the cessation of PB and stress exposure, effects on plasma cytokines have normalized at basal conditions. However, three months following the cessation of PB and stress exposure, a different picture emerges (Table 2.3). At this delayed time point, PB and stress interact to affect plasma levels of IL-1α [$F(1, 22) = 5.09, p = 0.03$], IL-2 [$F(1, 22) = 4.47, p = 0.046$], IL-4 [$F(1, 22) = 4.42, p = 0.047$], IL-6 [$F(1, 22) = 5.70, p = 0.03$], IL-10 [$F(1, 22) = 4.88, p = 0.04$], IL-12 [$F(1, 22) = 4.34, p = 0.049$], and IFN-γ [$F(1, 22) = 4.87, p = 0.04$]. Specifically, PB-NSC rats exhibit a decrease in IL-1α ($p = 0.041$), IL-2 ($p = 0.008$), IL-4 ($p = 0.019$), IL-6 ($p = 0.020$), IL-12 ($p = 0.038$), and IFN-γ ($p = 0.045$) relative to vehicle-NSC rats. There is a crossover interaction between PB and stress on levels of IL-10, where PB-NSC and vehicle-stressed rats exhibit a decrease in these cytokines relative to vehicle-NSC and PB-stressed rats ($p = 0.072$ and 0.086, respectively). In addition, there is a main effect of PB on TNF-α such that a prior history of PB regardless of stress history, decreases plasma levels of TNF-α [$F(1, 22) = 5.40, p = 0.03$].

Collectively, these data suggest that the effects of PB and stress are highly contingent upon time. During treatment, PB in the absence of stress produces significant increases in a variety of cytokines – both pro- and anti-inflammatory. Any effect of PB or stress on basal levels of plasma cytokines have disappeared by one-week
post-treatment. Conversely, three months post treatment, rats which received PB but not stress exhibit reduced levels of circulating cytokines. This could be an over-compensatory adaptation by the immune system in response to the acute effects of PB, suggesting that PB progressively disrupts homeostasis of plasma cytokines.

### Table 2.1 Cytokine Levels at Day 14 of Treatment as a Percent of Vehicle—NSC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-Stressed</th>
<th>PB-NSC</th>
<th>PB-Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>100.0 ± 13.5</td>
<td>196.4 ± 24.3*</td>
<td>218.2 ± 39.7*</td>
<td>88.1 ± 19.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0 ± 15.2</td>
<td>207.4 ± 34.8*</td>
<td>249.1 ± 49.0*</td>
<td>72.8 ± 14.9</td>
</tr>
<tr>
<td>IL-2</td>
<td>100.0 ± 15.3</td>
<td>163.6 ± 23.2*</td>
<td>173.9 ± 32.0*</td>
<td>63.3 ± 14.2</td>
</tr>
<tr>
<td>IL-4</td>
<td>100.0 ± 18.4</td>
<td>143.9 ± 22.6</td>
<td>125.8 ± 29.5</td>
<td>96.7 ± 3.3</td>
</tr>
<tr>
<td>IL-5</td>
<td>100.0 ± 15.4</td>
<td>192.0 ± 20.7*</td>
<td>209.3 ± 32.1*</td>
<td>83.4 ± 15.7</td>
</tr>
<tr>
<td>IL-6</td>
<td>100.0 ± 9.5</td>
<td>293.8 ± 85.4</td>
<td>493.8 ± 134.2*</td>
<td>124.5 ± 35.8</td>
</tr>
<tr>
<td>IL-10</td>
<td>100.0 ± 14.2</td>
<td>202.9 ± 38.3*</td>
<td>267.1 ± 59.8*</td>
<td>80.2 ± 17.0</td>
</tr>
<tr>
<td>IL-12</td>
<td>100.0 ± 21.3</td>
<td>238.9 ± 49.1*</td>
<td>284.0 ± 69.6*</td>
<td>72.3 ± 19.6</td>
</tr>
<tr>
<td>IL-13</td>
<td>100.0 ± 23.5</td>
<td>356.7 ± 82.5*</td>
<td>487.9 ± 122.6*</td>
<td>131.9 ± 40.8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100.0 ± 16.0</td>
<td>203.6 ± 49.9</td>
<td>265.6 ± 67.5*</td>
<td>91.3 ± 15.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100.0 ± 19.6</td>
<td>249.6 ± 48.9*</td>
<td>346.7 ± 84.5*</td>
<td>120.9 ± 33.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>100.0 ± 22.2</td>
<td>261.8 ± 45.2*</td>
<td>303.6 ± 72.3*</td>
<td>87.3 ± 23.5</td>
</tr>
</tbody>
</table>

* values are significantly different from Vehicle-NSC

### Table 2.2 Cytokine Levels at Ten Days Post Treatment as a Percent of Vehicle—NSC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-Stressed</th>
<th>PB-NSC</th>
<th>PB-Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>100.0 ± 7.9</td>
<td>99.3 ± 14.7</td>
<td>100.3 ± 8.0</td>
<td>94.2 ± 5.2</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0 ± 10.1</td>
<td>94.0 ± 17.6</td>
<td>97.8 ± 10.2</td>
<td>94.9 ± 8.3</td>
</tr>
<tr>
<td>IL-2</td>
<td>100.0 ± 8.0</td>
<td>94.1 ± 13.7</td>
<td>93.8 ± 6.0</td>
<td>95.6 ± 5.1</td>
</tr>
<tr>
<td>IL-4</td>
<td>100.0 ± 12.2</td>
<td>94.3 ± 21.4</td>
<td>96.9 ± 9.9</td>
<td>91.9 ± 8.5</td>
</tr>
<tr>
<td>IL-5</td>
<td>100.0 ± 5.7</td>
<td>91.7 ± 11.5</td>
<td>95.3 ± 4.9</td>
<td>91.7 ± 4.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>100.0 ± 11.4</td>
<td>101.6 ± 18.8</td>
<td>105.4 ± 10.5</td>
<td>91.0 ± 8.6</td>
</tr>
<tr>
<td>IL-10</td>
<td>100.0 ± 7.1</td>
<td>95.5 ± 12.0</td>
<td>94.4 ± 8.4</td>
<td>95.5 ± 5.3</td>
</tr>
<tr>
<td>IL-12</td>
<td>100.0 ± 9.9</td>
<td>94.1 ± 18.3</td>
<td>95.4 ± 9.6</td>
<td>91.5 ± 6.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>100.0 ± 8.7</td>
<td>92.1 ± 17.0</td>
<td>95.8 ± 9.3</td>
<td>91.2 ± 7.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100.0 ± 12.9</td>
<td>99.0 ± 20.3</td>
<td>104.3 ± 12.6</td>
<td>95.3 ± 9.9</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100.0 ± 12.6</td>
<td>99.0 ± 20.6</td>
<td>99.1 ± 11.8</td>
<td>84.1 ± 11.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>100.0 ± 12.4</td>
<td>91.6 ± 18.3</td>
<td>92.6 ± 9.3</td>
<td>94.2 ± 6.8</td>
</tr>
</tbody>
</table>
Table 2.3 Cytokine Levels at Three Months Post Treatment as a Percent of Vehicle--NSC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-Stressed</th>
<th>PB-NSC</th>
<th>PB-Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>100.0 ± 14.3</td>
<td>85.9 ± 10.3</td>
<td>61.8 ± 9.2*</td>
<td>95.8 ± 14.6</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0 ± 17.5</td>
<td>83.5 ± 10.8</td>
<td>60.0 ± 10.7</td>
<td>82.7 ± 11.8</td>
</tr>
<tr>
<td>IL-2</td>
<td>100.0 ± 13.6</td>
<td>79.7 ± 2.7</td>
<td>60.5 ± 10.2*</td>
<td>80.5 ± 8.4</td>
</tr>
<tr>
<td>IL-4</td>
<td>100.0 ± 16.1</td>
<td>77.3 ± 5.2</td>
<td>58.2 ± 11.5*</td>
<td>84.3 ± 11.0</td>
</tr>
<tr>
<td>IL-5</td>
<td>100.0 ± 8.0</td>
<td>90.9 ± 4.7</td>
<td>79.2 ± 6.5</td>
<td>95.1 ± 8.1</td>
</tr>
<tr>
<td>IL-6</td>
<td>100.0 ± 14.0</td>
<td>87.8 ± 10.0</td>
<td>56.5 ± 10.0*</td>
<td>96.0 ± 14.2</td>
</tr>
<tr>
<td>IL-10</td>
<td>100.0 ± 12.9</td>
<td>76.3 ± 3.7#</td>
<td>70.0 ± 8.6#</td>
<td>97.7 ± 14.5</td>
</tr>
<tr>
<td>IL-12</td>
<td>100.0 ± 16.4</td>
<td>79.6 ± 9.5</td>
<td>60.1 ± 11.5*</td>
<td>89.2 ± 13.4</td>
</tr>
<tr>
<td>IL-13</td>
<td>100.0 ± 16.0</td>
<td>83.9 ± 10.5</td>
<td>61.8 ± 10.2</td>
<td>81.6 ± 11.3</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100.0 ± 17.5</td>
<td>75.0 ± 10.3</td>
<td>55.2 ± 10.1*</td>
<td>68.4 ± 7.0*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100.0 ± 15.3</td>
<td>88.1 ± 11.5</td>
<td>59.0 ± 9.8*</td>
<td>99.0 ± 16.3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>100.0 ± 14.3</td>
<td>78.3 ± 9.9</td>
<td>63.4 ± 11.2</td>
<td>85.3 ± 15.4</td>
</tr>
</tbody>
</table>

* values are significantly different from Vehicle-NSC; # significant cross-over interaction

### 2.3 DISCUSSION

This study supports two important conclusions: 1) time is a crucial factor to consider when regarding physiological and behavioral effects in response to stress and PB, and 2) the peripheral physiological effects and behavioral effects do not always progress in parallel. By detailing the etiology of these effects over time in a rodent model of Gulf War Illness, these data provide critical insight as to how stress and PB may have interacted to produce some of the well-characterized physiological and behavioral symptoms in veterans with GWI.

**PB Disrupts Homeostasis of Cholinergic Systems in GWI**

The acute effects of PB and stress on plasma cholinesterase and plasma corticosterone are both straightforward and consistent with clinical and preclinical literature. As a cholinesterase inhibitor, PB deceases cholinesterase activity when measured on the last day of drug treatment. Similarly, rats undergoing restraint stress...
exhibit an increase in adrenal release of corticosterone levels relative to non-stressed rats. While there was no interaction between PB and stress at this time point, there was a selective interaction between PB and stress on both corticosterone and cholinesterase activity at the delayed time point. Only rats which received PB and were concurrently exposed to restraint stress demonstrated elevations in corticosterone and cholinesterase relative to controls three months following treatment-cessation. This provides important evidence that stress and PB interact to disrupt homeostasis of physiological systems and that these physiological changes emerge slowly over time.

Although to our knowledge no human clinical study has directly assessed plasma AChE levels in GWI, BChE levels have been examined. Even though BChE is not the primary enzyme which metabolizes acetylcholine, individuals with a genotype for low-functioning BChE are at a significantly greater risk for developing GWI if they were administered PB (Lea Steele, Lockridge, Gerkovich, Cook, & Sastre, 2015). However, there was no difference in BChE activity between veterans with GWI and veteran controls when collapsed across genotype. Given that our results determined that prior exposure to stress and PB increase cholinesterase over time, it is possible that this effect is driven by changes in AChE and not BChE. As AChE is the primary enzyme which metabolizes acetylcholine, changes in AChE activity would impact acetylcholine turnover in both the parasympathetic nervous system and neuromuscular junction (Macintosh, 1941).

Further studies will need to be performed to see if increases in plasma cholinesterase activity corresponds to increased or decreased acetylcholine levels in the
periphery, although it is tempting to speculate that increases in cholinesterase activity are associated with decreases in basal acetylcholine levels. Decreased acetylcholine levels paralleled by increased acetylcholine metabolism could explain some of the symptoms in GWI such as muscular fatigue and respiratory dysfunction. Despite muscular fatigue being a commonly reported symptom of GWI, clinical studies have struggled to find a direct link between changes in neuromuscular innervation and symptoms of muscular fatigue in GWI (Brown & Jason, 2007). However, these studies have relied primarily on physical grip strength or receptor expression. Cholinergic effects on muscular fatigue may be subtler and linked to the temporal dynamics associated with acetylcholine release and metabolism.

As the cholinergic anti-inflammatory pathway of the vagus nerve also regulates the peripheral innate immune response, changes in cholinesterase activity would also impact regulation of inflammation. The innate immune response is the first of two arms of the immune system and provides a general defense against a broad range of general infectious organisms (e.g. LPS). In contrast, the adaptive immune response is a targeted and antibody driven defense managed by helper T-cells. Cytokines provide an important means of communication in both the innate and adaptive immune responses with some cytokines primarily associated with innate immunity (e.g. TNF-α) and others primarily associated with adaptive immunity (e.g. IL-4). Following stimulation of the vagus nerve, acetylcholine activates nicotinic receptors on macrophages which inhibit the release of TNF-α, thus regulating innate immunity (Bernik et al., 2002; Borovikova et al., 2000; H. Wang et al., 2003). As such, a PB-induced shift in the homeostasis of cholinergic
metabolism can have far-reaching consequences. One possible mechanism is that PB-induced changes in cholinesterase activity disrupt TNF-α synthesis and release, thereby directly contributing to the chronic depression in basal levels of TNF-α at three months post-treatment. Although high levels of TNF-α are associated with a variety of disorders including depression and diabetes (Y. Liu, Ho, & Mak, 2012; McAfoose & Baune, 2009; Moller, 2000), low levels of TNF-α can also be detrimental as this can impair the body’s ability to respond effectively to infections. Interestingly, some studies have indicated higher levels of bacterial infections in soldiers returning from the Gulf War who develop GWI (Nicolson, Nicolson, & Nasralla, 1998; Nicolson & Nicolson, 1996).

**PB and Stress Interact to Increase Corticosterone in GWI**

Alterations of the HPA axis in veterans with GWI are well documented – including enhanced cortisol suppression to a dexamethasone challenge (Golier et al., 2006) and elevations of cortisol relative to ACTH when compared to non-deployed controls (Golier et al., 2007). This shift in the ACTH:cortisol ratio was positively correlated with cognitive dysfunction in veterans. Our study indicates that PB and stress interact and may have caused some of the perturbations in cortisol levels evidenced in human clinical populations. These elevations of cortisol could contribute to some of the neurological changes in GWI which may in turn contribute to the cognitive dysfunction – particularly learning and memory impairments. Glucocorticoids easily cross the blood-brain barrier, and as the hippocampus robustly expresses glucocorticoid receptors (Bruce S McEwen, Davis, Parsons, & Pfaff, 1979), chronic elevations of cortisol/corticosterone have profound effects on this brain region. Elevations of cortisol
in other diseases such as recurrent major depression (Bremner et al., 2000) and Cushing’s syndrome (Starkman, Gebarski, Berent, & Schteingart, 1992) are associated with decreases in hippocampal volume and function on learning and memory tests.

In support of interactions between cholinesterase inhibitors and hippocampal volume, veterans with suspected exposure to low-doses of Sarin following the weapons demolition at Khamisiyah have significantly reduced gray matter and hippocampal volume relative to non-exposed veteran peers (Chao, Rothlind, Cardenas, Meyerhoff, & Weiner, 2010). This link between cholinesterase inhibition and hippocampal volume has also been supported by preclinical studies. Animal models exposed to a combination of PB, DEET and permethrin in combination with mild chronic stress over the course of 4 weeks also exhibit reduced hippocampal volume along with decreases in neurogenesis and inflammation (Parihar et al., 2013). While our results provide evidence that PB in the absence of other chemical exposures is sufficient to contribute to endocrine disruption, future studies will need to verify if PB in combination with stress is also sufficient to produce chronic decreases in hippocampal volume. It is possible that the interactions between stress and PB on chronic corticosterone production contribute to many of the hippocampal changes evidenced in both clinical and preclinical studies. In summary, stress and PB interact over time to disrupt homeostasis of endocrine and cholinergic systems, providing insight into how these factors may have contributed to the pathogenesis of GWI.

**PB Suppresses the Immune Systems in GWI over Time**

In the search for biomarkers for GWI, shifts in immune activation have been the
most extensively investigated over the last decade. There has been a focus as to whether veterans with GWI exhibit a Th1 or Th2 cytokine shift. Th1 cytokines are primarily associated with stimulating phagocytic responses through the innate immune system whereas Th2 cytokines tend to be associated with antibody production (Romagnani, 1999). The importance of a balance between Th1 and Th2 cytokines emerged in the late 1980s following studies demonstrating that T-helper cells can produce specific cytokine profiles with Th1-producing cells predominantly expressing mRNA for IL-2, IFN-γ, TNF and GM-CSF and Th2-producing cells predominantly expressing mRNA for IL-4 and IL-5 (Cherwinski, Schumacher, Brown, & Mosmann, 1987). The idea that an optimal balance of these cytokines is critical for normal immune function and imbalanced in disease states is one that has gathered considerable interest over the last few decades (Kidd, 2003). An exaggerated Th1 cytokine profile has predominated literature on rheumatoid arthritis, diabetes, and multiple sclerosis whereas an exaggerated Th2 cytokine profile has been associated with the development of allergies and asthma, although some inconsistency within the literature has suggested the issue is more complicated than a binary division suggests (Kidd, 2003). Regardless, a core principle of immune system is that an appropriate immune response is not necessarily the largest or the most cytokine-specific, but rather one that succinctly and effectively responds to and then terminates the infectious cycle. For Gulf War Illness, an early hypothesis which emerged was that veterans with GWI exhibit a shift towards a Th2 cytokine profile (Rook & Zumla, 1997). This hypothesis was driven primarily by 1) similarities in symptoms of GWI with chronic fatigue
syndrome, which has evidence of a dominant Th2 cytokine profile (Skowera, Cleare, et al., 2004), and 2) the impact of stress-induced increases in cortisol on cytokine expression. However, support for this cytokine profile in the GWI literature is inconsistent. One clinical study testing this hypothesis failed to find a Th2 dominant profile in GWI (Skowera, Hotopf, et al., 2004). Another study partially supported it, suggesting that the most important cytokines for distinguishing GWI from diseases with similar symptoms are IL-7, IL-4, TNF-α, IL-13, and IL-17F (Khaiboullina et al., 2015). In this study, IL-4 and TNF-α were down-regulated in GWI relative to controls. Our study supports this finding as PB decreased both IL-4 and TNF-α chronically. However, due to the heterogeneity of the illness, it is likely that different populations of veterans with GWI present different cytokine profiles and that the presentation of these cytokine profiles is driven by different exposures. For example, veterans in combat zones who took PB may be more prone to changes in IL-4 and TNF-α whereas veterans who were exposed to low levels of nerve gas following the demolition of Khamisiyah or served off-shore in the Navy may exhibit different cytokine profiles, thus contributing to the variability of cytokine profiles evidenced in clinical studies of GWI. It is also possible that as the veterans continue to age, their cytokine profile is continuing to evolve, or that their current situational context is continuing to evolve and further influencing their cytokine profiles. The challenge, then, is to determine which exposures influence which cytokine profiles in veterans, and how these profiles change over time.

Our study demonstrated that PB significantly impacts cytokine profiles over time. Acutely, PB exhibits a dramatic inflammatory effect. This effect was surprising given the
interactions of cholinesterase inhibitors at cholinergic terminals in the parasympathetic nervous system, but it could be that chronic administration of a drug which disrupts the parasympathetic nervous system may lead to increases in inflammation. Future studies would need to determine if PB also exhibits this pro-inflammatory effect on the first day of treatment. In our study, PB did exhibit anti-inflammatory properties, but only in the presence of another pro-inflammatory event such as chronic stress. Three months later, rats with a prior history of PB demonstrate a depressed cytokine profile with significant decreases in IL-2, IL-4, IL-6, IL-10, and TNF-α under non-stimulated conditions, suggesting that combinations of PB and stress can differentially disrupt homeostasis of the immune system. This downregulation of IL-4 and TNF-α is important as it parallels findings in clinical studies by Khaiboullina et al. (2015) as well as other preclinical models where combinations of restraint stress, PB, and various pesticides saw a reduction in the expression of IL-4 and IL-10 in the hippocampus six months following the cessation of treatment (Shetty et al., 2017). As such, while we measured levels of peripheral cytokines, this PB-induced suppression of cytokine levels may also be evident in the brain. Future studies will need to confirm this.

Under normal regulatory conditions, increases in IL-4 expression can provide feedback to attenuate the TNF-α response, but our model suggests that both IL-4 and TNF-α are suppressed several months after PB treatment. This could suggest that PB dysregulates homeostasis of the immune system by potentially disrupting negative-feedback signals, rendering it ill-prepared to respond appropriately to threats. Clinical data support this hypothesis as veterans with GWI exhibit an impaired coordination of
the Th1 and Th2 immune response following an exercise challenge which stimulates a mild inflammatory response (Broderick et al., 2011, 2013). Other studies have confirmed these findings, indicating that male veterans with GWI can be differentiated from healthy males based on their cytokine profiles at rest and following recovery from an exercise challenge, but not during the peak of the challenge (Smylie et al., 2013). This suggests that it is the ability of the immune system to recover from the challenge and not the peak immune response which is impaired in veterans with GWI. Although the cytokine profiles in the current study were under non-stimulated conditions, future studies should examine whether PB and stress produce similar deficits in the Th1: Th2 coordinated response to a state of challenge, either using a mild dose of LPS or in response to exercise. In addition, although our study did not examine females, clinical studies have suggested that under basal conditions, there are no sex differences in veterans with GWI (Smylie et al., 2013). However, there are sex differences when placed under an exercise challenge. As such, future preclinical studies should verify if PB differentially impacts males and females in response to a challenge.

Reduced levels of IL-4 and TNF-α may also contribute to some of the clinical symptoms which characterize GWI. Low levels of IL-4 are significantly correlated to chronic pain in some clinical studies (Üçeyler et al., 2006), and preclinical studies have shown that administration of IL-4 decreases hyperalgesia evoked by painful stimuli (Cunha, Poole, Lorenzetti, Veiga, & Ferreira, 1999). However, understanding the intricacies of cytokine-cytokine interactions, coordination of appropriate immune responses, and associated physiological symptoms is extremely complex as these
systems act in concert, not in isolation. Further clinical and preclinical studies are necessary to elaborate on these emerging findings.

**PB Exacerbates Stress-Induced Short-Term Deficits in Contextual Fear Conditioning**

Although veterans with GWI exhibit a host of memory problems which have persisted over several decades, our model demonstrated only transient deficits in contextual fear conditioning following PB and stress. One week following the cessation of treatment, rats with a prior history of restraint stress exhibited deficits in contextual-fear conditioning, and this effect was exacerbated by PB, suggesting that PB and stress interact to impair fear memory. The hippocampus is critically involved in contextual fear conditioning, and rats with hippocampal lesions show severe deficits in the acquisition (Phillips & LeDoux, 1992) as well as the recall of contextual fear memory if the hippocampus is lesioned shortly after conditioning (J. J. Kim & Jung, 2006). One possible mechanism is that elevations in corticosterone at this time point contributed to this memory deficit. Although whether corticosterone facilitates or impairs context-learning depends on surrounding conditions, some studies have suggested that long-term-depression/long-term-facilitation (LTD/LTP) of memories is dependent on the relative occupancy of minerocorticoid (MR) and glucocorticoid receptors (GR) (de Kloet, Oitzl, & Joëls, 1999). Elevations in basal corticosterone in rats with a previous stress history may have tipped this balance during fear conditioning, contributing to an impairment in contextual fear learning as evidenced by decreases in freezing behavior.

Another possibility is that shifts in immune function in PB-treated rats are contributing to some of the deficits in contextual fear memory. Cytokines are important
facilitators of normal cognitive function (McAfoose & Baune, 2009), and TNF-α in particular plays an important role in the strength and permanence of LTP in the hippocampus (Beattie et al., 2002). If brain levels of TNF-α parallel the chronic decreases in peripheral TNF-α, then this could partially explain why PB-stress rats fail to show the same increases in contextual-freezing as vehicle-stressed rats at three-months post-treatment. However, it is important to note that we did not measure cytokines immediately following fear conditioning. As PB and stress could further exacerbate disruptions in cytokine signaling following a stressful challenge, measuring the cytokine response directly following fear conditioning or another memory-test would be an important future study.

These impairments in fear conditioning following combinations of PB and stress one week after the end of treatment have important clinical implications, particularly regarding PTSD rates in Gulf War veterans. PTSD is undoubtedly a prevalent condition in veterans from the Gulf War with higher rates in deployed versus non-deployed veterans (Ikin et al., 2004; Perconte, Wilson, Pontius, Dietrick, & Spiro, 1993). However, when Gulf War veterans are compared with other actively deployed veterans, prevalence of PTSD is reduced, suggesting that stress from active deployment may be a greater risk factor for PTSD than specific Gulf War conditions (Ikin et al., 2004).

Another important consideration is that the prevalence of PTSD in Gulf War veterans changes over time. Immediately following the Gulf War, several studies suggested low rates of PTSD with estimates between 2% and 6% (Perconte et al., 1993; Wolfe, Erickson, Sharkansky, King, & King, 1999). Two years after the war, PTSD rates
doubled (Wolfe et al., 1999). Similarly, in the current study, we saw an increased risk for contextual fear conditioning three months following the cessation of treatment selectively in rats which were stressed but did not receive PB. This suggests that repeated psychological stress may exert some latent effects on fear conditioning, possibly explaining in part some of the increases in prevalence in PTSD in the years following the return from war. Rates of conditioned freezing were not increased in the PB-stressed rats or PB-non-stressed rats relative to vehicle-NSC rats, suggesting that PB in the presence of stress may mitigate this latent effect on fear conditioning. Alternatively, these data could indicate that continued post-deployment stress or infection are crucial factors mediating the progression of GWI.

Corticosterone levels were also increased selectively in the PB-stressed rats at this time point. Either 1) corticosterone differentially contributes to fear conditioning at these time points, or 2) by the delayed time point the homeostatic balance between multiple systems have been sufficiently disrupted to complicate these interactions. However, veterans returning from the Gulf War did not return to stress-free conditions. Therefore, an important consideration is whether intermittent recurring stressors during that three-month post-treatment stretch would have exacerbated effects in PB conditions.

**Conclusions**

In sum, this study provides evidence that combinations of PB and stress contribute to the development of various physiological and psychological symptoms evidenced in veterans with GWI, and that the development of these symptoms change
over time. Corticosterone levels, like cytokines and cholinesterase activity, exhibit a homeostatic shift over time following chronic stress and PB. In addition, PB and stress interact to impair fear conditioning ten days following the cessation of treatment, providing further support that combinations of PB and stress impair memory networks. A history of stress increases fear conditioning at the delayed time point, but only in the absence of PB. This suggests that while stress may increase some PTSD-like symptoms, PB mitigates this effect.

As soldiers were exposed to a variety of factors, it is likely that the motley of physiological changes contributes to a range of these symptoms. Further studies will be needed to investigate whether PB and stress-induced changes in peripheral cytokines and cholinesterase contribute to chronic pain and muscular fatigue as opposed to cognitive dysfunction. Examining the progression of these symptoms over time is critical as evidence continues to mount that severity and prevalence of GWI symptomology is likewise progressing over the last several decades.
CHAPTER 3

EXPERIMENT TWO: ACETYLCHOLINE IN GULF WAR ILLNESS

Veterans with GWI exhibit a host of central nervous system deficits, including chronic headaches, depression, anxiety, sleep disturbances, chronic pain, attentional deficits, and working and long-term memory impairments (Haley et al., 1997; Steele et al., 2012; Sullivan et al., 2003; White et al., 2016). When paired with the peripheral deficits also evidenced by these veterans – including respiratory problems, rashes, chronic diarrhea, and muscular fatigue – there is a strong parallel to symptoms documented in cases of cholinergic toxicity (Pope et al., 2005). This correlation supports a plethora of epidemiological and preclinical studies suggesting that PB contributed to both peripheral and central symptoms in this population of veterans (Parihar et al., 2013; L Steele et al., 2012; Lea Steele et al., 2015; Sullivan et al., 2003).

Whether PB impacts the central cholinergic system is highly controversial. Some studies reported that chronic stress exposure creates a “leaky” blood-brain barrier (Friedman et al., 1996), allowing PB to cross and directly impact central cholinergic function, but other studies failed to replicate this finding (Amourette et al., 2009; Grauer et al., 2000; Kant et al., 2001; Song et al., 2002; Tian et al., 2002). To address whether stress and PB interact to either directly or indirectly influence central cholinergic function, the current study used a rodent model of GWI in combination with in vivo
microdialysis to examine the neurochemical response to an immune challenge and an acute stress challenge. Acetylcholine levels were assessed in two brain regions: the PFC and the hippocampus. These brain regions were targeted due to their robust cholinergic innervation and critical contribution to cognitive processes which exhibit deficits in veterans with GWI (Fadel, 2011; Lange et al., 2001; Mesulam, Mufson, Wainer, & Levey, 1983).

An immune challenge was selected as some clinical studies have suggested that adverse responses to bacterial infections may be an important component instigating the progression of GWI (Nicolson et al., 1998; Nicolson & Nicolson, 1998). In addition, clinical studies have suggested that veterans with GWI exhibit aberrant immune function, particularly in response to a challenge (e.g. exercise) (Broderick et al., 2013; Whistler et al., 2009). This finding has been corroborated with preclinical models which have suggested that immune function in the peripheral and central nervous system may be altered in models of GWI (Shetty et al., 2017; Whistler et al., 2009). In contrast, a restraint stress challenge was selected to assess neurochemical shifts to psychological stressors as veterans experienced many recurrent psychological post-deployment stressors. Altered reactions to systemic infection and psychological stress following the unique conditions in theater may influence the chronic progression of GWI symptoms.

We tested the hypothesis that combinations of PB and stress alter the cholinergic response to an innate immune challenge, thus potentially contributing to the cognitive deficits evidenced in veterans with GWI. In addition, we tested whether an acute psychological stressor (restraint stress) produced similar deficits in the cholinergic
response in rats with a prior history of PB and repeated stress. Although PB is not hypothesized to impact basal levels of cholinergic function, PB is hypothesized to interact with stress to produce deficits in the cholinergic response in the PFC and hippocampus.

3.1 METHODS AND MATERIALS

As in Experiment One, young adult Sprague Dawley rats were acclimated to housing conditions for one week following transport, prior to the onset of experiments. After a week, rats in the pilot study underwent surgery for in vivo microdialysis. Rats in the GWI study went through the GWI paradigm as previously described in Chapter 1 (Figure 1.2).

In vivo microdialysis was performed to assess how PB and stress interacted to influence how the PFC and hippocampus responded to both (a) an LPS challenge and (b) a restraint stress session. However, in order to determine the optimal dose of LPS for the GWI animals, a pilot study was first performed to assess 0 µg/kg, 10 µg /kg, 30 µg /kg, 100 µg /kg, and 300 µg /kg LPS impacted acetylcholine in the PFC and hippocampus.

Surgical Procedures

All rats underwent stereotaxic surgery to unilaterally implant two guide cannulae into the PFC and dorsal hippocampus for in vivo microdialysis. Each rat was induced with 5% isoflurane and 1.5 L/min oxygen, after which 2% isoflurane was used for anesthesia maintenance. The rat’s head was shaved and scrubbed with alternating betadine and alcohol wipes, following which each rat received three pericranial injections of 0.05 mL
carbocaine, a local anesthetic. A scalpel was then used to make a midline incision down the skull. Sterile q-tips were used to remove the fascia and connective tissue, and bregma was located and marked. Target coordinates were marked relative to bregma, and holes for the guide cannulae and anchoring screws were marked and drilled with a Dremel tool. BASi interlocking intracerebral guide cannula and stylet (MD-2251) were placed at the following coordinates (Table 3.1) and secured with Lang Ortho-Jet fast-drying orthodontic acrylic resin:

Table 3.1. Stereotaxic Coordinates for Guide Cannulae

<table>
<thead>
<tr>
<th>Coordinates relative to Bregma</th>
<th>PFC</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior-Posterior (AP)</td>
<td>+3.0 mm</td>
<td>-5.2 mm</td>
</tr>
<tr>
<td>Lateral (L)</td>
<td>±0.5 mm</td>
<td>±3.8 mm</td>
</tr>
<tr>
<td>Dorsoventral (DV)</td>
<td>-2.5 mm</td>
<td>-3.6 mm</td>
</tr>
<tr>
<td>Angle</td>
<td>0 %</td>
<td>10 %</td>
</tr>
</tbody>
</table>

Coordinates for the guide cannulae were selected based on the Paxinos and Watson rat brain atlas (1998). Left and right hemispheres were counterbalanced across rats. Postoperatively, each rat received a 0.5 mg/kg subcutaneous injection of buprenorphine and was then allowed to recover in his home cage with bacon softies for forty-eight hours prior to habituation for microdialysis procedures.

In Vivo Microdialysis

Two days after surgery, rats began habituation for in vivo microdialysis. The microdialysis room was kept under low lighting conditions (50 lux) with continuous white noise. In the center of the microdialysis room is the Raturn™ apparatus. The Raturn™ apparatus is a movement-sensitive apparatus which consists of four round-bottomed bowls on rotating plates, which critically maintains the integrity of the
microdialysis lines while enabling the rat to move freely at will. Each rat was provided his own bowl with fresh bedding at the onset of the first day of habituation. Dividers separated each bowl so that each rat was visually separated from the next. Rats were habituated to the microdialysis apparatus for four days and a total of 20 hours. Starting on the second day of habituation, each rat was provided a collar and tethered to the Raturn™ system, allowing the animal to freely move around the bowl as it gently counter-rotated in response. On the day of microdialysis, animals were brought up to the microdialysis room shortly after lights-on (7:00 AM). 2 mm probes from BASi (MD-2200) were flushed for thirty minutes with a 0.005% proclin solution to eliminate the glycerol coating, followed by infusion with a solution of artificial cerebral spinal fluid (aCSF) and 100 nM neostigmine. aCSF simulates the natural ionic composition of the brain and contains the following: 150 mM NaCl, 3 mM KCl, 1.7 mM CaCl₂·H₂O, 0.183 mM MgCl₂·6H₂O, 5 mM D-glucose. The addition of the neostigmine to the aCSF solution is critical as collection of in vivo extracellular acetylcholine levels approximate the detection limit of high performance liquid chromatography (HPLC) machines (Chang, Savage, & Gold, 2006). Neostigmine prevents the rapid enzymatic degradation of acetylcholine by cholinesterases, thus boosting collectible levels of acetylcholine.

The probe was then inserted into guide cannula in the PFC and hippocampus in place of the stylets. Probes were infused with the neostigmine/aCSF solution at a rate of 2 µL/min. The probe consists of a semi-permeable membrane with a 30 kDa molecular weight cutoff which extends 2 mm beyond the tip of the guide cannula. This membrane allows the free passage of small-molecule neurotransmitters in the absence of larger
molecules such as neuropeptides or hormones. Because the probe extends beyond the
guide cannula, three hours following the insertion of the probe are collected as discard.
Discard collection began at approximately 9:00 AM each dialysis day. This period is
critical for equilibration of the extracellular milieu following probe damage.
Sample collection began at noon, following the discard period. Samples were
collected in 15 minute intervals, yielding 30 µL of dialysis per collection, and
immediately stored at -80°C until analysis. Four collections were taken prior to any
stimulus presentation to assess basal neurotransmitter levels. After the cessation of one
microdialysis session, rats had a 48-hour recovery period before the following session to
enable the restoration of any depleted neurotransmitters. No animal went through
more than two microdialysis sessions.

*Pilot study: in vivo microdialysis with LPS challenge*

Prior to the start of the GWI studies, a pilot study was performed to assess the
dose-response effect of an intraperitoneal injection of LPS on acetylcholine levels in the
PFC and hippocampus. Each microdialysis session consisted of a total of sixteen
collections: four baseline collections, followed by an intraperitoneal injection of LPS and
12 post-injection collections. During each session, rats received one of the following
doses of LPS in a counter-balanced design: 0, 10, 30, 100, or 300 µg/kg LPS. LPS was
diluted daily immediately prior to use from 1 mg/mL frozen stock solution in sterile
saline. After the second microdialysis session, rats were anesthetized with isoflurane
and intracardially perfused with 100 mL of 0.1 M PBS followed by 300 mL of 4%
paraformaldehyde in 0.1M PB. Brains were removed and placed in a 30% sucrose/0.1 M
PB solution at 4°C where they were allowed to sink over several days. After sinking, brains were rapidly frozen using isopentane on dry ice and stored at -80°C. A sliding microtome was then used to cut 40 µm sections to verify probe placement in each rat.

For this pilot study, in the PFC, the LPS significantly increased acetylcholine levels in a dose-response manner, $F(4, 15)=3.48, p = .03$. Specifically, both 30 and 100 µg/kg LPS increased acetylcholine levels relative to rats injected with vehicle, $p = .04$ and $p = .01$ respectively. An injection of 300 µg/kg LPS resulted in a 16% greater increase in acetylcholine relative to an injection of 30 µg/kg LPS, although the two doses were not statistically significantly different from one another, $p = 0.29$. In the hippocampus, there is a non-significant trend for LPS dose to impact acetylcholine levels, $F(1, 4)=2.36, p = 0.09$, as well as a trend for a dose × time interaction, $F(60, 270)=1.37, p = 0.05$. While 30 µg/kg LPS did increase acetylcholine levels in the hippocampus, there did not appear to be larger response with increasing the dose beyond this amount. Based off these parameters, 30 µg/kg LPS was selected for the following GWI studies as 1) it increased acetylcholine levels in both the PFC and hippocampus, and 2) in the PFC, 30 µg/kg LPS exhibited a sub-maximal effect on acetylcholine levels.

**GWI: in vivo microdialysis with LPS challenge**

The LPS challenge in the GWI rats was performed on the first of two microdialysis days (Figure 3.1). This session consisted of four baseline collections, followed by an intraperitoneal injection of 30 µg/kg LPS. This dose of LPS was selected based on data from pilot studies to yield a significant but sub-maximal increase in acetylcholine. As with the pilot study, 12 collections followed the injection of LPS,
Figure 3.1. Dose-response of LPS on Acetylcholine in the PFC and Hippocampus.

One of five different doses of LPS (0, 10, 30, 100, 300 µg/kg) was administered immediately prior to the fifth collection. **Panel A.** LPS exhibited a dose-response effect on acetylcholine levels in the PFC with 300 µg/kg producing the largest effect. As 30 µg/kg increased acetylcholine levels beyond saline injections in the PFC while still being sub-maximal to the 300 µg/kg. As such, 30 µg/kg LPS was used for all subsequent experiments. **Panel B.** Although LPS also increased acetylcholine in the hippocampus, a dose-response effect was not discernible.
making a total of 16×30 µL collections. Immediately after the end of the last microdialysis collection, rats were returned to their home cage and their tails were bled. Blood was collected on ice in a microcentrifuge tube and then spun down in a microcentrifuge at 4°C for 15 minutes × 30,000 g. The supernatant (plasma) was removed and stored at -80°C for later analysis.

**GWl: in vivo microdialysis with acute restraint challenge and tissue collection**

The acute restraint challenge was performed on the second day of microdialysis, 48 hours following the LPS challenge (Figure 3.2). This session consisted of four baseline collections followed by four collections of restraint stress and then four collections following the release from restraint. For restraint, rats were returned to their home cages and secured in plastic restrainers specifically designed to not interfere with the microdialysis tubing. Rats went into restrainers at approximately 1:00 PM and were released from restrainers at 2:00 PM. When rats were released from the restrainers, they were returned to the microdialysis bowls. While this restraint challenge exhibits similar psychological parameters to the repeated restraint stress in the GWl paradigm, there are several notable differences: 1) the timing of the onset of restraint stress is shifted, 2) the room in which restraint stress occurred is different, and 2) the plastic restrainers are physically unique from the wire mesh restrainers. Therefore, while some aspects of this restraint challenge may be considered parallel to previous restraint conditions (homotypic stress), others are unique (heterotypic stress).
Figure 3.2. Timeline for Experiment Two. All rats underwent the GWI paradigm of ± drug treatment and ± repeated restraint stress. The day following the end of the treatment paradigm, rats were prepared for surgery where a guide cannula was unilaterally implanted into the prefrontal cortex and hippocampus. After two recovery days, rats began habituation to the microdialysis chamber. Rats were habituated for four days and a total of twenty hours. The first microdialysis session was performed on Day 22 where each rat received a 30 μg/kg injection of LPS. Tail bleeds were performed immediately following the end of the session. Forty-eight hours later, rats were returned to the microdialysis chamber for a restraint stress challenge. Rats were euthanized immediately following the end of the second microdialysis session, at approximately 4:00 PM on Day 24.
**High Performance Liquid Chromatography: Acetylcholine**

In random order from sample collection, 20 µL of previously frozen microdialysate sample was loaded onto an EiCom AC-GEL reverse-phase analytical column, packed with a polymer resin, where choline and acetylcholine were isolated from other biogenic compounds in interaction with a mobile phase consisting of 50 mM potassium bicarbonate, 300 mg/L sodium decanesulfonate, and 50 mg/mL 2Na EDTA, pH 8.4. Afterwards, a dual enzymatic column AC-ENZYM II from Eicom first metabolized acetylcholine into choline and acetyl-CoA by AChE, and then choline was further metabolized into hydrogen peroxide by choline oxidase. The hydrogen peroxide was oxidized at the platinum electrochemical detector with an applied current of +450 mV, resulting in O₂, 2H⁺, and 2e. The potential was read using the EiCom HT-500 detector system. Concentration of acetylcholine in samples was interpolated against a three-point standard curve of 2, 0.4, and 0.1 pmol/20 µL acetylcholine.

**Euthanasia**

After the second microdialysis session, rats were immediately anesthetized with isoflurane and transcardially perfused with 100 mL of 0.1 M PBS followed by 300 mL of 4% paraformaldehyde in 0.1M PB. Brains were removed and placed in a 30% sucrose/0.1 M PB solution at 4°C where they were allowed to sink over several days. After sinking, brains were rapidly frozen using isopentane on dry ice and stored at -80°C. A sliding microtome was then used to cut 40 µm sections to verify probe placement in each rat.
At the start of the perfusion, trunk blood was collected from the right atrium and kept on ice. Blood was spun down in a microcentrifuge at 4°C for 15 minutes × 30,000 g. The plasma supernatant was aspirated and stored in centrifuge tubes at -80°C for later analysis.

**Statistical Analyses**

Results were calculated as a 2×2×16 mixed ANOVA for the LPS session and a 2×2×12 mixed ANOVA for the restraint challenge session. For between subjects, this experiment had 2 levels of drug treatment (vehicle, PB), 2 levels of stress (NSC, stressed). Within-subjects repeated measures consisted of 16 levels, representing the 16 consecutive collections during microdialysis for the LPS challenge. The restraint stress challenge had a total of 12 collections: 4 baseline, 4 during restraint, 4 post-restraint. Following significant interactions, simple main effects post hoc tests were performed with Bonferroni post-hoc corrections for family-wise error.

Power analyses for in vivo microdialysis were performed a priori with an effect size of 0.8 and α=0.05; analyses suggested that a sample size of n=8 per group with a total N = 32 would be sufficient for achieving statistical significance. After correcting for statistical outliers and other complications during microdialysis and HPLC (e.g. erratic baseline, undetectable analytes, loss of cannula, broken probe), actual sample size for each condition was as follows:
Table 3.2 Sample Size for Each Condition in GWI Paradigm

<table>
<thead>
<tr>
<th></th>
<th>PFC</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>Restraint</td>
<td>LPS</td>
</tr>
<tr>
<td>Vehicle-NSC</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Vehicle-Stressed</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PB-Stressed</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

3.2 RESULTS

GWI In Vivo Microdialysis: Acetylcholine

Basal Levels of Acetylcholine

On the first day of microdialysis, prior to the LPS challenge, there was no effect of either a prior history of PB or restraint stress on basal acetylcholine levels in the PFC, $F(1, 25) = 0.25, p = 0.62$, or hippocampus, $F(1, 27) = 2.37, p = 0.13$ (Figure 3.3). On the second day of microdialysis, prior to the restraint challenge, the PFC still did not exhibit any differences in basal acetylcholine levels between any groups, $F(1, 22) = 0.53, p = 0.48$. However, on the second day of microdialysis, there was a significant cross-over interaction between PB and stress in the hippocampus, $F(1, 21) = 12.32, p = 0.002$ (Table 3.3). In this cross-over interaction, acetylcholine was elevated in PB-NSC and vehicle-stressed rats relative to vehicle-NSC and PB-stressed counterparts. This shift in basal acetylcholine levels in the hippocampal on the second day of microdialysis is difficult to interpret as it could be a function of several factors including differential recovery from probe damage, residual effects of LPS, or self-selection as the second day of microdialysis had a lower n-size due to experimental complications (e.g. probe breaking).
Figure 3.3. Basal Levels of Acetylcholine. On the first day of microdialysis, prior to the LPS challenge, there was no effect of either PB or stress on basal acetylcholine levels in the PFC (Panel A) or hippocampus (Panel B). This suggests that neither a history of PB nor restraint stress either independently or in interaction impact affect basal acetylcholine levels in either brain region.
Table 3.3 Basal Acetylcholine Levels on Day Two of Microdialysis

<table>
<thead>
<tr>
<th></th>
<th>PFC</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-NSC</td>
<td>0.09 ± 0.03</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>0.14 ± 0.02</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>Vehicle-Stressed</td>
<td>0.10 ± 0.03</td>
<td>0.14 ± 0.03*</td>
</tr>
<tr>
<td>PB-Stressed</td>
<td>0.09 ± 0.02</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

*significant cross-over interaction, $p = 0.002$; Data expressed as mean ±SEM (p mol/20 μL)

LPS challenge

LPS significantly increased acetylcholine levels in the PFC over time in all rats, $F(15, 405) = 12.0, p < 0.001$ (Figure 3.4). However, there was also a main effect of stress where a prior stress history attenuated the cholinergic response to LPS in the PFC, $F(1, 27) = 6.52, p = 0.017$. Additionally, there was a stress by time interaction, $F(15, 405) = 1.79, p = 0.034$. Specifically, while LPS increased acetylcholine relative to baseline at every collection starting at collection 5, prior stress history attenuated this effect relative to NSC rats at collections 5 and 6, ($p = 0.004$ and 0.009, respectively). There was no effect of PB on acetylcholine levels in response to an LPS challenge in the PFC, suggesting that 1) while prior stress history attenuates the cholinergic response to an immune challenge suggesting cross-over between these two heterotypic events, 2) stress and PB do not interact to influence the prefrontal cortical cholinergic response to an immune challenge.
Figure 3.4. Cholinergic Response to an LPS Challenge in the PFC. In rats without a prior restraint stress history, LPS increased acetylcholine levels over time with peak increases at collection 5 and a secondary, gradual increase starting an hour after the injection. In rats with a prior restraint stress history, the cholinergic response to LPS was significantly blunted relative to vehicle-NSC rats at collections 5 and 6, $p < 0.05$.

*: effect of stress relative to NSC rats
Figure 3.5. Cholinergic Response to an LPS Challenge in the Hippocampus. Although LPS increased acetylcholine levels in the hippocampus in vehicle-NSC rats, either a prior history of PB or a prior history of stress attenuated this effect between collections 11-14. Rats which received PB but had no prior restraint stress history had a similar, attenuated cholinergic response to LPS as did rats with a prior restraint stress history. This suggests that the hippocampus is particularly sensitive to the cholinergic effects of PB in the context of an immune challenge.

*: significant effect of PB in NSC rats
In the hippocampus, there was a main effect of time such that LPS significantly increased acetylcholine levels over time in all rats, $F(15, 390) = 7.52, p < 0.001$ (Figure 3.5). There was also an interaction between prior history of PB and stress on the cholinergic response to LPS in the hippocampus, $F(1, 26) = 6.22, p = 0.019$. In vehicle-treated rats, a prior stress history attenuated the cholinergic response to LPS relative to NSC rats, $p = 0.031$. Additionally, in NSC rats, PB attenuated the acetylcholine response relative to vehicle-treated rats at collections 11-14, ($p = 0.037, 0.016, 0.049, \text{ and } 0.025$, respectively). This attenuation of the cholinergic response to LPS during collections 11-14 in PB-NSC rats parallels the cholinergic response of rats with a prior stress history, suggesting that in response to a novel systemic stressful challenge, either a prior history of PB or stress can significantly dampen the response cholinergic systems in the hippocampus.

**Acute Restraint Challenge**

In response to an acute restraint challenge, there was a significant three-way interaction between prior stress history, prior drug history, and time on acetylcholine levels in the PFC, $F(11, 264) = 1.83, p = 0.049$ (Figure 3.6). In vehicle-treated rats, a prior stress history attenuated the cholinergic response to both restraint and release from restraint (collections 5-12) relative to NSC rats, ($p = 0.010, 0.034, 0.010, 0.021, 0.024, 0.002, 0.001, \text{ and } 0.004$, respectively). In contrast, PB-NSC rats exhibited a blunted cholinergic response to termination of the restraint stress challenge with decreased acetylcholine levels at collections 9 and 10 relative to vehicle-NSC rats, ($p = 0.01$ and 0.013, respectively). For rats with a prior stress history, PB treatment did not
Figure 3.6. Cholinergic Response to a Restraint Stress Challenge in the PFC. Either a prior history of restraint stress or PB attenuated the cholinergic response to an acute restraint challenge. While a prior history of restraint stress decreased the cholinergic response to both restraint and the release from restraint, a prior history of PB in the absence of stress selectively decreased the cholinergic response to the release from restraint. This suggests that PB may influence cholinergic processing of the removal of a stressful stimulus.

*: Significant effect of repeated restraint stress relative to vehicle-NSC rats

$: Significant PB effect relative to vehicle-NSC rats
Figure 3.7. Cholinergic Response to a Restraint Stress Challenge in the Hippocampus. Restraint stress increased acetylcholine levels in the hippocampus in Vehicle-NSC rats. Similar to results in the PFC, Vehicle-treated rats with a prior history of restraint stress exhibit an attenuated cholinergic response to both the restraint and release from restraint within the hippocampus. However, PB blocks the habituation of the cholinergic response in repeated restraint stress conditions. As such, cholinergic systems may fail to adapt to repeated psychological stress in individuals with a history of PB.

*: Significant effect of repeated restraint stress relative to Vehicle-NSC rats

#: Significant effect of PB within the repeated restraint stress condition
significantly alter the cholinergic response to an acute restraint challenge. These data suggest that in the PFC, PB treatment attenuates the cholinergic response to an acute novel stressor selectively in rats with no prior stress history.

In the hippocampus, there was a significant three-way interaction between prior stress history, prior drug history, and time on acetylcholine levels in response to an acute restraint challenge, $F(11, 220)=2.17, p = 0.02$ (Figure 3.7). In vehicle-treated rats, a prior history of restraint stress attenuated the cholinergic response to a restraint challenge and its termination (collections 6, 8, 11), ($p = 0.042, 0.029, \text{ and } 0.024,$ respectively). However, rats with a prior history of PB in conjunction to repeated restraint stress failed to show this attenuated stress effect during restraint, instead exhibiting a response parallel to that of vehicle-NSC rats at collections 5 and 8, ($p = 0.011 \text{ and } 0.038, \text{ respectively}$). These data suggest that PB impairs the habituation of the hippocampal cholinergic response to an acute stress challenge selectively in rats with a prior stress history such that they exhibit a similar cholinergic response to stress as that of stress-naïve rats.

3.3 DISCUSSION

This study demonstrated that a prior history of PB and stress differentially impact the cholinergic response to LPS and an acute restraint challenge across two brain regions: the prefrontal cortex and hippocampus. In the PFC, in rats with a no prior stress history, PB attenuates the cholinergic response to a restraint stress challenge but does not impact the response to an LPS challenge, suggesting that the effect of PB on
cholinergic function may be stressor-specific. In contrast, in rats with no prior stress
history, PB attenuates the cholinergic response to both restraint and LPS in the
hippocampus. Although a prior stress history attenuates the cholinergic response to
both LPS and restraint stress in both brain regions, only the hippocampus exhibits
synergistic effects between PB in stress. In response to a restraint challenge in the
hippocampus, PB-stressed rats fail to exhibit an habituation of the cholinergic response
to the restraint challenge, thus exhibiting a cholinergic response that parallels vehicle-
NSC rats as opposed to vehicle-stressed rats. The hippocampus appears to be a critical
region where the effects of PB and stress interact to influence acetylcholine levels.
These findings could have important implications for how recurrent life-stressors impact
neural function in veterans with GWI.

On a broader note, this is also the first in vivo study to demonstrate the effects
of an LPS challenge on acetylcholine levels in the brain. Our results agree with previous
studies which have demonstrated that acetylcholine levels in the PFC and dorsal
hippocampus are sensitive to acute stress, increasing both in response to the stress
challenge and again with its termination (Imperato, Puglisi-Allegra, Casolini, & Angelucci,
1991; Mark, Rada, & Shors, 1996; Mitsushima, Masuda, & Kimura, 2003; Tajima et al.,
1996). While previous studies have demonstrated that restricted housing conditions
attenuates the cholinergic response to an acute stress challenge in the hippocampus
(Mitsushima, Funabashi, Shinohara, & Kimura, 2003), this is the first study to show that
a prior history of repeated restraint stress also attenuates the cholinergic response to an
acute stress challenge in the PFC. Therefore, this study provides valuable information on
how the brain responds to stressful stimuli both under normal physiological conditions and in a disease state.

**Cholinergic Role in Cognitive Processing of Stressful Stimuli**

Altered cholinergic function following a history of PB pre-treatment and stress could have important consequences for the processing of stressful stimuli as the cholinergic system in the brain is associated with a variety of factors in the stress-response. For example, this sensitivity of cholinergic systems to acute stress have been attributed to processing of sensory stimuli (Inglis & Fibiger, 1995; Parikh, Kozak, Martinez, & Sarter, 2007), desynchronization of neural networks (Chen, Sugihara, & Sur, 2015), influencing cognitive processing during high-arousal states (Teles-Grilo Ruivo et al., 2017), cortical processing of anxiety-responses to stress (Hart, Sarter, & Berntson, 1999), and top-down modulation of the cardiovascular response to stress (Hart et al., 1999).

Interestingly, prior history of PB attenuates the cholinergic response to an acute restraint challenge in the PFC and hippocampus in rats without a prior history of restraint. This could suggest that PB impairs processing of novel stressful stimuli, which could provide insight to some of the executive functioning deficits in soldiers with GWI who reported taking PB (Sullivan et al., 2003). In contrast, while a prior history of stress decreased the cholinergic response to a restraint stress and LPS challenge in the hippocampus, PB pretreatment disrupts this adaptation of cholinergic systems selectively to the restraint challenge. One possibility for this discrepancy is that PB disrupts the cognitive processing of stressful stimuli as well as the ability of hippocampal
circuits to encode those stimuli into memories. As such, cholinergic systems in the hippocampus exhibit divergent effects for challenges which exhibit heterotypic (novel) versus homotypic (parallel) stress-aspects to their prior history of stress. However, future studies will need to determine if this effect of PB on the neurochemical response in the hippocampus is selective for acetylcholine or encompasses a broader array of neurotransmitters (e.g. glutamate). Future studies will also need to examine if PB-induced neurochemical deficits are stress-specific or if they are also evident during cognitive processing of non-stressful stimuli (e.g. novel object recognition task).

**Cholinergic Modulation of Inflammation**

While increased cholinergic signaling is often associated with facilitation of cognitive function, the cholinergic system also regulates inflammatory states in the brain. The relationship between acetylcholine and inflammation is particularly evident following LPS. In our study, the cholinergic system exhibits a two-phase response to LPS in both the PFC and hippocampus. The first phase is rapid, peaking in the first post-LPS collection. While part of this increase in acetylcholine levels is likely attributable to a stress-response to the injection, the dose-response effect of LPS on acetylcholine at that time in addition to the magnitude of the response is unlikely to be attributable to the injection alone. One possible mechanism is that this early response is reflective of neurological feedback from the vagus nerve, which is a critical and rapid point of communication between peripheral inflammation and the brain through its afferent projections to the nucleus tractus solitarius (NTS) (V. Pavlov et al., 2003). LPS activates toll-like receptor-4 on macrophages (Hoshino et al., 1999), which then stimulates
nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and releases IL-1 (Tracey, 2002). Vagus afferents, which have receptors for IL-1, send signals to the brain rapidly in response. In contrast, the latent hippocampal and PFC response to a peripheral injection of LPS may be more closely associated with peaking cytokine levels in the periphery and brain and shifting microglial phenotypes, which exhibit slower responses to infectious stimuli, ranging anywhere from 1-48 hours later (Erickson & Banks, 2011). As such, a decreased cholinergic response to LPS in PB-treated rats in the hippocampus at 2-3 hours post-LPS could indicate that PB shifts the brain’s cytokine response to LPS, which is then influencing acetylcholine levels. This could indicate, that the hippocampus is particularly sensitive to inflammatory changes following LPS in PB-treated rats. However, future studies would need to confirm this by measuring cytokine levels directly in the brain at this time point.

Although cytokines may be mediating the cholinergic response to LPS, acetylcholine also plays a critical regulatory role in mediating the cytokine response in the brain through interactions with microglial nicotinic α7 receptors. Nicotinic α7 receptors are essential negative regulators of inflammation in both the peripheral and central systems. In the brain, α7 activation maintains the senescent state of microglia by inhibiting microglial expression pro-inflammatory factors (Shytle et al., 2004; H. Wang et al., 2003). The phenotypic states of microglia parallel that of peripheral macrophages and are termed M0 (senescent), M1 and M2 (Franco & Fernández-Suárez, 2015). M1 microglia are associated with the innate immune system and respond to stimuli by releasing a variety of cytokines (e.g. TNF-α) that respond to an infection or neural
damage by stimulating phagocytosis. In contrast, M2 microglia are associated with the adaptive immune response through their expression of cytokines that facilitate antibody presentation (e.g. IL-4).

Following exposure to LPS, microglia and macrophages both increase production of pro-inflammatory cytokines to facilitate phagocytosis of the endotoxin. Either an over-expression or under-expression of these inflammatory markers can have detrimental consequences in the ability of the innate immune system to respond to a challenge. Thus, the failure of LPS to elicit a cholinergic response in the hippocampus of PB-NSC rats could indicate an over-reaction of the innate immune response in the brain, resulting in a shift from senescent toward a pro-inflammatory and phagocytotic microglial phenotype. While other preclinical models of GWI have demonstrated that a chemical cocktail with PB induces increased density of microglial M1 phenotype (Parihar et al., 2013), our model provides a potential underlying mechanism for how this phenotypic shift in microglia could occur.

Increased neuroinflammation has multiple detrimental consequences on cognitive function, including sensitivity to neuronal loss and an increased risk for dementia (Van Gool, Van De Beek, & Eikelenboom, 2010). This increased risk for cognitive deficits is largely associated with the inability of microglia to perform appropriate neuroprotective roles to later insults. As such, veterans who were administered PB during the Gulf War may have experienced impairments in the cholinergic negative regulatory state of microglia, sensitizing them towards later insults which could exacerbate neural damage and contribute to cognitive dysfunction. Indeed,
some animal models of GWI have demonstrated both reduced hippocampal volume, mild inflammatory states, and increased density of microglia relative to controls (Parihar et al., 2013). Addressing PB and stress induced disruptions in cholinergic function and the inflammatory response is increasingly critical for veterans with GWI as aging is also associated with a decline in cholinergic transmission and chronic low-grade inflammation (Norden, Muccigrosso, & Godbout, 2015; Wong, 2013). As such, conditions during the Gulf War may accelerate neural decline associated with aging.

Conclusions

In sum, the current study demonstrated that PB changes the response of the central cholinergic system in the brain to various stimuli. This is critical as it demonstrates that regardless of whether PB did or did not cross the blood-brain barrier, PB compromises the function of central cholinergic systems in a brain-region specific manner. In the current study, a prior history of stress failed to exhibit the secondary increase in the cholinergic response to LPS in the hippocampus. In addition, rats with a prior history of PB but not stress also fail to exhibit this latent cholinergic response to LPS, mimicking the effects evidenced in rats with a prior repeated stress history. As these PB-effects were specific for the hippocampus, this suggests that the hippocampal cholinergic system may be particularly sensitive to PB-induced changes. Moreover, these PB-induced changes may be reflective of impairments in the appropriate neural response to cytokine-induced inflammatory signals in the brain as opposed to the vagal response. Deficits in the ability of the cholinergic system to adequately respond to stressful stimuli may directly impact cognitive function, contributing to cognitive deficits.
evidenced in veterans with GWI. In addition, the inability of the cholinergic system to adequately inhibit activation of microglia could make the brain vulnerable to secondary insults, contributing to the progressive nature of GWI in veterans. As cholinergic systems also become compromised with age, it is possible that veterans with GWI could exhibit accelerated cognitive decline with age and age-related diseases such as Alzheimer’s.
CHAPTER 4

EXPERIMENT THREE: GLUTAMATE IN GULF WAR ILLNESS

Although cholinergic systems in both the central and peripheral nervous system are clearly impacted by repeated exposure to PB, glutamatergic systems may be more sensitive to the interactive effects between PB and stress in GWI. Repeated stress alters the immune profile of the adult rodent brain as well as glutamatergic tone and glial cell functionality, creating compounding effects and sensitizing the system to later damage (Banasr et al., 2010; Hinwood, Morandini, Day, & Walker, 2012; Macht & Reagan, 2017; Mayhew, Beart, & Walker, 2015; Tynan et al., 2013). This is likely partially due to the unique juxtaposition of glutamatergic synapses with glia. Two primary glial cells which are closely associated with glutamatergic transmission and actively respond to immune challenges (e.g. stress, bacterial exposure, etc.) by producing and reacting to various immune signals (e.g. cytokines and chemokines) are astrocytes and microglia (Lee, Liu, Dickson, Brosnan, & Berman, 1993; Sugama, Takenouchi, Sekiyama, Kitani, & Hashimoto, 2011). The following sections will discuss the relationship between astrocytes, microglia, glutamate, and their intersection between immune function and stress as this may also be an intersection between PB, stress, and cognitive dysfunction in veterans with GWI.
The quad-partite synapse: Astrocytes, Microglia, and Glutamate

PB and stress may interact at glutamatergic synapses, interfering with plasticity of glutamatergic systems and driving several of the neurocognitive deficits evidenced in veterans with GWI. The potential for glutamatergic systems to be sensitive to stress and PB interactions is largely associated with the unique juxtaposition of glutamatergic synapses with surrounding glia. Glutamatergic synapses are quad-partite, consisting of glutamatergic pre- and post-synaptic terminals, astrocytes, and microglia (Schafer, Lehrman, & Stevens, 2013). Astrocytes modulate glutamate homeostasis, conditionally release vesicular glutamate, modulate blood-brain barrier permeability, coordinate neuronal activity via intracellular calcium waves, and regulate local cytokine release (Abbott, Rönnbäck, & Hansson, 2006; Bezzi et al., 2004; Chung & Benveniste, 1990; Cornell-Bell, Finkbeiner, Cooper, & Smith, 1990; Sugama et al., 2011). In contrast, microglia are a type of macrophage with highly ramified processes that rapidly extend and retract to survey the surrounding environment (Nimmerjahn, Kirchhoff, & Helmchen, 2005). They can shift between M₀, M₁ and M₂ phenotypes which are associated with a variety of functions including facilitation of long-term potentiation, cell surveillance, neurotransmitter release, phagocytosis, tissue repair, and production of both pro- and anti-inflammatory cytokines (Cherry, Olschowka, & O’Banion, 2014; Franco & Fernández-Suárez, 2015; Hanisch & Kettenmann, 2007; Kettenmann, Kirchhoff, & Verkhratsky, 2013). As such, glutamate may be a key intersection between neurotransmission, cognitive function, and the immune systems through its dynamic relationship with surrounding glia.
Perturbations in immune function by PB could have downstream consequences on glia and glutamatergic transmission. As glutamatergic systems and glia in the PFC and hippocampus are also sensitive to repeated stress, the glutamatergic quad-partite synapse may be a critical point where stress and PB collide, resulting in synergistic and detrimental consequences on neural transmission and cognitive function.

**Interactions between Stress and Glutamate**

Perhaps some of the most well-documented effects of stress on glutamatergic neurons are the effects of stress on dendritic morphology (S. Cook & Wellman, 2004; Gould, Woolley, Frankfurt, & Mcewen, 1990; Liston et al., 2006; R.J. Liu & Aghajanian, 2008; Jason J. Radley et al., 2008; Jason J Radley et al., 2006; Vyas, Mitra, Rao, & Chattarji, 2002; Watanabe et al., 1992; Woolley, Gould, & Mcewen, 1990) In males, chronic stress generally produces reductions in morphological complexity and density of mushroom spines across a variety of nonsocial (e.g. unpredictable or restraint stress) and social (e.g. resident-intruder and social isolation) stressors, suggesting that decreased glutamatergic tone is a common underlying consequence for the PFC and hippocampus in response to chronic stress paradigms during adulthood.

Repeated stress also produces functional deficits in glutamate. For example, five days of social defeat stress produces reductions in synaptic excitability in the PFC (Urban & Valentino, 2017), and fourteen days of unpredictable stress decreases excitatory afferent activation as well as glutamatergic responses to an acute immobilization stressor (Jett, Bulin, Hatherall, McCartney, & Morilak, 2017). Chronic restraint stress reduces basal glutamate levels in the mPFC, while augmenting the stress response to a
novel tail pinch (Luczynski, Moquin, & Gratton, 2015). In contrast, there is no increase in the glutamatergic response to an acute immobilization stress challenge in rats with a prior history of chronic unpredictable stress relative to stress-naïve rats, suggesting that chronic stress attenuates the glutamatergic response to homotypic but not heterotypic stressors (Jett et al., 2017). Collectively, these studies highlight the versatility of the glutamatergic response to stress. As such, examining more than one type of stressor is helpful in determining stress-induced changes in glutamatergic transmission. Examining more than one type of stressor is particularly important for a model of GWI as 1) PB may further complicate the glutamatergic response to stress, and 2) humans regularly experience both psychological and physical stressors.

**Interactions between LPS and Glutamate**

Because of the well-documented disruptions in immune function in veterans with GWI, testing the neurological consequences of an immune challenge in a model of GWI may provide insight into how the brain’s response to bacteria could exacerbate cognitive dysfunction in veterans with GWI. LPS exerts divergent effects on various brain regions. For example, while the PFC and hippocampus are sensitive to the effects of restraint stress, they are particularly resistant to LPS-induced inflammatory effects during adulthood. Up to 10 µg/mL LPS failed to elicit either a cytokine response or shifts towards “activated” microglial phenotypes in neuro-glial cultures from the PFC or hippocampus (W.-G. Kim et al., 2000).

However, stress can prime the immune system to a second inflammatory insult (Dantzer & Kelley, 1989; Dhabhar, 2014; Simmons & Broderick, 2005). Although a single
stress session is sufficient to induce this cross-sensitization in plasma cytokine response to an LPS challenge (J. D. Johnson et al., 2002), these effects on the brain are particularly variable depending on the region being examined. For example, a single exposure to inescapable tail shock sensitizes the hypothalamic, pituitary and cerebellar but not the cortical or hippocampal cytokine response to LPS challenge (J. D. Johnson et al., 2002), suggesting that the adult PFC and hippocampus are resistant to immune sensitization following an acute challenge of either LPS or stress. In addition, some evidence suggests that the PFC and hippocampus have different susceptibilities to oxidative stress following chronic isolation stress with the PFC being the more sensitive of the two (Zlatković et al., 2014). Due to the particular variations in resistance of the PFC and hippocampus to an LPS challenge, PB-induced disruptions in the quad-partite synapse may selectively sensitize these regions to an LPS challenge with the hippocampus being the more sensitive of the two regions. As such, veterans who took PB during deployment could be more sensitive to hippocampal glutamatergic changes following a bacterial infection with consequential disruption of proper functioning in learning and memory circuits.

Current Experiment and Hypotheses

To address whether PB and repeated restraint stress interact to prime the glutamatergic response to inflammatory stimuli, the current study used a rodent model of GWI in combination with *in vivo* microdialysis to examine the neurochemical response to an immune challenge and an acute stress challenge. The glutamatergic response was assessed in two brain regions: the PFC and the hippocampus. These brain
regions were targeted due to 1) the critical roles of glutamate in their facilitation of cognitive function, and 2) their different susceptibilities to stress-induced inflammation.

We tested the hypothesis that combinations of PB and stress increased the glutamatergic response to an innate immune challenge, thus potentially contributing cognitive deficits evidenced in veterans with GWI. Specifically, while neither the PFC nor hippocampus were hypothesized to react to LPS in vehicle-NSC rats, both regions were hypothesized to exhibit increases in the glutamatergic response following repeated restraint stress. PB was hypothesized to exacerbate this effect, and the PFC was hypothesized to be the most sensitive to the combined effects of PB and stress.

In addition, we tested whether an acute psychological stressor (restraint stress) produced similar deficits in the glutamatergic response in rats with a prior history of PB and repeated stress. Although PB is not hypothesized to impact basal levels of glutamatergic function, PB is hypothesized to interact with stress to produce impairments in the habituation of the glutamatergic response to the restraint stress challenge in both the PFC and hippocampus.

4.1 METHODS AND MATERIALS

Microdialysis samples were the same collections from the same rats used for acetylcholine analysis, and as such, all procedures including animal handling, the GWI paradigm, surgery, sample collection, and euthanasia were identical to Experiment Two. In each sample, acetylcholine was analyzed first, and glutamate was analyzed second. As such, samples went through one additional freeze-thaw cycle prior to glutamate
analysis. This order of analysis was performed as acetylcholine is more sensitive to
freeze-thaw cycles than glutamate. Results from the plasma collected during the first
and second microdialysis sessions will be discussed in this section.

**Cytokine Analysis**

Plasma cytokines in response to LPS were analyzed using a 12-Plex Th1/Th2 Bio-
Plex kit. Tail bleeds were performed immediately at the termination of the first
microdialysis session, approximately 3 hours after LPS administration. Blood was
collected on ice and then immediately spun down in a microcentrifuge at 4°C for 15
minutes \( \times 30,000 \text{ g} \). The plasma supernatant was aspirated and stored in centrifuge
tubes at -80°C for later analysis.

The Bio-Plex assay uses premixed coupled magnetic beads and detection
antibodies to detect 12 different rat cytokines. Solutions for the assay were brought to
room temperature 30 minutes prior to initiation of assay procedures. Eight four-fold
dilution standards were prepared using the provided diluent and reconstituted
standard, and samples were thawed and diluted 1:4 with diluent. 288 µL of 20x beads
were vortexed for 30 seconds and then diluted with 5472 µL of assay buffer for a total
volume of 5760 µL. 50 µL of the diluted beads were then added to be bottom of a 96-
well plate, followed by 50 µL of standards and samples. The assay plate incubated on an
orbital shaker plate for 1 hour at room temperature. After 3 washes with wash buffer,
25 µL of detection antibody was added to each well, followed by a 30 minute incubation
at room temperature on an orbital shaker. The detection antibody was washed from the
plate with 3 washes using wash buffer. 50 µL of SA-PE will be added to each well and the
plate was incubated on an orbital shaker at room temperature for 10 minutes. After the incubation, the plate was washed 3 times with wash buffer and then the beads were re-suspended in 125 µL assay buffer. After 30 seconds, the plate was read on a plate reader using high photomultiplier voltage. Results were interpreted from a logarithmic standard curve. Due to assay to assay variability, data at each time point were expressed as a percentage of the mean of their respective vehicle-NSC groups in order to allow clear interpretations of the data.

High Performance Liquid Chromatography for Glutamate

In random order from sample collection, 7.5 µL of previously frozen microdialysate sample was loaded onto an EiCom GU-GEL polymer resin based analytical column where glutamate was isolated from other biogenic compounds in interaction with a mobile phase consisting of 50 mM ammonium chloride-ammonia, 250 mg/L hexadecyltrimethylammonium bromide, and 10 µL/L of 5 mg/mL 2Na EDTA, pH 7.2. Afterwards, a glutamate oxidase enzyme column metabolized glutamate into hydrogen peroxide and 2-ketoglutalate. The hydrogen peroxide was oxidized at the platinum electrochemical detector with an applied current of +500 mV, resulting in O₂, 2H⁺, and 2e. The potential was read using the Epsilon computer controlled detector system from BASi. Concentration of glutamate in samples was interpolated against a three-point standard curve of 3, 1, and 0.1 µM glutamate.

Statistical Analyses

Results were calculated as a 2×2×16 mixed ANOVA for the LPS session and a 2×2×12 mixed ANOVA for the restraint challenge session. For between subjects, this
experiment had 2 levels of drug treatment (vehicle, PB), 2 levels of stress (NSC, stressed). Within-subjects repeated measures consisted of 16 levels, representing the 16 consecutive collections during microdialysis for the LPS challenge. The restraint stress challenge had a total of 12 collections: 4 baseline, 4 during restraint, 4 post-restraint. Following significant interactions, simple main effects post hoc tests were performed with Bonferroni post-hoc corrections for family-wise error.

Power analyses for in vivo microdialysis were performed a priori with an effect size of 0.8 and \( \alpha = 0.05 \); analyses suggested that a sample size of \( n=8 \) per group with a total \( N = 32 \) would be sufficient for achieving statistical significance. After correcting for statistical outliers and other complications during microdialysis and HPLC (e.g. erratic baseline, undetectable analytes, loss of cannula, broken probe), actual sample size for each condition was as follows:

<table>
<thead>
<tr>
<th>Condition</th>
<th>PFC LPS</th>
<th>PFC Restraint</th>
<th>Hippocampus LPS</th>
<th>Hippocampus Restraint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-NSC</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>9</td>
<td>6</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Vehicle-Stressed</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>PB-Stressed</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>

4.2 RESULTS

GW1 In Vivo Microdialysis: Glutamate

Basal Levels of Glutamate

On the first day of microdialysis, prior to the LPS challenge, neither prior history of PB nor restraint stress significantly impacted basal glutamate levels in the PFC, \( F(1, \)
Figure 4.1 Basal Levels of Glutamate on the First Day of Microdialysis. On the first day of microdialysis, prior to the LPS challenge, there was no effect of either PB or stress on basal glutamate levels in the PFC or hippocampus. This suggests that neither a history of PB nor restraint stress either independently or in interaction impact affect basal glutamate levels in either brain region.
22) = 0.62, \( p = 0.44 \), or hippocampus, \( F(1, 24) = 0.18, p = 0.67 \) (Figure 4.1). On the second day of microdialysis, prior to the restraint challenge, the hippocampus still did not exhibit any differences in basal acetylcholine levels between any groups, \( F(1, 18) = 0.03, p = 0.87 \). However, on the second day of microdialysis, there was a significant cross-over interaction between PB and stress on basal glutamate levels in the PFC, \( F(1, 21) = 12.32, p = 0.045 \) (Table 4.2). In this cross-over interaction, glutamate was reduced in PB-NSC and vehicle-stressed rats relative to vehicle-NSC and PB-stressed counterparts. This shift in basal glutamate levels in the PFC on the second day of microdialysis is difficult to interpret as it could be a function of several factors including differential recovery from probe damage, residual effects of LPS, or self-selection as the second day of microdialysis had a lower n-size due to experimental complications (e.g. probe breaking).

<table>
<thead>
<tr>
<th></th>
<th>PFC</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-NSC</td>
<td>1.18 ± 0.40</td>
<td>1.83 ± 0.88</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>0.58 ± 0.07*</td>
<td>0.55 ± 0.21</td>
</tr>
<tr>
<td>Vehicle-Stressed</td>
<td>0.58 ± 0.09*</td>
<td>3.37 ± 0.46</td>
</tr>
<tr>
<td>PB-Stressed</td>
<td>1.11 ± 0.29</td>
<td>2.38 ± 1.07</td>
</tr>
</tbody>
</table>

*significant cross-over interaction, \( p = 0.045 \); Data expressed as mean ±SEM (µM)
Figure 4.2. Glutamate Response to an LPS Challenge in the PFC. There was a main effect of drug treatment on glutamate levels following LPS in the PFC. Specifically, although glutamatergic systems showed no response to LPS in vehicle-NSC conditions, there was decreased glutamatergic signaling in response to LPS selectively in PB-treated rats starting at the 8th collection with the exception of collection 10. This suggests that PB treatment primes glutamatergic systems for later immune challenges. Decreased glutamate levels in the PFC following LPS could indicate sensitivity of this brain region to LPS-induced deficits in synaptic plasticity.

*: Significant effect of PB relative to vehicle-treated rats, $p < 0.05$
Figure 4.3. Glutamate Response to an LPS Challenge in the Hippocampus. Unlike in the PFC, vehicle-NSC rats exhibit a depression in glutamatergic levels in response to LPS, suggesting that this brain region may be more sensitive to the effects of LPS. PB-stressed rats fail to show this LPS-induced depression in glutamate, suggesting that PB and stress interact to influence how the hippocampus responds to a systemic stressor.

*: PB-stressed rats significantly different from vehicle-treated rats, $p < 0.05$
**LPS Challenge**

In the PFC, there is a significant interaction between time and PB treatment on the glutamatergic response to an LPS challenge, \( F(15, 360) = 3.82, p < 0.001 \) (Figure 4.2). Specifically, after intraperitoneal injection of LPS, glutamate levels significantly decreased in PB compared to vehicle-treated rats at collections 8-9 and 12-16, \( (p = 0.022, 0.008, 0.006, 0.010, 0.037, 0.016, \) respectively). These data suggest that PB impacts the glutamatergic response to an LPS challenge in the PFC, resulting in a significant reduction in glutamate levels.

In the hippocampus, there is a significant interaction between a prior history of PB treatment and time on the glutamatergic response to LPS, \( F(15, 360)=1.77, p = 0.037 \) (Figure 4.3). There is also a trend for an interaction between prior stress history and drug treatment on the glutamatergic response to LPS, \( F(1, 24)=4.00, p = 0.057 \). Specifically, while LPS reduces glutamate levels in vehicle-treated rats with a prior history of restraint stress, glutamate fails to decrease in response to LPS in rats PB-stressed rats relative to their vehicle-stressed counterparts at collections 9, 13, and 16, \( (p = 0.04, 0.037, \) and 0.004, respectively). This suggests that PB interacts with stress to influence the glutamatergic response to a novel systemic stressor in the hippocampus.

**Acute Restraint Challenge**

Prior stress history and prior drug history interacted over time to influence the glutamatergic response to an acute restraint challenge in the PFC, \( F(11, 209)=1.98, p=0.031 \) (Figure 4.4). Specifically, rats which experienced a prior restraint stress history failed to exhibit a glutamatergic response to the acute restraint challenge (collections 5
Figure 4.4. Glutamate Response to a Restraint Stress Challenge in the PFC. Restraint stress produces an acute increase in glutamatergic levels in the PFC during the restraint challenge, followed by a rapid return to baseline after the stressor is removed. There is no increase in glutamate levels in rats with a prior stress history in this brain region. In contrast, rats with a prior history of PB but not restraint stress exhibit climbing levels of glutamate even after removal of the stressor, possibly suggesting an inability of glutamatergic systems to recover from this experience.

*: significant effect of stress, $p < 0.05$

$: PB-NSC rats significantly different from vehicle-NSC rats, $p < 0.05$
Figure 4.5. Glutamate Response to a Restraint Stress Challenge in the Hippocampus. A prior history of restraint stress increases the glutamatergic response to a restraint stress challenge in non-stressed vehicle-treated rats but depresses the glutamatergic response to a restraint stress challenge in vehicle-treated rats. However, PB blocks this stress-adaptation of the glutamatergic response in rats with a prior stress history. This suggests that PB impairs the ability of glutamatergic circuits to adapt to prior experience, providing a potential underlying mechanism for the memory impairments evidenced in veterans with GWI.

*: Significant effect of prior stress history in vehicle-treated rats, $p < 0.05$

#: Significant effect of PB in rats with a prior stress history, $p < 0.05$
and 7), unlike vehicle-NSC rats, ($p = 0.008$ and 0.022, respectively). In contrast, glutamatergic levels in response to restraint remained elevated following release from restraints selectively in the PB-NSC condition (collections 11 and 12), suggesting that PB interferes with the ability of the glutamatergic system to recover from an acute stress challenge in the PFC, ($p = 0.015$ and 0.011, respectively).

In the hippocampus, there was a significant time × drug treatment, $F(11, 198) = 2.28, p = 0.01$, and time × stress interaction, $F(11, 198) = 3.87, p < 0.001$. Specifically, in rats with a prior history of restraint stress, there is a significant effect of drug treatment at collection 5, 6, 7, 8, and 9, ($p = 0.019$, 0.005, 0.041, 0.035, and 0.037, respectively). At each of these time points, a prior stress history decreased the glutamatergic response to restraint, but a combined history with PB blocked this stress-effect. This suggests that PB impairs the adaptation of the glutamatergic response in the hippocampus to psychological stress (Figure 4.5).

**Cytokine Response to LPS**

A prior history of PB selectively attenuated the LPS-induced increase in IL-6, $F(1, 24) = 7.30, p = 0.01$ (Figure 4.6). No other cytokines were significantly impacted by either stress or drug treatment, although there was a trend for TNF-α levels to be attenuated in PB-treated rats, $F(1, 24) = 4.19, p = 0.05$ (Table 4.3). This suggests that 1) IL-6 may be particularly sensitive to the effects of PB, and 2) a prior history of PB impairs innate immune system’s ability to mount an appropriate response to an LPS challenge.
### Table 4.3 Cytokine Levels Three Hours after LPS as a Percent of Vehicle—NSC

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-Stressed</th>
<th>PB-NSC</th>
<th>PB-Stressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>100.0 ± 4.1</td>
<td>76.7 ± 13.9</td>
<td>98.3 ± 7.4</td>
<td>95.22 ± 9.4</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0 ± 6.4</td>
<td>94.1 ± 19.3</td>
<td>78.8 ± 8.0</td>
<td>77.9 ± 11.6</td>
</tr>
<tr>
<td>IL-2</td>
<td>100.0 ± 4.2</td>
<td>75.9 ± 19.7</td>
<td>91.0 ± 7.8</td>
<td>94.3 ± 8.0</td>
</tr>
<tr>
<td>IL-4</td>
<td>100.0 ± 3.7</td>
<td>72.5 ± 20.5</td>
<td>88.1 ± 8.6</td>
<td>90.0 ± 7.7</td>
</tr>
<tr>
<td>IL-5</td>
<td>100.0 ± 2.1</td>
<td>79.8 ± 14.6</td>
<td>94.3 ± 5.6</td>
<td>95.5 ± 4.9</td>
</tr>
<tr>
<td>IL-6</td>
<td>100.0 ± 14.2</td>
<td>105.1 ± 32.2</td>
<td>56.0 ± 7.2*</td>
<td>65.2 ± 9.3*</td>
</tr>
<tr>
<td>IL-10</td>
<td>100.0 ± 4.0</td>
<td>76.8 ± 16.2</td>
<td>85.9 ± 7.3</td>
<td>87.9 ± 6.5</td>
</tr>
<tr>
<td>IL-12</td>
<td>100.0 ± 5.6</td>
<td>73.0 ± 21.7</td>
<td>86.4 ± 10.2</td>
<td>88.5 ± 11.1</td>
</tr>
<tr>
<td>IL-13</td>
<td>100.0 ± 6.8</td>
<td>76.4 ± 21.8</td>
<td>92.6 ± 11.2</td>
<td>98.9 ± 11.6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>100.0 ± 10.9</td>
<td>108.9 ± 23.2</td>
<td>66.2 ± 7.7#</td>
<td>88.1 ± 14.9#</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>100.0 ± 3.4</td>
<td>79.1 ± 15.4</td>
<td>83.4 ± 7.0</td>
<td>92.0 ± 8.2</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>100.0 ± 6.4</td>
<td>71.8 ± 22.7</td>
<td>86.9 ± 10.3</td>
<td>93.3 ± 11.0</td>
</tr>
</tbody>
</table>

* values are significantly different from Vehicle-NSC; #: trend for a significant effect relative to Vehicle-NSC rats

### 4.3 DISCUSSION

The current study used a rodent model of GWI to demonstrate that 1) the response of glutamatergic systems to systemic or psychological stress is disrupted synergistically by combinations of PB and repeated restraint stress, and 2) the effect of PB on glutamatergic systems is dependent upon the brain region, the type of challenge, and the stress history. In rats with no history of restraint stress, PB preferentially increases the glutamatergic response relative to vehicle-NSC rats following a restraint stress challenge in the PFC but not the hippocampus. In contrast, when LPS was administered as a systemic stress challenge, there was a decrease in the glutamatergic response to LPS in PB-treated rats regardless of their stress history. This suggests that PB disrupts how the glutamatergic system in the PFC processes novel stressful stimuli.

Because cortical activity has important consequences for how the hippocampus encodes...
Figure 4.6. Plasma IL-6 Response to an LPS Challenge: 3 hours Post-Injection. LPS increased cytokine levels in all rats relative to a sample of Day 14 tail bleeds from vehicle-NSC rats in the absence of LPS. However, the LPS-induced increase in IL-6 was significantly attenuated by a prior history of PB. This suggests that PB impairs the ability of the innate immune system to mount an appropriate response to a systemic stressor.

*: Significant effect of PB relative to vehicle-treated rats, $p < 0.05$

#: Significant effect of LPS, $p < 0.05$
a stressful event, shifts in cortical processing of stressful stimuli could have profound consequences on the hippocampal response following repeated exposures to stress.

When the stressor is unique to prior stressful experiences (e.g. LPS challenge is heterotypic to restraint stress), then the hippocampal glutamatergic response to that challenge is similar in vehicle-NSC rats to both vehicle-stressed and PB-NSC rats. However, in rats with both a history of PB and repeated restraint stress, LPS fails to induce a change in glutamatergic levels. This suggests that PB and repeated restraint stress interact to selectively attenuate how the hippocampus responds to a systemic stressor. This selective effect of PB and repeated restraint stress on glutamatergic systems is also evident following a restraint stress challenge. In this case, rats with a prior history of PB and restraint stress exhibit a parallel glutamatergic response to the restraint stress challenge as restraint stress naïve rats. That is, glutamatergic levels in the hippocampus are similar in PB-stressed rats as vehicle or PB-NSC rats following a restraint challenge. The failure of glutamatergic systems to adapt in the hippocampus to repeated exposures of a stressful stimulus could be attributable to a combination of factors. One possible mechanism is that PB-driven shifts in the cortical processing of stressful stimuli could impair the ability of the hippocampus to form an appropriate memory circuit to encode the stressful stimulus. Another possibility is that PB-driven changes in the cytokine response to stress may impair the ability of the glutamatergic systems to facilitate long-term potentiation in the hippocampus. The subsequent sections will address each of these possibilities.
**Cortical Processing of Stressful Stimuli**

In the PFC, a prior history of PB accentuates the glutamatergic response to restraint stress but attenuates the glutamatergic response to LPS in rats with no prior stress history. An important distinction between the glutamatergic response for each of these stressors is that the PFC is typically resistant to the effects of LPS but sensitive to the effects of restraint stress (Pablos et al., 2006; Moghaddam, 1993). As such, a decrease in the glutamatergic response following LPS administration in PB-treated rats could indicate that PB increases the sensitivity of the PFC to inflammatory effects. In addition, glutamatergic levels in the PFC fail to recover following the termination of restraint stress in rats with no prior restraint history. This suggests that PB alters the cortical processing of novel stressful stimuli such that when veterans returning from the Gulf War are subjected to new stressors, glutamatergic systems may not respond in an adaptive manner. For example, climbing glutamate levels following the termination of restraint stress in PB-treated rats could create a predisposition to neurotoxicity following psychological stress. In contrast, depression of glutamatergic levels in response to LPS may indicate sensitivity to sickness behaviors, including impairments in complex cognitive tasks (Moghaddam, Adams, Verma, & Daly, 1997; Verma et al., 1996; Walker et al., 2013). Further studies would need to be conducted to discern whether LPS exacerbates cognitive deficits following PB and whether PFC systems are sensitized to excitotoxicity following a restraint challenge.

**Cytokines and Glutamate**

Following an LPS challenge, rats with a prior history of PB treatment exhibit an
attenuated response of IL-6 to this challenge. Although we measured IL-6 levels in the periphery, studies have demonstrated that brain levels of IL-6 follow a similar pattern to plasma levels at this time point after LPS administration (Erickson & Banks, 2011). IL-6 is intimately linked with memory formation in the hippocampus, although its role in the facilitation or suppression of memory formation is extremely complex. For example, early studies using ex vivo hippocampal slice electrophysiology demonstrated that high levels of IL-6 impair LTP (A.-J. Li, Katafuchi, Oda, Hori, & Oomura, 1997). Other studies used knockout models for IL-6 to demonstrate that IL-6 -/- mice are protected against LPS-induced deficits in Morris water maze performance (Sparkman et al., 2006). This idea that IL-6 impairs learning and memory predominated the literature for several years.

However, a more complex role of IL-6 in health and disease began to emerge in the late 1990s (Gadient & Otten, 1997). This evolution of pleiotropic roles of cytokines parallels the expansion of our understanding for the complex relationships between cytokines and neurotransmitter systems. IL-6, among other cytokines, remains an important point of communication between neurons and glia in normal development (Prieto & Cotman, 2017). For example, mouse IL-6 knockout models show profound deficits in recognition memory and spatial memory relative to wild-type mice (Baier, May, Scheller, Rose-John, & Schifflerholz, 2009; Hryniewicz, Bialuk, Kamiński, & Winnicka, 2007). In addition, other ex vivo and in vivo studies have demonstrated that LTP induces IL-6 expression (Balschun et al., 2004), and that the induction of IL-6 is critical for modulating the temporal specificity of LTP within local circuits. As the
induction of IL-6 following a tetanus is mediated by NMDA receptors, this suggests that changes in glutamatergic signaling in response to a stressful stimulus could directly impact the local cytokine response, which consequently would impact the specificity of the plasticity of local circuits. Other studies have found a similar relationship between glutamate and IL-6, with one study suggesting that genetic overexpression of IL-6 produces an excitatory-inhibitory shift by stimulating the formation of excitatory synapses and interfering with the formation of inhibitory synapses in the hippocampus (Wei et al., 2012).

This expanding view of the role of IL-6 in memory suggests that under normal conditions, IL-6 is an important mediator of memory formation through its interaction with excitatory and inhibitory circuits, and deviations in the relationship between glutamate and IL-6 may impair the formation of appropriate memory-dependent plasticity in circuits. Following an LPS challenge, rats with a history of both stress and PB exhibit no change in glutamate levels in the hippocampus in addition to deficits in levels of peripheral IL-6. If these peripheral levels of IL-6 parallel that in the hippocampus, it is possible that the cellular dynamics between IL-6 and glutamate in facilitating the appropriate formation of memory-circuits has been disrupted, resulting in deficits in plasticity in this system.

However, cytokines often work in concert to achieve different effects. As such, it is important to note that we also saw a trend for decreased TNF-α ($p = 0.05$) in PB-treated rats following LPS administration. Like IL-6, TNF-α is also intimately linked with glutamate and LTP. However, whereas IL-6 primarily interacts with NMDA receptors,
TNF-α upregulates AMPA receptors to influence synaptic scaling (Beattle et al., 2002). Thus, TNF-α is also an important mediator of cellular mechanisms underlying memory formation. Combined deficits in levels of IL-6 and TNF-α would produce compounding effects on the efficacy of glutamatergic systems to facilitate event-driven plasticity in the central nervous system. As such, this shift in the signaling between cytokines and excitatory neurotransmission may underlie some of the memory-deficits evidenced in veterans with GWI. Future studies will need to verify 1) if these peripheral changes in IL-6 match brain levels in this model and 2) if manipulation of either IL-6, TNF-α, or glutamate through agonists and antagonists can shift memory performance in a model of GWI.

**Conclusions**

In sum, a history of PB and repeated restraint stress interact to preferentially impact the glutamatergic response in the hippocampus to either a systemic or psychological stress challenge. In contrast, PB preferentially impacts how the PFC processes novel stressful stimuli. These results suggest that alterations in the ability of excitatory neurotransmitter systems to respond appropriately to stressful events may underlie memory consolidation problems in veterans with GWI.
Collectively, these studies provide insight as to how PB and stress may have independently and in combination adversely impacted peripheral and central physiology, thus providing a potential mechanism by which PB and stress may have caused impairments in veterans from the Gulf War. This discussion will therefore first summarize the independent effects that PB and stress each exhibited on physiology and behavior. Secondly, the synergistic effects between PB and stress will be discussed in the context of modeling the pathogenesis of GWI.

5.1 EFFECTS OF PB ALONE

There has been a long-standing debate about the effects of PB on physiology. As an effective treatment for the muscular disorder myasthenia gravis with decades of history, long-term detrimental effects of PB when given to soldiers was not anticipated (J. E. Cook, Kolka, & Wenger, 1992). In addition, PB was not thought to cross the blood brain barrier and therefore not anticipated to have any cognitive effects. However, our study demonstrates that PB, even in the absence of stress, changes both peripheral physiology and central neurochemistry (Figure 5.1).
Figure 5.1. Impact of PB in NSC Rats. On the last day of treatment (day 14), PB decreases AChE activity but produces robust increases in both Th1 and Th2 cytokines. Although PB in the absence of stress does not impact freezing behavior in a conditioned freezing paradigm at any time point, PB does produce several central neurological changes one-week post treatment. Generally, cholinergic systems exhibit an attenuated response to challenges in both brain regions. The effect of PB on glutamate levels is mixed with selective effects on the glutamatergic response in the PFC. As such, PB may prime cortical systems to shift processing of novel stressful events, which could have consequences for how veterans processed stressful stimuli upon returning from deployment.
**PB Disrupts the Innate Immune System**

One of the most surprising peripheral effects evidenced in PB-treated rats was the effect on the immune system. Cholinesterase inhibitors, like PB, have typically been considered anti-inflammatory through their ability to augment acetylcholine levels in the parasympathetic nervous system and increasing activity at nicotinic α7 cholinergic receptors on tissue macrophages as part of the vagus anti-inflammatory pathway (V. A. Pavlov et al., 2009; Tracey, 2002). However, this anti-inflammatory effect of cholinesterase inhibitors is typically examined in the context of another pro-inflammatory stimulus such as LPS or a disease state such as delirium, encephalomyelitis, or Alzheimer’s (Nizri et al., 2008; V. A. Pavlov et al., 2009; Reale et al., 2004; Van Gool et al., 2010). While we saw an anti-inflammatory effect of PB in the context of stress, our study also demonstrated that in the absence of another inflammatory stimulus, repeated administration of PB disrupts the innate immune response, increasing levels of both pro- and anti-inflammatory cytokines. At basal levels, this effect of PB on inflammation appears transitory as it disappears within a week of the cessation of treatment. However, an LPS challenge at one-week post treatment demonstrates that PB continues to disrupt the ability of the innate immune system to adaptively respond to an immune challenge: PB-treated rats exhibit an attenuated response of IL-6 and TNF-α relative to controls. These results suggest that it is likely that the effects of PB on the innate immune system provides the first catalyst to tip the homeostatic balance of physiological systems, thereby priming the organism to the effects of a later stressor.
**PB Disrupts the Central Cholinergic System**

A major point of contention with the administration of PB in soldiers is whether it crossed the blood brain barrier. While some evidence suggests that stress creates a leaky blood brain barrier, allowing PB to cross directly into the central nervous system (Abdel-Rahman et al., 2002), other studies have failed to replicate these findings (Amourette et al., 2009; Grauer et al., 2000; Kant et al., 2001; Song et al., 2002; Tian et al., 2002). Our study suggests that regardless of whether PB does or does not cross the blood-brain barrier, it produces profound effects on central cholinergic function. Importantly, the effects of PB on the central nervous system are not evident in the absence of a stimulus which would challenge the cholinergic system. This could explain some of the inconsistent results with previous literature on how PB impacts brain acetylcholine levels.

Generally, following either an immune or stress challenge, a prior history of PB attenuates the cholinergic response. As a history of restraint stress also attenuates the cholinergic response to a challenge, these data suggest that PB mimics the effect of stress on cholinergic systems but does not exacerbate the effects of stress in rats with a history of both PB and restraint stress. These effects of PB on central cholinergic function have multiple consequences for cognition and could provide insight as to how central cholinergic systems respond differently in veterans with GWI, thus providing a mechanism explaining their cognitive deficits. In particular, these results suggest that PB may impair cortical cholinergic processing of novel stressful stimuli.
Post-deployment in veterans is associated with a variety of novel stressors including underemployment, financial decline, marital stress, substance abuse, and stress associated with post-deployment care and hospitalization from war-related injuries (Spelman, Hunt, Seal, & Burgo-Black, 2012). Veterans administered PB during deployment may process these stressors differently. Not only would an attenuated cholinergic response to these types of stressors impair cognitive functioning, but it would also decrease the ability of central cholinergic systems to properly regulate microglial inflammatory phenotypes. As such, veterans who were administered PB during the war may also be more sensitive to the inflammatory consequences of post-war stressors. As rates of GWI continue to climb and symptoms in veterans become more exacerbated over time (B. Li, Mahan, Kang, Eisen, & Engel, 2011), shifts in how the cholinergic system responds to new stressful events may be the catalyst for the progressive state of this illness.

5.2 EFFECTS OF STRESS ALONE

Repeated restraint stress produces several changes in the periphery, the brain, and behavior (Figure 5.2). One week after the end of restraint stress, rats with prior stress histories exhibit impairments in contextual fear conditioning, suggesting a memory deficit. This deficit in conditioned freezing is paralleled by shifts in both cholinergic and glutamatergic responses to stressful challenges in the PFC and hippocampus. Our results parallel a plethora of literature which suggests that
Figure 5.2. Impact of Restraint Stress in Vehicle-treated Rats. Stress increases corticosterone levels following repeated restraint stress in vehicle-treated rats and increases IL-1α and IL-5 on the last day of treatment. Conditioned freezing is also attenuated one week following the end of treatment but increased three months post-treatment. Cholinergic and glutamatergic systems also demonstrated robust stress-induced adaptations to LPS and restraint stress challenges with parallel responses between the PFC and hippocampus. A prior history of restraint stress reduces the cholinergic response to stress ubiquitously. However, glutamate decreases in response to restraint stress but responds like vehicle-NSC rats to a novel stressor (LPS).
neurological systems adapt to repeated stress. Interestingly, glutamatergic and cholinergic systems exhibit divergent responses depending on the type of stress challenge (i.e. LPS versus restraint stress). A prior history of restraint stress reduces the cholinergic response to a novel (LPS) or restraint stress challenge. The glutamatergic response to a restraint stress challenge is also decreased in vehicle-stressed rats relative to vehicle-NSC rats. However, glutamatergic systems do not exhibit this attenuated response to a novel challenge, such as LPS. This divergent response of glutamate following repeated restraint stress closely parallels the glucocorticoid response to homotypic and heterotypic stressors (Kopp, Wick, & Herman, 2013; Lui et al., 2012). As pyramidal neurons are extremely sensitive to the effects of repeated stress, exhibiting highly reproducible glucocorticoid-induced shifts in morphology (B. McEwen & Morrison, 2013), it is possible that glucocorticoids in part mediate these divergent patterns in the glutamatergic response to an LPS and restraint stress challenge in vehicle-treated rats with a prior history of repeated restraint.

5.3 SYNERGISTIC EFFECTS OF PB AND STRESS

Independently, PB and stress produce several changes in both peripheral markers, the central nervous system, and behavior (Figure 5.3). PB and stress exhibit synergistic effects on conditioned freezing one week following the cessation of treatment, suggesting that PB exacerbates memory deficits of repeated stress. These memory deficits are paralleled by shifts in neurochemical processing of an LPS or restraint stress challenge. However, while the shifts in the neurochemical response to
Figure 5.3. Interaction between Restraint Stress and PB. Acutely, PB decrease AChE while stress increases corticosterone. PB in the context of stress exhibits anti-inflammatory effects on cytokines. Conditioned freezing is also transiently decreased one week following the end of treatment. Hippocampal systems are particularly sensitive to the synergistic effects of PB and stress at this time. For example, glutamatergic levels in the hippocampus fail to respond to an LPS challenge, unlike both vehicle-NSC and vehicle-stressed rats which exhibit a decrease in glutamate in response to LPS in the hippocampus. In addition, while glutamatergic and cholinergic systems in vehicle-stressed rats exhibit an attenuated response to restraint stress, PB-stressed rats respond in parallel to vehicle-NSC rats.
these challenges in the PFC are associated with the effects of PB or stress independently, PB and stress synergistically impact hippocampal neurochemistry so that the combined effects of PB and stress on hippocampal function diverge from vehicle-stressed counterparts. For example, following a restraint stress challenge, both cholinergic and glutamatergic systems in PB-stressed rats respond like the systems in vehicle-NSC rats instead of vehicle-treated stress counterparts. In addition, glutamatergic but not cholinergic systems in the hippocampus are uniquely impacted following a restraint stress challenge, where unlike all other groups, glutamatergic systems in PB-stressed rats fail to respond to an LPS challenge in the hippocampus. These results suggest that glutamatergic systems in the hippocampus are the most robustly affected by the combined effects of PB and repeated restraint stress. Because glutamatergic systems are also sensitive to inflammation due to the close juxtaposition of their synapses with microglia and astrocytes, dysregulation of glutamate may be compounded by the robust effects of PB and stress on immune function, exacerbating deficits in neural plasticity and increasing sensitivity to damage from oxidative stress. This could suggest that targeting either cytokines or glutamate may be an optimal treatment strategy for reversing cognitive deficits in veterans with GWI or slowing the progression of the illness.

**Modeling Peripheral Interactions between PB and Stress**

The most likely point for PB and stress to have interacted peripherally to produce these changes in brain neurochemistry and behavior is the immune system (Figure 5.4).
PB increases peripheral acetylcholine levels through inhibition of AChE. Increased acetylcholine would increase activity at nicotinic receptors on macrophages, which would inhibit cytokine release, and chromaffin granules in the adrenal medulla, which would release epinephrine and norepinephrine to stimulate adrenergic receptors on macrophages and increase cytokine release. Stress also activates the sympathetic nervous system to release epinephrine and norepinephrine from the adrenal medulla as well as activate the HPA axis to increase glucocorticoid release from the adrenal cortex. Glucocorticoids inhibit the inflammatory response through activity on glucocorticoid receptors within the macrophage cytoplasm. These conflicting points of interaction between PB and stress would dysregulate macrophage-mediated inflammatory responses, which would provide feedback on the state of peripheral inflammation to the central nervous system via afferents of the vagus.
By inhibiting cholinesterases and thereby augmenting acetylcholine levels, PB would increase interactions between acetylcholine and cholinergic receptors across the parasympathetic nervous system, including via receptors on both macrophages and on the adrenal glands (H. Wang et al., 2003; S. P. Wilson & Kirshner, 1977). Cholinergic stimulation of chromaffin granules in the adrenal medulla results in the release of the catecholamines epinephrine and norepinephrine as part of the sympathetic nervous system, also colloquially termed the “fight or flight response.” Because stress also activates the sympathetic nervous system, it is possible that the noradrenergic response to stress would be augmented following PB. Norepinephrine activates α-adrenergic receptors on macrophages to stimulate NF-κB and consequently release a variety of cytokines including TNF-α, IL-6, and IL-1β (Bierhaus et al., 2003). However, increased acetylcholine would also interact with nicotinic α7 receptors on macrophages which would inhibit NF-κB and attenuate inflammation. Stress further complicates regulation of immune function as it also has a bimodal influence on inflammation, first increasing cytokines through noradrenergic signaling but also inhibiting NF-κB through glucocorticoid signaling in macrophages (Raison, Capuron, & Miller, 2006). Because of the complicated relationship between macrophages and the appropriate modulation of inflammation, PB may exacerbate the effects of stress and further disrupt homeostatic mechanisms modulating proper inflammatory responses. This hypothesis is supported by the fact that in response to an LPS challenge, IL-6 and TNF-α are attenuated in plasma of PB-treated rats relative to vehicle-treated rats, suggesting that PB impairs the ability of the immune system to mount an appropriate inflammatory response.
Dysregulation of inflammatory networks has important implications for neural function, as cytokines act directly on vagus afferents to provide neural feedback of the peripheral inflammatory state. In addition, cytokines can directly influence neural activity by either crossing the blood-brain barrier or stimulating glial cells by the blood-brain barrier. As such, interactions between stress and cytokines may provide the first step towards cognitive dysfunction evidenced in veterans with GWI. This hypothesis is supported by clinical studies which consistently demonstrate that immune dysfunction is a hallmark feature of GWI (Broderick et al., 2011, 2013; Khaiboullina et al., 2015; Zhang et al., 1999).

**Modeling PB and Stress and the Cellular Mechanisms for Learning and Memory**

In addition to interacting at multiple points in peripheral systems, stress and PB also produce many convergent and synergistic changes how the brain responds neurochemically to either an LPS ([Figure 5.5](#)) or restraint stress challenge ([Figure 5.6](#)). As these models demonstrate, some of the neurochemical changes in PB-stressed rats are driven independently by PB or stress whereas others are a result of synergistic effects between PB and stress. For example, while PB and stress both independently and in combination produce decreases in the cholinergic and glutamatergic response to a challenge (either LPS or restraint stress) in the PFC, glutamatergic systems in the hippocampus continue to respond as if they have no prior stress history. This failure in hippocampal glutamatergic systems to adapt to repeated stressors could have multiple causes and consequences. For example, decreases in cortical processing of stressful
**Figure 5.5. Neurochemistry of LPS in a model of GWI.** **A.** Cholinergic terminals project diffusely across the PFC to modulate the excitatory-inhibitory balance via muscarinic (mACh) and nicotinic (nACh) receptors. A history of stress decreases the cholinergic response to LPS in both vehicle and PB-treated rats. **B.** PB decreases glutamate in the PFC in response to LPS, irrespective of stress-history. This decrease in glutamate could be attributable to reductions in local signaling or afferents from distal brain regions, such as the Thalamus. **C.** Decreases in cholinergic and glutamatergic processing of new stressful stimuli could provide an underlying mechanism for impairments in PFC-driven cognitive function in veterans with GWI. **D.** Both PB and stress decrease the cholinergic response to LPS in the hippocampus. PB also decreases plasma levels of IL-6 and TNF-α. In the brain, IL-6 is regulates the temporal specificity of LTP; TNF-α regulates stability of synaptic plasticity my mediating AMPA trafficking. **E.** Combinations of PB and stress produce synergistic effects on the glutamatergic response to LPS in the hippocampus. **F.** Combined deficits in cholinergic, glutamatergic, and cytokine systems could impair the stability of LTP and thus decrease memory function.
Figure 5.6. Neurochemistry of Restraint Stress in a Model of GWI. A. A prior history of PB or restraint stress both independently and in combination decrease the cholinergic response to a stressful stimulus in the PFC. B. A history of stress decreases the glutamatergic response to stressful stimuli in the PFC. This decrease in glutamate could be attributable to reductions in local signaling or afferents from distal brain regions, such as the Thalamus. C. Decreases in cholinergic and glutamatergic processing of new stressful stimuli could provide an underlying mechanism for impairments in PFC-driven cognitive function in veterans with GWI. D. While both PB and stress decrease the cholinergic response to stressful stimuli in the hippocampus independently, combinations of PB with stress synergize to increase the cholinergic response to a restraint challenge. E. PB and stress also produce synergistic effects on the glutamatergic response to a stressful challenge. F. Failure in the cholinergic and glutamatergic systems' adaptation to stress in the hippocampus could either be reflective of impaired hippocampal-dependent memory in veterans with GWI.
stimuli induced by PB and stress could impair the salience of the stressful event which would have downstream effects on memory-formation.

In addition, if IL-6 and TNF-α are decreased in the hippocampus like they are in the plasma in response to LPS, then this would have important consequences for the appropriate assimilation of the cellular mechanisms for memory: LTP. IL-6 and TNF-α are constituentely expressed under normal physiological conditions. IL-6 is associated with proper temporal tuning of LTP, exhibiting a negative regulatory feedback. TNF-α facilitates AMPA receptor trafficking, influencing the stability of newly formed synapses. Either too much or too little IL-6 or TNF-α produces deficits in synaptic plasticity and the stability of new memories. As such, deficits in cortical processing of stressful stimuli in addition to deficits in cytokine-mediation of synaptic plasticity could underlie some of the surprising neurochemical responses in the hippocampus to PB and stress. These shifts in the neurochemical response to a stressful challenge provide a potential mechanism underlying cognitive deficits in soldiers with GWI.

Our studies provide insight as to how shifts in acetylcholine, glutamate, and cytokines may be influencing the cellular processes underlying learning and memory in a model of GWI. In addition, as deficits in contextual fear conditioning in PB-stressed rats appear transient, these acute shifts in cognitive processing of stressful stimuli may have important consequences for maintenance and progression of GWI as veterans returning from deployment face a host of stressors upon reintegrating into society. An inability of the central nervous system to adapt to repeated stressors may increase the sensitivity to neural damage from oxidative stress, thus exacerbating the effects of PB and stress. If
shifts in cognitive processing in response to recurrent stressors is a factor in perpetuating the progression of GWI, treatment strategies would need to emphasize two primary goals: 1) to stop the progression of GWI, and 2) to reverse physiological and cognitive deficits in GWI.

5.4 FUTURE DIRECTIONS AND TREATMENT STRATEGIES

The current experiments highlighted the importance for discerning interactions between stress and PB over time. As such, future microdialysis studies will need to determine if the effects of LPS and a restraint stress challenge shift at three-months post-treatment time point. In addition, while the current study examined changes in peripheral cytokines, future studies will need to determine if brain cytokine levels parallel those of plasma in this model of GWI. However, a priority of future studies should also be to examine potential treatment options. With veterans from the Gulf War continuing to age, the search for appropriate treatment strategies is becoming increasingly urgent.

This hunt for appropriate treatment strategies is complicated due to the multifactorial nature of the illness, and it is possible that different treatments will be necessary to target different symptoms. There has been some success in identifying potential interventions which modestly improve cognitive symptoms of GWI. For example, one study found that twelve weeks of cognitive behavioral therapy in combination with mild aerobic exercise provided a mild improvement in cognitive function, although these improvements were transient and declined post-therapy
(Donta et al., 2003). Other symptoms such as chronic pain proved to be treatment-resistant. Antioxidant treatment in Gulf War veterans yielded similar results with improvements in cognitive functioning but no improvements in other symptoms including fatigue or hyperalgesia (Baraniuk, El-Amin, Corey, Rayhan, & Timbol, 2013).

As preclinical and clinical studies have routinely suggested alterations in immune function in veterans with GWI, directly targeting immune function may be a successful treatment strategy. Our studies support this hypothesis as PB produced robust effects on the innate immune system over time, demonstrating shifts in both basal cytokine levels and an impaired cytokine response to LPS. However, because of the unique conditions of the Gulf War, restoring proper balance in the immune response may be complicated. For example, cholinesterase inhibitors have been used as anti-inflammatories in various disorders of neuroinflammation, but the chronic disruption of cholinesterase activity in PB-stressed rats suggests that use of a cholinesterase inhibitor may not be successful in these populations.

Because of the combined deficits in both central and peripheral systems, targeting the vagus nerve may be an alternative strategy. The vagus nerve provides a link between the brain and the body, and stimulation of the vagus nerve produces a strong anti-inflammatory effect (Borovikova et al., 2000). Stimulation of the vagus nerve has been proposed as an effective treatment strategy for a variety of inflammatory disorders, including treatment-resistant depression (Rush et al., 2000; Sackeim et al., 2001), and experimentally-induced pain (Kirchner, Birklein, Stefan, & Handwerker, 2000). Because hyperalgesia is one of the more difficult symptoms to treat in GWI, vagal
stimulation could potentially provide a unified treatment for cognitive deficits as well as fatigue and hyperalgesia.

Another potential treatment strategy for GWI would be to target the kynurenine pathway. Although kynurenic acid was originally considered an inert metabolite of tryptophan degradation in the kynurenine pathway, it is now associated with a wide range of physiological functions in both the central and peripheral nervous systems (Moroni, Cozzi, Sili, & Mannaioni, 2012). Centrally, kynurenic acid competitively antagonizes hippocampal glutamatergic NMDA (Perkins & Stone, 1985) and noncompetitively antagonizes cholinergic α7nACh receptors (Hilmas et al., 2001). In addition, kynurenic acid acts as an endogenous antioxidant in a mechanism that is independent from its glutamatergic or cholinergic interactions (Lugo-Huitrón et al., 2011). Due to the widespread physiological effects of kynurenic acid in the brain, its unsurprising that kynurenic acid has been linked to a variety of disorders. For example, increasing kynurenic acid by inhibiting kynurenine 3-monooxygenase (KMO), an enzyme which regulates a divergent metabolic pathway, is neuroprotective in a mouse model for Alzheimer’s and Huntington’s disease (Zwilling et al., 2011). Peripherally, decreased production of kynurenic acid and overproduction of 3-hydroxykynurenine by KMO is positively associated with IL-6 and TNFα, oxidative stress, and cardiovascular disease (Q. Wang, Liu, Song, & Zou, 2016), all of which have been implicated in GWI. Therefore, increasing kynurenic acid by antagonizing KMO could provide a novel and unified treatment strategy for GWI that targets both peripheral and central symptoms.
5.5 CONCLUSIONS

The current studies provide insight into neurochemical, behavioral, and physiological effects of chronic PB treatment and stress. Although the primary hypotheses was that PB and stress interact to cause deficits in GWI, our study found that some effects were primarily driven by PB, some by stress, and others by synergistic combinations of PB and stress. The current studies also provided insight towards the controversial debate of whether PB can impact the central nervous system. Although these studies did not assess whether PB crossed the blood-brain barrier, they did demonstrate that PB impacts central cholinergic function regardless of this fact.

The effect of PB on central cognitive function is complex, varying by both stimulus and brain region. Importantly, since PB did not impact basal levels of acetylcholine, this suggests that the effects of PB are stimulus-dependent. In general, while these effects of PB on acetylcholine were not synergistic with stress, PB and stress did produce synergistic effects on glutamatergic function with the hippocampus being particularly sensitive to these interactions. This would suggest that in GWI, glutamatergic systems – particularly hippocampal glutamatergic systems – may be a prime target for intervention and treatment strategies in veterans with GWI.

Currently, GWI remains treatment-resistant with symptoms continuing to become exacerbated with time. Our study highlights that PB and stress interact to produce unique changes in the brain’s neurochemistry, thus potentially providing a mechanism underlying cognitive dysfunction in veterans with GWI. Understanding the neurochemical deficits in Gulf War Illness is an important precursor to providing
effective treatment strategies for this treatment-resistant population. This treatment-centered objective is urgent as veterans from the Gulf War continue to age. Thus, finding successful treatment strategies to either halt or reverse the progression of GWI is paramount in a race-against time. Moreover, as PB remains approved as a nerve-agent pretreatment drug, these studies highlight the importance of using this drug cautiously in soldiers as it produces several long-term detrimental changes to vertebrate physiology.


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