Progressive Neurochemical, Neuroinflammatory and Cognitive Deficits in an Experimental Model of Gulf War Illness

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PROGRESSIVE NEUROCHEMICAL, NEUROINFLAMMATORY AND COGNITIVE DEFICITS IN AN EXPERIMENTAL MODEL OF GULF WAR ILLNESS

by

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DEDICATION

This work is dedicated to my mom. I am forever grateful for your selflessness and unconditional love.
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ABSTRACT

Gulf War Illness (GWI) is a multi-symptom illness that presents with cognitive disturbances and immune dysregulation and continues to affect over 25% of Gulf War veterans. While soldiers were exposed to several hazards in the Gulf, the prophylactic use of the acetylcholinesterase inhibitor, pyridostigmine bromide (PB) and war-related stress have been identified as chief factors in GWI pathology. As both PB and stress alter acetylcholine, a critical mediator of cognition and inflammation, the focus of my dissertation work was investigating the lasting effects of PB and stress on peripheral and central cholinergic signaling. Specifically, I assessed hippocampal-dependent learning and memory, inflammatory markers, and cholinergic neurochemistry in a previously established rat model of GWI consisting of PB treatment and repeated restraint stress (RRS). Interestingly, many clinical studies have found that GWI patients exhibit exaggerated cognitive deficits following a stressful stimulus such as an exercise challenge. Thus, I also investigated how an innate immune or stress challenge may alter cholinergic responses and behavior. Results indicate that a history of PB treatment elicits 24-hour hippocampal-dependent memory deficits when challenged with low-dose lipopolysaccharide (LPS, 30 µg/kg) or the stress of swimming. However, PB-treated rats did not exhibit any impairments in memory acquisition. By assessing peripheral and central inflammatory responses to LPS, I report that PB treatment produces exaggerated inflammatory responses that emerge long-after treatment cessation and likely contribute to the observed 24-hour memory impairments. Lastly, using in vivo microdialysis, I found
that within the hippocampus, PB-treated rats exhibited potentiated cholinergic responses to both immune and stress challenges over three months after treatment cessation. Conversely, this potentiated cholinergic response was only observed in the PFC of rats previously subjected to PB and RRS and challenged with LPS. These findings suggest that PB has lasting effects on hippocampal-dependent memory and dysregulates the cholinergic anti-inflammatory pathway in this region, but not the PFC. My studies also illustrate the latent phenotype of GWI and how deficits are best observed following a physiological stressor. Lastly, my results identify the cholinergic anti-inflammatory pathway as a potential site of therapeutic intervention to alleviate the progressive cognitive deficits affecting GWI patients.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotrophin hormone</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BF</td>
<td>basal forebrain</td>
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<tr>
<td>BW</td>
<td>body weight</td>
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<tr>
<td>ChE</td>
<td>cholinesterase</td>
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<tr>
<td>CINC-3</td>
<td>cytokine-induced neutrophil chemo-attractant 3</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CORT</td>
<td>corticosterone</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>DoD</td>
<td>Department of Defense</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GW</td>
<td>Gulf War</td>
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<tr>
<td>GWI</td>
<td>Gulf War Illness</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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</table>
IFN-γ ................................................................. interferon-gamma
IL ................................................................. interleukin
ITI ................................................................. intertrial interval
LPS ................................................................. lipopolysaccharide
LTP ................................................................. long-term potentiation
mAChR ........................................................... muscarinic acetylcholine receptor
MG ................................................................. Myasthenia gravis
MIP-2 .............................................................. macrophage inflammatory protein 2
MWM ............................................................... Morris water maze
nAChR ............................................................. nicotinic acetylcholine receptor
NF-κB ............................................................. nuclear factor kappa B
NOR ............................................................... novel object recognition
NSC ................................................................. non-stressed control
NTS ................................................................. nucleus tractus solitarius
OP ................................................................. organophosphate
PAM ............................................................... positive allosteric modulator
PB ................................................................. pyridostigmine bromide
PFC ................................................................. prefrontal cortex
PTSD .............................................................. post-traumatic stress disorder
RRS ................................................................. repeated restraint stress
TNF-α ............................................................. tumor necrosis factor alpha
U.S. ............................................................... United States
CHAPTER 1

GENERAL INTRODUCTION

1.1 Gulf War Illness

The Gulf War (GW) began on August 2, 1990, after Iraqi leader, Saddam Hussein, called for the invasion and occupation of Kuwait (Kerr, 2015). Hussein sought to acquire Kuwait’s large oil reserves as tensions had risen over oil exports from the neighboring nations and Kuwait’s large debt to Iraq (Britannica, 2023). The initial occupation of Kuwait by the Iraqi military was swift, and there were concerns that this invasion would continue into Saudi Arabia and beyond (Kerr, 2015). As a countermeasure, the United States (U.S.) military deployed Operation Desert Shield on August 7, 1990, which successfully prevented the invasion of Iraqi troops into Saudi Arabia (Bulloch & Morris, 2016). Beginning in January of 1991, a coalition of 700,000 troops from several countries including the U.S., the United Kingdom, France, and Canada then turned their attention to ending Iraq’s control of Kuwait with Operation Desert Storm (Bulloch & Morris, 2016). The ground campaign lasted only 4 days with less than 200 casualties sustained before a cease-fire was accepted on February 28, 1991 (Britannica, 2023).

While the U.S. considers the GW a military success, approximately one third (~250,000) of GW veterans are currently suffering from a progressive, multi-symptom illness that is unique to this veteran population (Arkin, 1998; Kerr, 2015; Nettleman, 2015). Initially, the Department of Defense (DoD) and Department of Veterans Affairs denied that such symptoms were exclusive to GW soldiers and attributed these complaints to post-
traumatic stress disorder (PTSD) (Britannica, 2023). Though PTSD is common among veterans (Norris & Slone, 2013), rates of PTSD in GW veterans are lower than other veteran populations (Jeffrey et al., 2021) and many studies have concluded that the symptomology of GW veterans is distinct from PTSD (Group, 1997; Proctor et al., 1998). After years of advocacy work by veterans, the U.S. government began investigating the potential for a “Gulf War Illness” (GWI) in October of 1993 (Program, 1996). The DoD launched numerous studies and congressional hearings between 1993 and 1997 before finally recognizing that GW veterans are suffering from a unique combination of symptoms (Kerr, 2015).

Once recognized, GWI was defined as a chronic, multi-symptom illness that includes symptoms of general fatigue, mood and cognitive abnormalities, and musculoskeletal pain (Arkin, 1998). Many soldiers began experiencing such symptoms while deployed, including Staff Sergeant Chris Kornkven, an Army Reservist, who described his extensive symptomology to a 1997 Congressional committee:

“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 breathing problems . . . no diagnosis. 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" (Committee, 1997).

Unfortunately, 10-year and 20-year follow-up surveys reported that many GWI symptoms had worsened in affected veterans and implicated additional organ systems not included in the initial definition (Blanchard et al., 2005; Kang et al., 2009; Mawson & Croft, 2019). Akin to Staff Sergeant Kornkven’s testimony, GW veterans reported cardiovascular abnormalities such as tachycardia and hypertension, gastrointestinal (GI) disturbances including diarrhea and irritable bowel syndrome, and respiratory impairments including emphysema and chronic bronchitis (Auxémery, 2013; Nettleman, 2015). This was also true for the neurological disturbances experienced by many veterans as chronic headaches, sleep disturbances, persistent depression and progressive memory and attentional impairments were also reported in these follow-up surveys (Auxémery, 2013; Blanchard et al., 2005).

While this complex disease remains poorly understood, immune dysfunction is emerging as a hallmark feature of GWI, and numerous studies have identified elevated inflammatory markers in samples obtained from GWI patients. Specifically, GW veterans exhibit elevations in circulating immune cells including lymphocytes, monocytes, B-cells, and T-cells, and reduced levels of natural killer cells (Johnson et al., 2016; Joshi et al., 2018; Vojdani & Thrasher, 2004; Zhang et al., 1999). Other studies found elevated levels of pro-inflammatory markers such as interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-2, and C-reactive protein (CRP) in the plasma of GWI patients (Johnson et al., 2014; Skowera et al., 2004). Additionally, metabolic analysis revealed an upregulation of pathways
involved in mitochondrial function and cellular energetics, which also mediate immune
responses (Naviaux et al., 2019). These findings suggest that aberrant immune signaling is
occurring in GWI and likely contributes to disease presentation as many GWI symptoms
have been associated with chronic inflammation.

Along with immune dysfunction, dysregulated endocrine signaling has also been
implicated in GWI (Blanchard et al., 2005; Lindheimer et al., 2020; Nettleman, 2015)
These impairments have been associated with abnormal hypothalamic-pituitary-adrenal
(HPA) axis activity, which regulates the stress response by producing cortisol through the
release of corticotropin releasing factor (CRF) and adrenocorticotrophin hormone (ACTH)
(Papadimitriou & Priftis, 2009). When stress responses were tested in GW veterans using
a dexamethasone suppression test, this group showed significantly greater suppression of
cortisol levels than controls (Golier et al., 2006). The dexamethasone suppression test
assesses the feedback activity of the HPA axis by mimicking cortisol release which in turn
should suppress ACTH and the further release of cortisol (Oei, 1988). Thus, a greater
cortisol suppression in GW veterans may be indicative of impaired pituitary or adrenal
responses (Golier et al., 2006). Golier and colleagues expanded upon these findings and
reported that GW veterans exhibit significantly lower levels of plasma ACTH and a higher
cortisol:ACTH ratio which they attributed to an enhanced sensitivity to cortisol’s feedback
effects and reduced HPA axis activity (Golier et al., 2007). Importantly, cortisol and other
glucocorticoids released by the HPA axis perform critical anti-inflammatory actions and
the attenuation of this axis in GW veterans may be exacerbating the observed immune
dysfunction (Liberman et al., 2018; Tapp et al., 2019).
Perhaps the best illustration of disrupted immune and endocrine signaling in GWI is when patients are subjected to an exercise challenge. It is well established that fatigue and post-exertional malaise are common symptoms of GWI (Lindheimer et al., 2020), but exercise has also been shown to exacerbate other GWI symptoms including musculoskeletal pain and cognitive impairments (Boruch et al., 2023; Cook et al., 2010; Washington et al., 2020). Various imaging studies have been performed to understand this latent phenotype of GWI and why exercise can worsen these symptoms. One group reported that while performing a working memory task after bicycling, the dorsal midbrain and cerebella dermis became deactivated in GWI patients, but not healthy controls (Washington et al., 2020). Additionally, this bicycling challenge separated GWI patients into two phenotypes, with one group exhibiting orthostatic tachycardia and brainstem atrophy, and the other group experiencing severe pain and exhibiting cortical atrophy (Rayhan et al., 2013). Another study that analyzed cerebral spinal fluid after bicycling found elevations in glutamate in a subset of GWI patients, which may be indicative of exercise-induced excitotoxicity (Baraniuk et al., 2021).

It has been suggested that such negative responses to exercise may be due to sensitized immune and endocrine responses as GWI patients subjected to a bicycle test showed abnormal immune responses. Specifically, exercise induced a heightened pro-inflammatory state in GWI patients relative to healthy controls as the inflammatory activities of nuclear factor kappa B (NF-κB) and IL-6 were increased. Other studies from this lab report that exercise reduced the expression of genes associated with the activity of natural killer cells in GWI patients (Whistler et al., 2009). Interestingly, many of these genes are also involved in glucocorticoid receptor signaling pathways and salivary cortisol
was significantly lower in GWI patients after exercise (Whistler et al., 2009). These reductions provide further evidence that diminished HPA axis activity may be a key feature of GWI. Collectively, these studies highlight that GWI presents with a latent phenotype as most symptoms are exacerbated following an exercise challenge. Thus, the pathophysiology of GWI may be due to sensitized responses to stressors, however the mechanism responsible for these aberrant immune and endocrine responses has not been fully elucidated.

1.2 Potential causes of Gulf War Illness

After GWI was defined as a unique illness solely affecting GW veterans, researchers looked for potential exposures during deployment that could cause such complex symptomology. Soldiers were exposed to several hazards in the Gulf including pesticides on their uniforms, airborne particulates from dust and sand, depleted uranium in military vehicles, smoke from oil well fires, and severe stress from combat (White et al., 2016). Additionally, soldiers were administered various vaccines and pyridostigmine bromide (PB), a pharmacological prophylaxis against potential nerve gas attacks (Gordon et al., 1978; von Bredow et al., 1991). While all of these exposures can produce negative side effects, the use of PB and war-related stress have been identified as chief factors in GWI pathology (Golomb, 2008; Haley et al., 1997; Steele et al., 2012; Sullivan et al., 2003; White et al., 2001; White et al., 2016).

1.2.1 Pyridostigmine bromide

PB was originally synthesized in 1945 by Hoffman-La Roche laboratories and sold under the name Mestinon® for the treatment of myasthenia gravis (MG) (Keesey, 2004). MG is an autoimmune disease characterized by muscle weakness due to the production of
autoantibodies that block acetylcholine (ACh) receptors at the neuromuscular junction (Gilhus, 2016). ACh is the neurotransmitter that must bind to cholinergic receptors on muscle fibers to cause their contraction, thus, reduced cholinergic receptors in MG results in weaker muscles that fatigue quicker (Rodríguez Cruz et al., 2020). PB is the dimethylcarbamate ester of 3-hydroxy-1methylpyridinium bromide and acts as an acetylcholinesterase (AChE) inhibitor (Sox et al., 2000). AChE is a serine hydrolase that catalyzes the hydrolysis of ACh into choline and acetic acid in order to terminate its action (Pohanka, 2011). AChE is an extremely efficient enzyme that tightly regulates cholinergic neurotransmission in both the periphery and central nervous system (CNS) by inactivating ACh within seconds to milliseconds of its release (Pope & Brimijoin, 2018). PB blocks this enzymatic activity by binding to the serine residue in the enzyme active site, rendering it inactive (Pohanka, 2011). Being a carbamate compound, PB is considered a reversible AChE inhibitor as the binding of the AChE-carbamate complex that inhibits AChE action is quite unstable and will spontaneously split from the enzyme by hydrolysis (Colović et al., 2013). Importantly, PB’s inhibitory actions are confined to the periphery due to its quaternary ammonium group that limits lipid solubility and prevents passage through the blood-brain barrier (BBB) (Golomb, 2008). As PB can increase available ACh by reversibly inhibiting AChE activity in the neuromuscular junction, Mestinon® was approved by the Food and Drug Administration (FDA) in 1955 for the treatment of MG and proved successful in reducing muscle weakness (Sox et al., 2000).

While PB is well known for its therapeutic properties, many other AChE inhibitors are better known for their deleterious actions. Organophosphate (OP) AChE inhibitors are much more toxic than PB as they permanently inhibit the enzyme by non-reversible
phosphorylation (Colović et al., 2013). Such irreversible AChE inhibitors produce a dangerous accumulation of ACh in the synaptic cleft that compromises normal muscle control, which becomes lethal if the respiratory muscles are affected and asphyxiation occurs (Sidell & Wilde, 1998). OP AChE inhibitors have been used as chemical warfare agents as they can be vaporized or aerosolized to attack large groups through their inhalation and absorption into the skin (Pohanka, 2011). Hussein was thought to possess large stockpiles of these OP AChE inhibitors, namely the G-series nerve agents, sarin and soman (Mukherjee & Gupta, 2020). Due to this serious threat, military officials sought out various protective measures before deploying soldiers to the Gulf War (Kerr, 2015). Protective suits and respirators are the most effective way to prevent nerve agent exposure, however, pharmacologic antidotes are available if exposure is unavoidable (Colović et al., 2013). Atropine, a competitive antagonist of ACh receptors, has been utilized as an antidote in nerve gas attacks as it can block the harmful actions of the accumulated ACh (Smythies & Golomb, 2004). Additionally, oxime reactivators were created to rescue AChE activity by cleaving the bond between the inhibitor and AChE and freeing the enzyme (Colović et al., 2013). While these antidotes have proven effective if administered shortly after nerve agent exposure, the AChE can become too damaged to be reactivated by the oxime if administered too late (Colović et al., 2013).

Rather than relying on protective equipment and antidotes that may not be readily deployed in a spontaneous nerve gas attack, military officials also sought to provide soldiers with pharmacologic prophylaxis (Golomb, 2008). As a reversible AChE inhibitor, PB was identified as a potential prophylactic against irreversible OP AChEs due to its ability to compete for AChE binding (Berry & Davies, 1970). Preclinical studies before
the GW showed that carbamate AChEs, such as PB, significantly improved survival rates of G-series nerve agent exposure when used as a pretreatment (Berry & Davies, 1970; Gordon et al., 1978; von Bredow et al., 1991). Thus, PB was used as an investigational drug in the GW, which was done without soldiers’ informed consent as the FDA had recently granted the DoD permission to waive this requirement (Rettig, 1999). Each soldier received 21-tablet blister packs and was instructed to take one 30 mg tablet every 8 hours to ensure continuous protection (Sox et al., 2000). This prescribed dose suppressed plasma AChE activity by approximately 50% (Marino et al., 1998), which replicated the dose tested in preclinical models (von Bredow et al., 1991). Many soldiers reported immediate side effects to PB, with most involving the GI system, but such symptoms were not considered incapacitating (Keeler et al., 1991). While the DoD reports that approximately 250,000 military personnel took PB during the GW, its self-administration led to a wide variety of dosing regimens (Lashof, 1997). Despite its varied use, PB exposure has been consistently correlated with GWI presentation as many of the aforementioned GWI symptoms can be attributed to cholinergic toxicity (Haley et al., 1997; Pope et al., 2005; Steele et al., 2012; White et al., 2016).

1.2.2 War-related stress

Though PB was prescribed to every GW soldier, the incidence of GWI is more prevalent in ground forces that faced combat compared to forces that remained in relief areas (Steele, 2000). This disparity led researchers to hypothesize that the physical and psychological stress of combat may also contribute to GWI presentation (Amourette et al., 2009; Burzynski et al., 2023; Burzynski et al., 2022; Kant et al., 2001; Macht et al., 2020; Macht et al., 2018; Macht et al., 2019). As described above, the stress response is primarily
mediated by the HPA axis, which releases glucocorticoids such as cortisol (Papadimitriou & Priftis, 2009). Chronic stress, such as the prolonged stress of deployment, can dysregulate the feedback mechanisms of the HPA axis and lead to chronic inflammation, which has been reported in GWI patients (Papadimitriou & Priftis, 2009; Skowera et al., 2004). Glucocorticoid receptors are also found within the CNS, and chronic stress can produce memory and attentional deficits like those observed in GWI (McEwen et al., 1968). Specifically, within the hippocampus, an essential integration center for learning and memory in the mammalian brain (McEwen, 2001), chronic stress reduces dendritic complexity and neurogenesis (Gould et al., 1998; Magariños & McEwen, 2000). Similar effects of chronic stress on neuronal structure and function are also observed in the prefrontal cortex (PFC), a region that mediates higher-order cognitive functions such as attention and decision making (Arnsten, 2009).

Another deleterious effect of stress on the CNS is compromising the BBB, a semipermeable layer of endothelial cells and immune cells that regulates the transport of substances into the CNS (Bang et al., 2017; Welcome & Mastorakis, 2020). Chronic stress can cause the downregulation of essential proteins that stabilize this barrier and increase its permeability to molecules that would normally be excluded (Hannocks et al., 2017; Welcome & Mastorakis, 2020). Given these findings, some researchers proposed that war-related stress compromised the BBB and allowed PB to directly alter central cholinergic signaling (Friedman et al., 1996). While some studies have demonstrated exposure to GW-related chemicals, including PB, inhibits brain AChE activity (Beck et al., 2003; Friedman et al., 1996; Kaufer et al., 1998; Tian et al., 2002) these studies were not replicated by other researchers (Amourette et al., 2009; Grauer et al., 2000; Kant et al., 2001; Song et al., 2002;
Regardless, both PB and stress are known to alter ACh (Golomb, 2008; Kaufer et al., 1998; Pohanka, 2011) and the higher prevalence of GWI in soldiers subjected to both exposures suggests that disruptions in cholinergic signaling may be a mechanistic mediator in GWI.

1.3 Acetylcholine

As stated above, ACh is an essential neurotransmitter best known for its actions within the neuromuscular junction and peripheral nervous system (Krnjevic & Miledi, 1958). ACh is synthesized in nerve terminals when choline and acetyl coenzyme A react with the catalyst choline acetyltransferase (Sam & Bordoni, 2022). ACh is then stored in vesicles via vesicular ACh transporter and released into the synaptic cleft when an action potential triggers an influx of calcium (Krnjevic & Miledi, 1958). Once released, cholinergic neurotransmission can occur through either nicotinic or muscarinic receptors (Picciotto et al., 2012). Nicotinic ACh receptors (nAChRs) are cys-loop ligand-gated ion channels that contain five subunits symmetrically arranged around the ion channel (Sam & Bordoni, 2022). These five subunits can be identical or a combination of the 17 nAChR subunits (ten α-subunits, four β-subunits, δ, ε and γ) that have been identified in mammals (Taly et al., 2009). nAChRs are classified based on their localization as N1 receptors are located on muscle cells at the neuromuscular junction and N2 receptors represent those found in neurons (Sam & Bordoni, 2022). While different nAChRs have distinct pharmacological and kinetic properties, neurotransmission through these ligand-gated ion channels is incredibly fast and excitatory (Taly et al., 2009). Conversely, ACh can bind to muscarinic receptors which produce a slower response that can be excitatory or inhibitory (Leach et al., 2012). Muscarinic ACh receptors (mAChRs) are G-protein coupled receptors
(GPCRs) and are comprised of M1, M2, M3, M4 and M5 subtypes. M1, M3 and M5 are Gq GPCRs, which are considered excitatory, while M2 and M4 are Gi GPCRs that act as inhibitors (Sam & Bordoni, 2022). Utilizing both nAChRs and mAChRs, ACh is a key regulator of the autonomic nervous system, especially parasympathetic responses in the periphery (Daroff & Aminoff, 2014). Specifically, ACh mediates gastric motility, bronchoconstriction, vasodilation, and other “rest and digest” actions (Sam & Bordoni, 2022).

1.3.1 Role of acetylcholine in cognition

Beyond the periphery, cholinergic neurotransmission is also extremely important in the CNS as it regulates cognitive processes including attention and memory (Friedman & Robbins, 2022; Hasselmo, 1999). A large portion of the cholinergic neurons in the CNS are found in the basal forebrain (BF) nuclei, a heterogeneous set of structures located in the rostroventral forebrain (Mesulam et al., 1983). These structures include the medial septum and the diagonal band of Broca which send cholinergic projections to the hippocampus (Woolf, 1991). Clinical studies have shown the importance of ACh neurotransmission in the hippocampus by using cholinergic antagonists to impair performance in memory tasks (Atri et al., 2004; Green et al., 2005). In addition to hippocampal projections, the BF also contains the nucleus basalis and substantia innominata, which house cholinergic projections that innervate the PFC (Woolf, 1991). It is well established that BF cholinergic neurons are involved in PFC-dependent cognitive processes as lesioning BF projections in rodents results in significant impairments in attentional tasks (Dalley et al., 2004; McGaughy et al., 1996; McGaughy & Sarter, 1998; Newman & McGaughy, 2008; Turchi & Sarter, 1997). Besides cholinergic neurons,
nAChRs and mAChRs are also found on other neurons in the PFC and hippocampus including glutamatergic, GABAergic, noradrenergic, and dopaminergic neurons (Picciotto et al., 2012). Thus, ACh is also considered a neuromodulator as it can mediate the release of these other neurotransmitters and regulate neuronal excitability, synaptic plasticity, and coordinate the firing of groups of neurons to adapt to environmental stimuli (Kawai et al., 2007; Rice & Cragg, 2004; Wonnacott, 1997; Zhang & Sulzer, 2004).

1.3.2 The cholinergic anti-inflammatory pathway

Among its modulatory actions, cholinergic neurotransmission has also been recognized as a key regulator of inflammation through the cholinergic anti-inflammatory pathway (Pavlov et al., 2003). The cholinergic anti-inflammatory pathway was first identified by Kevin Tracey’s group who discovered the presence of α7 nAChRs on macrophages. (Borovikova et al., 2000). These *in vitro* studies found that activation of α7 nAChRs by ACh reduced the expression of pro-inflammatory cytokines, specifically IL-1β, IL-6, IL-18 and tumor necrosis factor alpha (TNF-α), in macrophages exposed to the endotoxin lipopolysaccharide (LPS) (Borovikova et al., 2000). This was later confirmed *in vivo* when α7 nAChR deficient mice exhibited suppressed anti-inflammatory responses to an LPS challenge compared to wild type mice (Wang et al., 2003). Later studies identified α7 nAChRs on other immune cells including monocytes (Hamano et al., 2006; Yoshikawa et al., 2006), T-cells (De Rosa et al., 2009; Razani-Boroujerdi et al., 2007), and B-cells (Skok et al., 2003).

Peripherally, ACh’s anti-inflammatory actions are mediated by the vagus nerve as it is a chief producer of ACh (Pavlov et al., 2003). Vagal afferent fibers innervate a multitude of peripheral organs and alert the CNS about any homeostatic disturbances
through their projections to the nucleus tractus solitarius (NTS) in the brainstem (Tracey, 2002). Vagal afferents can detect such disruptions as they contain receptors for endotoxins and the pro-inflammatory cytokines they produce (Goehler et al., 2000). Once this information is relayed to the NTS, it is propagated to other brain regions involved in processing visceral information, such as the hypothalamus, amygdala, cortex, and locus coeruleus (Berthoud & Neuhuber, 2000; Grill & Hayes, 2012; Hachem et al., 2018). To restore homeostasis, vagal efferent fibers residing in the dorsal motor nucleus of the vagus within the brainstem release ACh into the periphery which binds to α7 nAChRs and dampens inflammation (Wang et al., 2003). This anti-inflammatory vagal reflex is best illustrated in vagotomized mice experiencing endotoxemia after high dose LPS administration while sham animals do not (Borovikova et al., 2000). Importantly, the immunosuppressive actions of the vagus nerve are incredibly efficient and occur much faster than other immune responses (Czura et al., 2003).

As α7 nAChRs are also in the CNS, more recent studies have investigated if a central cholinergic anti-inflammatory pathway exists. Evidence for such a pathway was first supported by studies that identified α7 nAChRs on microglia and astrocytes, key CNS immune cells (Sharma & Vijayaraghavan, 2001; Shytle et al., 2004). In vitro studies have used ACh and nicotine, a nAChR agonist, in microglial cultures to show that activation of α7 nAChRs suppresses LPS-induced TNF-α increases and this response is blocked by the α7 nAChR antagonist α-bungarotoxin (Shytle et al., 2004). The immunosuppressive role of microglial α7 nAChRs has also been observed in vivo as a mouse model of ischemic stroke exhibited smaller infarct size and less microglia-mediated inflammation when treated with the α7 nAChR agonist PNU282987 (Parada et al., 2013). Astrocytic α7
nAChRs elicit similar effects as nicotine also dampens the pro-inflammatory response of cultured astrocytes activated by IL-1β (Revathikumar et al., 2016). As neuroinflammation has been implicated in a variety of neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis, disruptions in the cholinergic anti-inflammatory pathway may be a mechanistic mediator of these diseases (Dumas & Newhouse, 2011; Gamage et al., 2020). Indeed, many preclinical studies have investigated α7 nAChR agonists as potential therapeutics for neurodegenerative diseases driven by neuroinflammation (Hua et al., 2019; Reale et al., 2015; Takata et al., 2018). The therapeutic effects of α7 nAChR agonists have also been investigated in chronic inflammatory diseases in the periphery including rheumatoid arthritis, ulcerative colitis, and diabetes (Marrero et al., 2010; Pu et al., 2022; Zhu et al., 2021). This area of research may be especially relevant for GWI studies as GW veterans experience similar symptoms and immune dysregulation is emerging as a hallmark feature (Johnson et al., 2016; Skowera et al., 2004). Furthermore, many GWI symptoms involve organ systems that are highly innervated by the vagus nerve (heart, lungs, GI tract) (Blanchard et al., 2005; Farmer et al., 2016), but the potential dysfunction of cholinergic signaling, including its anti-inflammatory actions in GWI has not been examined.

1.4 Summary and hypothesis

Given that many GWI symptoms include disruptions in both the periphery and CNS, it is important to understand how ACh neurotransmission, a key mediator of cognition and immune responses, is affected by PB and stress. As symptoms of GWI, especially the cognitive impairments, continue to worsen as veterans age, the focus of my dissertation work was investigating the lasting effects of PB and stress on peripheral and
central cholinergic signaling and how that may contribute to the pathophysiology of GWI. I hypothesized that PB and stress interact to progressively impair cognition by dysregulating inflammatory responses and cholinergic neurochemistry in a rat model of GWI. The overarching goal of this project was to gain mechanistic insight into GWI and its progression, which can help identify potential therapeutics to slow or stop this debilitating disease. Given the latent phenotype of GWI, it was also important to assess how PB and stress alter cholinergic responses to physiological stressors. I accomplished these goals through the completion of the following aims:

Aim 1: Determine if PB and stress have lasting effects on hippocampal-dependent learning and memory and if such deficits are exacerbated by an immune or stress challenge.

Aim 2: Determine if PB and stress produce long-term impairments in peripheral and central immune responses to LPS.

Aim 3: Determine if PB and stress alter central cholinergic responses to immune or stress challenges.
CHAPTER 2
MODELING GULF WAR ILLNESS IN RODENTS

2.1 Introduction

To study the synergistic effects of PB and stress, the Reagan lab has established an experimental model of GWI with adult male Sprague Dawley rats (Burzynski et al., 2023; Burzynski et al., 2022; Macht et al., 2020; Macht et al., 2018; Macht et al., 2019). Previous studies from the Reagan lab and those outlined in the following chapters focused on male rats as over 95% of GWI patients are males (Nettleman, 2015). PB (1.3 mg/kg body weight (BW)) was administered by oral gavage for 14 consecutive days to mirror the dosing regimen of PB treatment during the GW which aimed to suppress plasma AChE activity by approximately 50% (Marino et al., 1998). Repeated restraint stress (RRS) is a stress paradigm that places rodents in wire mesh restrainers to produce a psycho-social stressor as the rodents are unable to escape (Glavin et al., 1994; Servatius et al., 2007). This is highly relevant to GWI research as a majority of GW stressors were psychological, including the lack of social contact with family, threat of attack, and fear of a friend or self being killed (Gifford et al., 2006). RRS was also utilized in this model because of its well-established effects on hippocampal structure and function that are consistent with those observed in GWI patients (Chao et al., 2010; X. Li et al., 2011; O'Donovan et al., 2015). Numerous studies have found that RRS elicits dendritic retraction in the CA3 region of the hippocampus, which includes a decrease in branch points, branch length and synaptic suppression (Conrad et al., 1999; Magariños & McEwen, 2000). This reduction in
dendritic complexity is known to produce deficits in hippocampal-dependent behaviors, especially spatial memory (Bowman et al., 2003; Conrad et al., 1996; Kleen et al., 2006), but does not result in permanent cell death (McLaughlin et al., 2007). Importantly these hippocampal impairments only occur if a 6h/21-day RRS paradigm is used, as shorter paradigms do not produce significant structural and functional changes (McLaughlin et al., 2007). However, shorter RRS paradigms have been shown to produce deficits in HPA axis activity (Gądek-Michalska et al., 2013), which have also been implicated in GWI (Golier et al., 2006; Golier et al., 2007).

Based on these findings, the Reagan lab’s rat model of GWI combines 14 consecutive days of PB treatment with 10 consecutive days of RRS for 6 hours per day (Burzynski et al., 2023; Burzynski et al., 2022; Macht et al., 2020; Macht et al., 2018; Macht et al., 2019). The shorter 10-day RRS paradigm was chosen to ensure that any synergistic effects of PB and stress can be observed as a 21-day RRS paradigm may mask PB’s contributions. Furthermore, RRS-induced hippocampal deficits can be accelerated if secondary insults such as metabolic dysfunction are present (Grillo et al., 2003; Magariños & McEwen, 2000; Reagan et al., 2000). Thus, a 10-day RRS paradigm is better suited for these studies as PB treatment serves as a secondary insult. Lastly, to accurately replicate the prophylactic use of PB during the GW, the RRS paradigm begins 4 days after the start of PB treatment (day 5 of 14-day paradigm).

2.2 Materials and Methods

All studies described in subsequent chapters utilize the GWI treatment paradigm described below.
2.2.1 Animals

All procedures were approved and performed in accordance with all guidelines and regulations of the Dorn VA Animal Care and Use Committee. Adult male Sprague Dawley rats (Envigo, 200-225g, approximately six weeks old) were individually housed at the University of South Carolina School of Medicine animal facility with irradiated Sani-Chips wood bedding (P.J. Murphy Forest Products Corp.) and maintained on a 12/12hr light-dark cycle (lights on at 7:00A.M.) at 22°C. Rats were given *ad libitum* access to food and water and provided Nylabones® for enrichment. Animals were given one week to acclimate before the GWI treatment paradigm began.

2.2.2 GWI paradigm

The experimental design of this rat model of GWI includes the following 4 groups; 1) vehicle-treated (sterile water by gavage), non-stressed control rats (Vehicle-NSC); 2) PB-treated (1.3 mg/kg BW by gavage), non-stressed control rats (PB-NSC); 3) vehicle-treated rats subjected to repeated restraint stress (Vehicle-RRS); and 4) PB-treated rats subjected to repeated restraint stress (PB-RRS). Rats were gavaged daily from days 1-14 with either vehicle or PB (prepared daily, 1.3 mg/kg BW, Sigma-Aldrich). Restraint stress began on day 5; immediately following gavage, Vehicle-RRS and PB-RRS rats were placed in mesh restrainers for 6 hours (10:00 am-4:00 pm) for 10 consecutive days. Non-stressed rats were handled daily and returned to their home cage. Rats undergoing stress conditions were housed in a separate room for the 14-day treatment paradigm to ensure NSC rats were not exposed to any auditory or olfactory stressors. Both rooms were maintained at the same conditions described above. Restraint stress took place in the home cage to selectively examine the effects of this stressor without adding the additional stress of a cage change.
After the completion of the treatment paradigm, all animals were housed in the same room for the duration of the study. Endpoint measures were taken at two timepoints: approximately 10 days post treatment (early cohort, approximately 11-12 weeks old) and approximately 90-100 days post treatment (delayed cohort, approximately 6 months old). See Figure 2.1 for a general experimental timeline.

### 2.2.3 GWI Model Validation

Tail blood was collected on day 14 approximately 30 minutes after PB was administered and RRS was initiated. Plasma cholinesterase (ChE) activity was measured with a colorimetric assay (Abcam, ab#138871) according to manufacturer’s instructions. This assay measures both AChE as well as butyrylcholinesterase activity. Plasma corticosterone (CORT) levels were measured with an enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences, #ADI-900-097) according to manufacturer’s instructions and described previously (Macht et al., 2018). Both assays were analyzed using a BioTek Synergy microplate reader (BioTek Instruments Inc.).

### 2.2.4 Statistical analysis

Plasma measures for ChE activity and CORT were analyzed using a 2 × 2 analysis of variance (ANOVA) with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress history (NSC, RRS). Following a significant interaction, post-hoc follow-up analyses were analyzed with a Bonferroni correction. Post-hoc tests assessed all levels of drug treatment within each level of stress, and all levels of stress within each level of drug treatment.
Figure 2.1 General experimental timeline. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (NSC, RRS). Separate cohorts were used for endpoint measures at either early (days 24-30) or delayed (days 90-100) timepoints. Figure made with www.biorender.com

2.3 Results

To validate this rat model of GWI, plasma ChE activity and CORT levels were assessed to ensure that PB adequately suppressed plasma ChE activity and RRS increased plasma CORT levels appropriately. As described previously, tail blood collected on the last day of the treatment paradigm revealed that 14 days of PB treatment (1.3 mg/kg BW) reduces plasma ChE activity by approximately 50% relative to vehicle-treated animals \([F(1, 23) = 69.40, p < 0.0001\) (Figure 2.2, Panel A) \]. These samples also revealed that plasma CORT levels are significantly higher in animals subjected to a 6h/10-day RRS paradigm compared to NSCs \([F(1, 33) = 12.38, p < 0.01\) (Figure 2.2, Panel B) \] (Macht et al., 2018).
Figure 2.2 Plasma measures on day 14 GWI paradigm. On the last day of the GWI paradigm, PB treatment decreased plasma ChE activity by approximately 50% relative to vehicle-treated rats (Panel A) and RRS significantly increased plasma CORT levels relative to NSC rats (Panel B). All data are expressed as a percentage of Vehicle-NSC rats. Values are expressed as mean ± SEM, n = 5-10/group. [***: Significant effect of PB, p < 0.0001. #: Significant effect of RRS, p < 0.01].
2.4 Conclusions

These results confirm that this rat model of GWI effectively replicates the GW’s dosing regimen of PB as well as the psycho-social stress experienced by soldiers. Similar doses of PB have been used in studies that also reported approximately 50% suppression of plasma ChE activity in rodents (Amourette et al., 2009; Lamproglou et al., 2009), further emphasizing the validity of this model. Importantly, while these data are from a previous study (Macht et al., 2018), this validation procedure was performed for every cohort described in subsequent chapters, ensuring that this model is highly reproducible (Burzynski et al., 2023; Burzynski et al., 2022).
CHAPTER 3

ASSESSING HIPPOCAMPAL-DEPENDENT LEARNING AND MEMORY IN AN EXPERIMENTAL MODEL OF GWI

3.1 Introduction

While multiple organ systems are implicated in GWI, I sought to investigate the mechanistic basis of the CNS impairments as the progressive cognitive deficits affecting many GW veterans are especially devastating. As I hypothesize that PB and stress produce lasting cognitive impairments, I first assessed hippocampal-dependent learning and memory in our rat model of GWI with novel object recognition (NOR) and Morris water maze (MWM) testing. The NOR task utilizes rodents’ innate curiosity to measure their memory of familiar versus novel stimuli (Antunes & Biala, 2012). NOR can be used to test different phases of memory, which employ different brain regions, based on the amount of time between familiar and novel object presentation (intertrial interval; ITI) (Cohen & Stackman, 2015). Specifically, lesion studies have found that cortical structures facilitate object recognition with short ITIs (seconds to minutes) (Buckmaster et al., 2004), while the hippocampus facilitates object recognition with longer ITIs (up to 24-hours) (Clark et al., 2000). To best assess the effects of PB and stress on hippocampal-dependent memory, the following studies use a 24-hour ITI for all NOR testing. Due to the progressive nature of GWI, I performed NOR testing at early and delayed timepoints to determine if PB and stress produce immediate or delayed cognitive deficits. As GWI patients are known to
experience more severe memory impairments after a physiological stressor (Broderick et al., 2013; Broderick et al., 2011; Whistler et al., 2009), I also included an immune challenge (30 µg/kg BW LPS, i.p.) prior to familiar object presentation for one NOR session at the delayed timepoint. Comparing NOR performance with and without this immune challenge will determine if our experimental model of GWI accurately depicts the latent phenotype observed in GWI patients.

Along with NOR, the same cohort of rats also underwent MWM testing, which assesses spatial hippocampal-dependent learning and memory (Morris & Seifert, 1982). The MWM paradigm involves a swimming arena with a submerged escape platform that can be found using distal cues located around the arena (Morris, 1984). Animals learn the platform’s location through a series of training trials and their escape latency is indicative of their spatial acquisition capabilities (Vorhees & Williams, 2006). Probe trials assess spatial memory by removing the escape platform and observing the animals’ behavior (Vorhees & Williams, 2006). Generally, animals that accurately recall the platform’s location will spend a majority of the probe trial swimming in that location, while animals who have impaired memory will spend less time in the correct location (Brandeis et al., 1989). Similar to NOR testing, different phases of memory can be measured by increasing the amount of time between training and probe trials (D’Hooge & De Deyn, 2001). For these studies, I conducted both 1-hour and 24-hour probe trials to determine how PB and stress affect memory acquisition and consolidation. Unlike the NOR sessions, this behavioral paradigm was not paired with an immune challenge as the swimming component of this test serves as a physiological stressor (Harrison et al., 2009).
Collectively, the studies outlined in this chapter assessed the potential role of stressors, namely immune or stress challenges, in the development of cognitive-behavioral deficits in our GWI model. These results can provide insight into how PB and stress produce long-lasting hippocampal impairments which may contribute to the latent cognitive deficits observed in veterans with GWI.

3.2 Materials and Methods

This chapter describes a cohort of rats (cohort #1) that underwent the GWI treatment paradigm outlined in Chapter 2 and hippocampal-dependent learning and memory assessments at both early and delayed timepoints. See Figure 3.1 for an experimental timeline of these behavioral tests.

**Figure 3.1 Experimental timeline for GWI behavioral studies.** Cohort #1 underwent Session 1 of NOR testing approximately 10 days after treatment cessation which included 3 days of habituation, familiar object presentation on day 4, and novel object presentation on day 5 (24-hour intertrial interval). Cohort #1 underwent an additional 8 days of NOR testing approximately 3 months later which included an acute saline challenge before familiar object presentation (Session 2) and an acute LPS challenge before familiar object presentation (Session 3) the following week. Approximately two weeks after the final NOR session, the same cohort of rats underwent MWM testing for a total of 6 days. Illustration made with www.biorender.com.
3.2.1 Novel object recognition task

On days 24-29 (approximately three months old) and 100-110 (approximately 6 months old) of the GWI paradigm, cohort #1 underwent NOR testing during the early portion of the light cycle (9:00A.M.-12:00P.M.). The arena was 60 cm x 60 cm with 35 cm walls. Luminosity was maintained at 45 lux throughout the arena. All objects were made of ceramic or glass, approximately 7 cm x 12 cm and secured to the arena with magnets. Each set of familiar and novel objects had similar dimensions. The arena and objects were cleaned with 5% ammonium hydroxide between each animal. Behavior was recorded and locomotor activity was measured with EthoVision XT 15 software (Noldus, Leesburg, VA, USA).

NOR Habituation

Rats were given 3 days of acclimation to the testing arena for 5 minutes per day. Animals were also habituated to an i.p. injection by receiving 1 mL/kg sterile saline (i.p.) 15 minutes before each habituation session. These habitual injections were meant to remove any injection stress during the immune challenge presented in the third NOR session.

NOR Session 1

After habituation, the first NOR session occurred approximately 28 days post treatment. Animals received an i.p. injection of 1 mL/kg sterile saline 15 minutes prior to initiation of the test. The 5-minute test (trial 1) consisted of two identical objects placed in opposite corners, approximately 25 cm from wall. Twenty-four hours later, animals underwent trial 2 with no injection. This 5-minute test consisted of one familiar object from
the previous day and one novel object in the same locations as trial 1. Locations of familiar and novel objects were counterbalanced across groups.

**NOR Session 2**

Approximately three months after treatment cessation, cohort #1 underwent additional NOR testing following the same habituation and testing protocol as session 1. Briefly, animals underwent three days of habituation to the arena for 5 minutes per day. Animals were also habituated to an intraperitoneal (i.p.) injection by receiving 1 mL/kg i.p. sterile saline 15 minutes before each habituation session. On the fourth day of session 2, animals received an i.p. injection of 1 mL/kg sterile saline 15 minutes before the test began. The 5-minute test (trial 1) consisted of two identical objects (different object than those used in session 1) placed in opposite corners, approximately 25 cm from wall. Twenty-four hours later, animals underwent trial 2 with no injection. This 5-minute test consisted of one familiar object from the previous day and one novel object in the same locations as trial 1. Locations of familiar and novel objects were counterbalanced across groups.

**NOR Session 3**

Animals were given two days of rest after session 2 before undergoing another habituation session with 1 mL/kg i.p. saline injection. The following day, animals received an i.p. injection of 30 µg/kg LPS 15 minutes before the test began. The 5-minute test (trial 1) followed the same protocol as session 1, trial 1 with two new identical objects. Trial 2 occurred 24 hours after trial 1 and followed the same protocol as session 1, trial 2 with one familiar object from the previous day and a different novel object. Locations of familiar and novel objects were counterbalanced across groups.
NOR Scoring

The time spent exploring each object was scored manually by two lab members blinded to the animals’ treatment histories and the other member’s analysis. Exploration was defined as the nose of the rat actively touching the object or being in close proximity (approximately 2 cm or less) while the nose was oriented toward the object. If a rat used the object to rear but was looking around the arena, this interaction was not considered exploratory. The percent of time spent exploring the novel object was calculated by dividing the time spent exploring the novel object by the total time spent exploring both the novel and familiar object, multiplied by 100.

3.2.2 Morris water maze testing

MWM Apparatus

MWM testing took place approximately two weeks after Session 3 of NOR testing (roughly 135 days post treatment). MWM testing was not conducted at the early timepoint as this behavioral paradigm can only be used once per cohort (D’Hooge & De Deyn, 2001). Testing was conducted in a 1.76 m diameter pool filled with 27°C water made opaque with non-toxic, white, tempera paint. The pool was surrounded by a white curtain that contained black geometric patterns in each quadrant that served as visual cues. Behavior was recorded and performance measures were assessed with EthoVision XT 15 software (Noldus, Leesburg, VA, USA).

MWM Place learning procedure

Animals underwent four days of training to find the hidden platform, submerged 2 cm under the water in the middle of the southwest quadrant. Each trial began with the rat being placed in the pool at a different starting position (north, south, east, or west) facing
the wall of the pool. Animals were given 1-minute to find the hidden platform. If animals were unable to find the platform in the allotted time, they were guided to the platform where they remained for 15 seconds. Each day of training consisted of four trials, with 5-minutes between each trial. On the fourth day of training, animals also underwent a probe trial 1-hour after the fourth training trial. During the probe trial, the platform was lowered to the bottom of the pool, requiring the rat to swim for the entire 1-minute trial. After 1 minute, the platform was raised to allow the animal to escape and reinforce the trained behavior. A second probe trial was conducted 24-hours later, but animals did not undergo any training trials on this day. During training trials, escape latency was used to evaluate performance while the time spent swimming in the target quadrant was the metric used to evaluate performance during the probe trials. Swim speed was measured during all trials to ensure that there were no physical limitations of any animal or group.

**MWM Cue training procedure**

Following training and probe trials, animals in cohort #1 underwent cue training to exclude any animals that may have visual impairments. All animals underwent six cue trials in which the platform was raised 1 cm above the water, making it fully visible. The position of the raised platform alternated between three quadrants (northwest, northeast, southeast) and rats were placed at a different starting position (north, south, east, or west) for each trial. Escape latency and path length were recorded for each cue trial.

### 3.2.3 Statistical analysis

Comparisons of NOR performance between groups during the first session, as well as locomotor activity and total exploration, were assessed with 2 x 2 ANOVAs with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (NSC, RRS). NOR performance,
locomotor activity and total exploration in sessions 2 and 3 were assessed with $2 \times 2 \times 2$ mixed ANOVAs. Between-subjects factors consisted of 2 levels of drug treatment and 2 levels of stress. Within-subjects repeated measures consisted of 2 levels, representing the immune challenge presented prior to testing (saline, LPS). MWM performance during the training trials was assessed with a $2 \times 2 \times 4$ mixed ANOVA with between-subjects factors consisting of 2 levels of drug treatment and 2 levels of stress and the 4 days of training representing the within-subjects repeated measures. Performance in the probe trials was first assessed with a $2 \times 2 \times 4$ ANOVA with the 4 quadrants representing the within-subjects repeated measures. One-sample t-tests were performed as follow-up to determine if the time spent in the target quadrant was significantly different than chance (15 seconds or 25% of trial) in each group. Performance in the probe trials was then analyzed by a $2 \times 2$ ANOVA to observe group differences. Locomotion during the probe trials and cue trials was also assessed by a $2 \times 2$ ANOVA. For all analyses, statistical significance was set at $\alpha = 0.05$. Unless otherwise stated, following a significant interaction, post-hoc follow-ups were assessed with a Bonferroni-corrected simple main effects analyses. Post-hoc tests assessed all levels of drug treatment within each level of stress, and all levels of stress within each level of drug treatment across each level of immune challenge (NOR sessions 2 and 3) or time (MWM) when applicable.

3.3 Results

3.3.1 LPS administration elicits delayed impairments in NOR performance in PB-treated rats

Since GWI patients exhibit worse cognitive performance after a physiological stressor (Broderick et al., 2013; Broderick et al., 2011; Whistler et al., 2009), I examined
whether a mild immune challenge would adversely affect performance of a hippocampal-
dependent learning and memory task, namely the NOR task. I first conducted NOR testing
approximately 10 days after the cessation of the treatment paradigm to determine if animals
showed successful discrimination at this early timepoint. When given saline (1 mL/kg, i.p.)
and a 24-hour ITI, a two-way ANOVA revealed that there were no significant differences
in novel object exploration observed between treatment groups at this early timepoint
(Figure 3.2, Panel A) and paired t-tests confirmed that all groups successfully exhibited
recognition of the novel object ($p < 0.05$). In addition, neither PB nor stress impacted the
total distance traveled (Figure 3.2, Panel B) or total exploration time (Figure 3.2, Panel C)
during the session when assessed by two-way ANOVA.
Figure 3.2 Novel object recognition performance 10 days after treatment cessation. PB administration alone and in combination with RRS did not elicit any deficits in novel object recognition when animals were tested approximately 10 days after treatment with a 24-hour intertrial interval (Panel A). The total distance traveled (Panel B) and total exploration time did not differ between groups (Panel C). All data are expressed as mean + SEM, $n = 12-15$/group.
As I hypothesized that the interaction of PB and stress produces delayed effects on hippocampal-dependent memory, the same cohort of rats underwent additional NOR testing three months after the cessation of treatment. When rats were given saline (1 mL/kg, i.p.) and assessed following a 24-hour ITI, the Vehicle-RRS, PB-NSC and PB-RRS groups exhibited successful discrimination between the familiar and novel object ($p < 0.05$), but the Vehicle-NSC group failed to reach statistical significance ($p = 0.16$). Importantly, there were no significant differences in the time spent exploring the novel object observed between groups (Figure 3.3, Panel A). Two-way ANOVAs also revealed that there was no effect of treatment history on locomotion (Figure 3.3, Panel B) or total exploration time (Figure 3.3, Panel C) during this session. Interestingly, when rats were challenged with acute LPS administration (30 µg/kg BW, i.p.) one-week later, I observed a significant main effect of LPS [$F(1,49) = 8.950$, $p = 0.004$] and significant interaction of LPS and PB [$F(1,49), = 8.232, p = 0.006$] to decrease the time spent with the novel object 24 hours later (Figure 3.3, Panel A). Post-hoc pairwise comparisons revealed that PB-treated rats, irrespective of stress history, spent significantly less time with the novel object when challenged with LPS compared to their performance during the saline session (PB-NSC: $p = 0.007$, PB-RRS: $p = 0.003$). There were no significant interactions between LPS and RRS [$F(1,49) = 0.361, p = 0.551$] or LPS, PB and RRS [$F(1,49) = 0.202, p = 0.655$]. This LPS-induced deficit in PB-treated rats was not due to any locomotor impairments as there was a significant main effect of LPS to increase the distance traveled in all groups, relative to the saline session [$F(1,47) = 11.297, p = 0.002$] (Figure 3.3, Panel B). Post-hoc pairwise comparisons show that Vehicle-RRS rats travel significantly greater distances during the LPS NOR session compared to their total distance traveled during the saline NOR session.
(\(p = 0.002\)). The total exploration time (Figure 3.3, Panel C) during the LPS session did not differ between groups nor between the saline session.

**Figure 3.3 Novel object recognition performance with acute immune challenge at delayed timepoint.** Three months after treatment cessation, PB-treated rats spent significantly less time with the novel object when challenged with acute LPS (Blue hatched bars) compared to their performance when challenged with saline (Open bars; Panel A). All animals traveled more during the LPS session relative to the saline session, with the Vehicle-RRS animals reaching significance (Panel B). There was no effect of treatment history or LPS on the total exploration time of any group (Panel C). All data are expressed as mean + SEM, \(n = 12-14/\text{group}\). [**: Significant effect of LPS in PB-treated rats, \(p < 0.01\). %: Significant effect of LPS in Vehicle-RRS rats, \(p < 0.01\).]
Prior history of PB treatment produces deficits in long-term retention in hippocampal-dependent learning and memory

Beyond NOR, I also investigated if PB and stress produce delayed deficits in hippocampal-dependent learning and memory. Approximately two weeks after the final NOR session (approximately four months after PB and stress), the rats in cohort #1 underwent six days of MWM testing. After completing four training trials per day for four consecutive days, a 2 x 2 x 4 mixed ANOVA revealed that there was a significant effect of time on the latency to find the platform (Figure 3.4, Panel A) \[F(3,62) = 32.402, p < 0.001\], although there was no effect of prior PB treatment or stress on acquisition throughout the training trials. When the platform was removed 1-hour after the last training session, there was no effect of treatment history on the time spent swimming in the target quadrant (Figure 3.4, Panel B). A 2 x 2 x 4 mixed ANOVA revealed a significant effect of quadrant \[F(3, 162) = 63.02, p < 0.0001\] and one-sample t-tests revealed that the time spent swimming in the target quadrant was significantly greater than chance (15 seconds) for all groups (\(p < 0.05\)). Additionally, there were no differences in swim speed observed in any group during the 1-hour probe trial (Figure 3.4, Panel C). Similar to our NOR findings, a 24-hour probe trial conducted on day five revealed that PB-treated rats spent significantly less time in the target quadrant relative to vehicle-treated rats (Figure 3.4, Panel D) \[2 x 2 ANOVA, F(1,54) = 4.347, p = 0.042\]. A 2 x 2 x 4 mixed ANOVA revealed a significant effect of quadrant \[F(2.079, 112.30) = 35.95, p < 0.0001\]. One-sample t-tests revealed that the time spent swimming in the target quadrant was significantly greater than chance (15 seconds) in Vehicle-NSC, Vehicle-RRS and PB-RRS animals (\(p < 0.05\)), but PB-NSC animals failed to reach statistical significance (\(p = 0.070\)). This 24-hour memory deficit observed in PB-
treated rats was not due to any locomotor deficits as swim speed was not different between groups (Figure 3.4, Panel E). Cue training conducted the following week validated that treatment history did not result in any visual impairments as there were no differences in escape latency (Figure 3.4, Panel F) or path length (Figure 3.4, Panel G) when the platform was visible.
Figure 3.4 Morris water maze performance 3 months after treatment cessation. There was no effect of treatment history on acquisition across 4 training days at the delayed timepoint (Panel A). There is no effect of PB or RRS history on the time spent swimming in the target quadrant (Panel B) or swimming velocity (Panel C) during the 1-hour probe trial. Rats with a history of PB treatment spent significantly less time in the target quadrant during the 24-hour probe trial (Panel D) but swim speed did not differ between groups (Panel E). When the platform was visible, there was no effect of treatment history on escape latency (Panel F) or path length (Panel G). All data are expressed as mean ± SEM, n = 13-15/group. [*: Significant effect of PB, p < 0.05].
3.4 Conclusions

This study demonstrates lasting effects of PB treatment on hippocampal-dependent behaviors, particularly in response to an immune or stress challenge. Specifically, irrespective of PB or stress history, male rats do not exhibit 24-hour memory impairments in NOR approximately 10 days after treatment cessation. However, three months after treatment cessation, PB-treated rats exhibit 24-hour memory deficits in NOR, but only after an immune challenge. A history of PB treatment also produced 24-hour memory impairments in MWM performance but did not have any effect on learning or 1-hour memory. Conversely, a history of stress did not affect NOR or MWM performance at any timepoint. Thus, these findings demonstrate that PB treatment alone impairs hippocampal-dependent memory processes, especially memory consolidation, long after treatment cessation. These results have critical implications for GWI, which is characterized by its persistent and progressive nature (Blanchard et al., 2005; B. Li et al., 2011). Furthermore, the cognitive impairments observed in PB-treated rats are stimulus dependent, which recapitulates the latent cognitive impairments observed in GWI patients when challenged with physiological stressors (Broderick et al., 2013; Broderick et al., 2011; Whistler et al., 2009). Importantly, while this chapter focuses on hippocampal-dependent tasks, performing similar assessments with PFC-dependent tasks represents an important future direction for this project as attentional deficits have been reported in many GWI patients.
CHAPTER 4
PERIPHERAL AND CENTRAL IMMUNE RESPONSES IN AN EXPERIMENTAL MODEL OF GWI

4.1 Introduction

Given the cognitive deficits observed in our GWI model, I hypothesized that PB and stress increase inflammation in brain regions involved in cognition, namely the hippocampus and PFC. Neuroinflammation in these regions has been associated with cognitive decline in neurodegenerative diseases including Alzheimer’s disease and aging (Kalaria et al., 1989; Lee et al., 1999; Rozovsky et al., 1998). Specifically, studies have shown that increased levels of pro-inflammatory cytokines such as IL-1β and TNF-α can impair synaptic plasticity and trigger neuronal death if overproduced (Cacabelos et al., 1994; Raffaele et al., 2020). Peripheral inflammation is thought to contribute to neuroinflammation and subsequent cognitive decline as circulating cytokines can initiate the production of pro-inflammatory cytokines within the CNS (Banks, 2005).

Peripheral immune dysregulation has been observed in both preclinical and clinical GWI studies (Broderick et al., 2013; Broderick et al., 2011; Bryant et al., 2021; Parkitny et al., 2015; Whistler et al., 2009) including previous studies from the Reagan lab (Macht et al., 2019). However, most preclinical studies have analyzed the immediate effects of GW exposures on the immune system, not the long-term consequences. Thus, I first investigated if our GWI model exhibited long-lasting deficits in peripheral immune
responses. I then examined if similar immune disruptions were occurring in the CNS, which may be a mechanistic mediator of the delayed cognitive impairments seen in our model of GWI and GWI patients.

4.2 Materials and Methods

This chapter describes two separate cohorts of rats that underwent the GWI treatment paradigm outlined in Chapter 2 and received intraperitoneal (i.p.) saline or LPS injections 2 hours before euthanasia. Cohort #2 represents an early cohort and cohort #3 represents a delayed cohort. As in the previous chapter, LPS was used as an immune challenge to determine if a physiological stressor produces more robust differences between treatment groups.

4.2.1 Plasma endocrine measures

One day (cohort #2) or 90 days (cohort #3) after treatment cessation, rats were exposed to 3 habitual i.p. saline injections (0.1 mL/kg BW) over the course of 10 days. On days 24-30 (cohort #2) or 100-105 (cohort #3), rats were euthanized by rapid decapitation 2 hours following an i.p. injection of saline (0.1 mL/kg BW) or LPS (30μg/kg BW). This dose and time course would allow for more direct comparisons between LPS-induced changes in brain cytokine levels and the neurochemical studies discussed in Chapter 5. Tail blood collected on day 14 from cohort #2 (no stimulus) or day 90 from cohort #3 (2 hours following i.p. saline injection) was used to assess plasma ChE activity and leptin levels. Plasma ChE activity was measured with the ELISA protocol described in Chapter 2. ELISA analysis was used to measure leptin levels (Millipore, #EZRL-83K), according to manufacturer’s instructions. Both assays were analyzed using a BioTek Synergy microplate reader (BioTek Instruments Inc.).
4.2.2 Assessment of central and peripheral inflammatory markers

Following rapid decapitation, anterior, posterior and the hippocampal regions from cohorts #2 and #3 were dissected and immediately frozen on dry ice and stored at -80°C. The PFC was dissected from the frozen anterior section using a 1mm x 1mm disposable biopsy punch (Integra) on a sliding microtome and stored at -80°C. Two punches were taken from each hemisphere. To assess cytokine levels in brain homogenates, PFC and hippocampal dissections were removed from -80°C and homogenized with lysis buffer (137mM NaCl, 20mM Tris-HCl, 10% Glycerol, 1% Tergitol-type NP40). The ratio of lysis buffer to tissue was 50μL buffer per punch for PFC and 5μL buffer per 1mg tissue for hippocampus. 0.5mm Zirconium Oxide beads (Next Advance) were added to each sample at a ratio of 1-part beads to 2-parts buffer. Samples were mechanically homogenized by a Bullet Blender (Next Advance) at 4°C for 3 minutes at a speed of 8 and then centrifuged at 4°C for 15 minutes at 14,000 x g. Supernatant was collected and stored at -20°C until use. Th1/Th2 rat cytokines were quantified using a Bio-Plex cytokine assay (Bio-Rad, #171k1002M) according to manufacturer’s instructions. Hippocampal lysates were diluted 1:3 with diluent, PFC lysates were not diluted, and plasma was diluted 1:4 with diluent. The plate was read on a Luminex plate reader using high photomultiplier voltage and analyzed with Bio-plex manager software. Cytokine values in lysates were normalized for protein. Lowest limit of detection (i.e. lowest standard) for each cytokine is as follows: IL-1α = 31.48 pg/ml; IL-1β = 10.54 pg/ml; IL-2 = 95.69 pg/ml; IL-4 = 1.5 pg/ml; IL-5 = 34.51 pg/ml; IL-6 = 15.49 pg/ml; IL-10 = 11.34 pg/ml; IL-12 = 4.19 pg/ml; IL-13 = 4.23 pg/ml; GM-CSF = 10.32 pg/ml; IFN-γ = 12.9 pg/ml; TNF-α = 16.68 pg/ml. Cytokine-induced neutrophil chemo-attractant 3 (CINC-3) levels were measured in plasma.
isolated from trunk blood of cohorts #2 and #3 using an enzyme-linked immunosorbent assay (R&D Systems, #DY525), according to manufacturer’s instructions. Plasma samples were diluted 1:2 with diluent. The plate was read on a BioTek Synergy microplate reader (BioTek Instruments Inc.).

**4.2.3 Central ChE activity**

Central ChE activity was measured in saline-treated animals using the hippocampal homogenates and PFC punches prepared for the cytokine assay. 15 µg of protein was used for all samples and ChE activity was measured with the ELISA protocol described in Chapter 2.

**4.2.4 Immunoblot analysis**

Immunoblotting analysis was performed using the membrane fractions prepared for cytokine analysis. Briefly, fractions were separated by SDS/PAGE (10%), transferred to polyvinylidene difluoride (PVDF) membranes, and blocked in TBS plus 10% non-fat dry milk plus 0.05% Tween 20 for 1 hour. PVDF membranes were incubated with primary antisera selective for the α7 nAChR (Bioss Antibodies #bs-1049R, 1:5000) in TBS/2% non-fat dry milk/0.05% Tween 20. After overnight incubation at 4°C, blots were washed and incubated with IRDye 800 CW goat anti-rabbit 926-3211 secondary antibody (LICOR, 1:15,000) in 1% non-fat dry milk. PVDF membranes were then washed with TBS/0.05% Tween 20 and developed using LI-COR Odyssey system. Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for actin (Sigma Chemical cat#A5441; 1:150,000 dilution). α7 nAChR and actin bands were quantitated by densitometry using ImageJ (NIH).
4.2.5 Statistical analysis

Measures of ChE activity, leptin and CINC-3 were analyzed using a 2 × 2 ANOVA with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress history (NSC, RRS). Plasma and brain cytokines were analyzed using a 2 x 2 multivariate analysis of variance (MANOVA) with twelve different cytokines as dependent variables: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, TNF-α. Univariate ANOVAs were performed as follow-up. Expression of α7 nAChRs in tissue homogenates was assessed by a 2 x 2 ANOVA. Statistical significance was set at α = 0.05. Following a significant interaction, post-hoc follow-up analyses were analyzed with a Bonferroni correction. Post-hoc tests assessed all levels of drug treatment within each level of stress, and all levels of stress within each level of drug treatment.

4.3 Results

4.3.1. Prior history of PB treatment enhances peripheral endocrine and immune responses

At the completion of the 14-day GWI paradigm, plasma ChE activity exhibited the expected decrease in PB-treated rats, irrespective of stress conditions [F (1,36) = 125.6 (p < 0.0001] (Figure 4.1, Panel A), as shown previously (Macht et al., 2020; Macht et al., 2018). Interestingly, plasma ChE activity was significantly elevated in PB-treated rats at the delayed timepoint, 90 days following completion of the GWI paradigm [F (1, 28) = 12.26, p = 0.002] (Figure 4.1, Panel B). Based on previous clinical studies (Johnson et al., 2016), we also measured plasma leptin levels in our four treatment groups. At the completion of the GWI paradigm (day 14), rats subjected to stress exhibited significant decreases in plasma leptin levels, irrespective of PB treatment [F (1,33) = 33.41, p <
0.0001] (Figure 4.1, Panel C). Such observations are consistent with previous studies demonstrating that the more immediate effects of chronic stress include reductions in body weight, body adiposity and plasma leptin levels (Tamashiro et al., 2007). Conversely, at the delayed timepoint (i.e., 90 days post treatment), plasma leptin levels were significantly increased in PB-treated rats, irrespective of stress exposure \([F(1,32) = 5.093, p = 0.031]\) (Figure 4.1, Panel D). These results are consistent with clinical studies that reported that plasma leptin levels are elevated in veterans with GWI (Johnson et al., 2016).
Figure 4.1 Plasma measures of saline-treated early and delayed cohorts. A history of PB treatment suppresses plasma ChE activity at the early timepoint (Panel A) but significantly increases plasma ChE activity at the delayed timepoint (Panel B) compared to vehicle-treated groups. At the early timepoint, a history of RRS elicits significantly lower plasma leptin levels compared to the NSC groups (Panel C). At the delayed timepoint, a history of PB treatment significantly increases plasma leptin levels compared to vehicle-treated groups (Panel D). All data are expressed as mean percent change from Vehicle-NSCs + SEM, n = 8-10. [*: Significant effect of PB, p < 0.05. #: Significant effect of RRS, p < 0.001].
Previous studies by the Reagan lab demonstrated that peripheral responses to an immune challenge, namely LPS administration (30 µg/kg BW i.p.), were dysregulated in PB-treated rats compared to vehicle-treated rats at the early timepoint (day 24) (Macht et al., 2019). To assess the long-term effects of RRS and/or PB administration, I measured LPS-induced changes in plasma inflammatory markers in a delayed cohort of rats (cohort #3). In the delayed cohort, LPS-induced increases in plasma TNF-α levels were significantly greater in PB-treated rats compared to vehicle-treated rats on days 100-105 [F (1,25) = 5.221, p = 0.031] (Figure 4.2, Panel A). In addition, LPS-induced increases in plasma levels of CINC-3 were significantly elevated in PB-treated rats compared to vehicle-treated rats [F (1,27) = 6.807, p = 0.015] (Figure 4.2, Panel B).
Figure 4.2 LPS-induced inflammatory markers in plasma of delayed cohort. Prior PB treatment elicits an increase in plasma TNF-α levels in response to LPS compared to vehicle-treated groups in the delayed cohort (Panel A). Prior PB treatment elicits an increase in plasma CINC-3 levels in response to LPS compared to vehicle-treated groups in the delayed cohort (Panel B). Data in Panel A are expressed as mean percent change from saline-treated controls from each respective group + SEM, n=7-9. Data in Panel B are expressed as mean percent change from LPS-treated, Vehicle-NSCs + SEM as CINC-3 was not detected in saline treated animals, n = 6-8. [*: Significant effect of PB, p < 0.05].
4.3.2 A prior history of PB treatment elicits potentiated pro-inflammatory responses in the hippocampus

Similar to basal plasma cytokine levels at the early timepoint (Macht et al., 2019), neither a history of PB treatment nor stress elicited significant changes in hippocampal cytokines in saline-treated rats at the early timepoint (cohort #2), (Table 4.1). When hippocampal cytokines of LPS-treated rats were compared to saline-treated rats in each respective group, PB treatment suppressed the IL-1α response to LPS \([F(1, 31) = 5.203, p = 0.030]\) (Figure 4.3, Panel A), compared to vehicle-treated rats. Conversely, PB treatment enhanced the IL-1β response to LPS \([F(1, 29) = 8.240, p = 0.008]\), within stressed rats (Figure 4.3, Panel C).
Table 4.1 Cytokine levels in the hippocampus of Vehicle-NSC, Vehicle-RRS, PB-NSC and PB-RRS rats in the early cohort 2 hours following i.p. saline or LPS injections.

<table>
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<tr>
<th>Cytokine</th>
<th>Saline</th>
<th>LPS</th>
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<td></td>
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<td>Vehicle-RRS</td>
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<td>PB-RRS</td>
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<td>IL-1α</td>
<td>2.05 ± 0.26</td>
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<td>IL-1β</td>
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<td>TNF-α</td>
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<td>GM-CSF</td>
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<td>1.64 ± 0.19</td>
<td>1.80 ± 0.44</td>
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<td>IL-1α</td>
<td>2.44 ± 0.35</td>
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<td>TNF-α</td>
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<td>1.46 ± 0.30</td>
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Data expressed as mean pg of cytokine per mg of protein ± SEM. [%: significant cross-over effect of PB and stress, p<0.05, n = 7-9]. The following cytokines were not detected: IL-2, IL-4, IL-5, IL-10, IL-13, IFN-γ.
Figure 4.3 Effects of PB on the hippocampal cytokine response to LPS. At the early timepoint, PB treatment suppresses the IL-1α response to LPS in the hippocampus compared to vehicle-treated controls (Panel A). Only the combination of PB and repeated restraint stress (RRS) elicits an increase in IL-1β levels compared to Vehicle-RRS animals at the early timepoint (Panel C). At the delayed timepoint, a history of PB treatment elicits increased IL-1β (Panel D), IL-12 (Panel F) and GM-CSF (Panel H) responses to LPS in the hippocampus compared to vehicle-treated controls. All data are expressed as mean percent change from saline treated controls from each respective group ± SEM, n = 7-9. See Tables 4.1 and 4.2 for complete list of hippocampal cytokines measured. [*: Significant effect of PB treatment compared to vehicle treatment].
At the delayed timepoint (cohort #3), a history of stress led to decreased hippocampal levels of IL-1β: \( F(1, 25) = 8.355, p = 0.008 \); IL-5 \( F(1, 21) = 16.97, p = 0.001 \); IL-6 \( F(1, 25) = 15.08, p = 0.001 \); and TNF-α \( F(1, 25) = 9.868, p = 0.004 \) following saline administration relative to non-stressed controls (Table 4.2). When hippocampal cytokines of LPS-treated rats were compared to saline-treated rats in each respective group, a history of PB treatment significantly enhanced IL-1β: \( F(1, 26) = 10.24, p = 0.004 \) (Figure 4.3, Panel D), IL-12: \( F(1, 28) = 5.270, p = 0.029 \) (Figure 4.3, Panel F) and GM-CSF: \( F(1, 26) = 10.82, p = 0.003 \) (Figure 4.3, Panel H) responses to LPS compared to vehicle-treated rats, increases that were not observed at the early timepoint (Figure 4.3, Panels C, E and G).
Table 4.2 Cytokine levels in the hippocampus of Vehicle-NSC, Vehicle-RRS, PB-NSC and PB-RRS rats in the delayed cohort 2 hours following i.p. saline or LPS injections.

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<td>7.14 ± 0.61##</td>
<td>11.73 ± 0.94*</td>
<td>8.25 ± 0.80***</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.76 ± 0.15</td>
<td>1.90 ± 0.38</td>
<td>1.79 ± 0.17</td>
<td>1.60 ± 0.11</td>
</tr>
<tr>
<td>IL-5</td>
<td>3.38 ± 0.22</td>
<td>3.01 ± 0.19</td>
<td>3.59 ± 0.45</td>
<td>3.13 ± 0.23</td>
</tr>
<tr>
<td>IL-6</td>
<td>20.07 ± 1.55</td>
<td>18.38 ± 1.31#</td>
<td>23.77 ± 1.76</td>
<td>19.36 ± 1.34#</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.88 ± 0.14</td>
<td>2.11 ± 0.29</td>
<td>2.47 ± 0.31</td>
<td>2.05 ± 0.12</td>
</tr>
<tr>
<td>IL-12</td>
<td>10.58 ± 0.87</td>
<td>8.87 ± 1.48#</td>
<td>11.81 ± 0.47</td>
<td>8.29 ± 0.91#</td>
</tr>
<tr>
<td>IL-13</td>
<td>3.61 ± 0.31</td>
<td>4.18 ± 0.28</td>
<td>3.47 ± 0.36</td>
<td>4.35 ± 0.57</td>
</tr>
<tr>
<td>TNF-α</td>
<td>17.59 ± 1.79</td>
<td>15.57 ± 1.10#</td>
<td>20.19 ± 1.76</td>
<td>15.87 ± 0.67#</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.39 ± 0.55</td>
<td>6.02 ± 1.14</td>
<td>5.84 ± 0.49</td>
<td>5.25 ± 0.39</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1.63 ± 0.11</td>
<td>1.64 ± 0.14</td>
<td>2.01 ± 0.26</td>
<td>1.45 ± 0.14</td>
</tr>
</tbody>
</table>

Data expressed as mean pg of cytokine per mg of protein ± SEM. [*: significant effect of PB, p<0.05. #: significant effect of stress, p<0.05. ##: significant effect of stress, p<0.01, n = 7-9]. IL-2 was not detected.
4.3.3 A prior history of PB treatment exaggerates the pro-inflammatory response in the PFC

I also assessed the early and delayed effects of PB and RRS on immune responses in PFC micropunches collected from cohorts #2 and #3. At the early timepoint, there were no significant differences in PFC cytokine levels following saline administration between any group (Table 4.3). Comparing PFC cytokines of LPS-treated rats with saline-treated rats from each respective group determined that PB treatment significantly suppresses the IL-1β response to LPS relative to vehicle-treated rats at the early timepoint \([F (1, 31) = 4.751, p = 0.037]\) (Figure 4.4, Panel A).

**Table 4.3 Cytokine levels in the PFC of Vehicle-NSC, Vehicle-RRS, PB-NSC and PB-RRS rats in the early cohort 2 hours following i.p. saline or LPS injections.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-RRS</th>
<th>PB-NSC</th>
<th>PB-RRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>1.46 ± 0.15</td>
<td>1.38 ± 0.11</td>
<td>1.42 ± 0.07</td>
<td>1.46 ± 0.14</td>
</tr>
<tr>
<td>IL-1β</td>
<td>7.64 ± 1.07</td>
<td>9.28 ± 0.40</td>
<td>9.45 ± 0.77</td>
<td>9.31 ± 1.01</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.84 ± 0.90</td>
<td>10.98 ± 0.64</td>
<td>10.04 ± 0.68</td>
<td>10.72 ± 1.47</td>
</tr>
<tr>
<td>IL-12</td>
<td>7.60 ± 0.47</td>
<td>7.60 ± 0.51</td>
<td>7.48 ± 0.48</td>
<td>8.17 ± 0.72</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.84 ± 0.21</td>
<td>2.92 ± 0.26</td>
<td>2.55 ± 0.19</td>
<td>3.04 ± 0.26</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.57 ± 0.52</td>
<td>4.08 ± 0.25</td>
<td>3.74 ± 0.38</td>
<td>3.30 ± 0.29</td>
</tr>
</tbody>
</table>

**Saline**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Vehicle-NSC</th>
<th>Vehicle-RRS</th>
<th>PB-NSC</th>
<th>PB-RRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>1.23 ± 0.10</td>
<td>1.36 ± 0.06</td>
<td>1.30 ± 0.14</td>
<td>1.38 ± 0.15</td>
</tr>
<tr>
<td>IL-1β</td>
<td>9.40 ± 0.63</td>
<td>9.74 ± 0.90</td>
<td>9.35 ± 0.62</td>
<td>8.16 ± 1.15</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.56 ± 0.65</td>
<td>11.11 ± 1.18</td>
<td>9.12 ± 0.79</td>
<td>9.78 ± 0.81</td>
</tr>
<tr>
<td>IL-12</td>
<td>8.31 ± 0.56</td>
<td>8.93 ± 0.50</td>
<td>8.15 ± 0.70</td>
<td>7.29 ± 0.41</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>2.95 ± 0.26</td>
<td>3.49 ± 0.62</td>
<td>2.57 ± 0.31</td>
<td>3.02 ± 0.26</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.60 ± 0.31</td>
<td>4.88 ± 0.54</td>
<td>3.58 ± 0.42</td>
<td>3.16 ± 0.41</td>
</tr>
</tbody>
</table>

**LPS**

Data expressed as mean pg of cytokine per mg of protein ± SEM, \(n = 6-9\). The following cytokines were not detected: IL-2, IL-4, IL-5, IL-10, IL-13, TNF-α.
Figure 4.4: Effects of PB on the PFC’s cytokine response to LPS. At the early timepoint, PB treatment suppresses the IL-1β response to LPS in the PFC compared to vehicle-treated controls (Panel A). At the delayed timepoint, a history of PB treatment elicits an increased IL-12 (Panel D) and GM-CSF (Panel F) response to LPS in the PFC compared to vehicle-treated controls. All data are expressed as mean percent change from saline treated controls from each respective group + SEM, n = 7-9. See Tables 4.3 and 4.4 for complete list of PFC cytokines measured. [*: Significant effect of PB treatment compared to vehicle treatment].
Similar to the early timepoint, saline administration did not elicit any differences in PFC cytokine levels between any group at the delayed timepoint (Table 4.4). I next examined PFC cytokines of LPS-treated rats compared to saline-treated rats from each respective group. As observed in the hippocampus, PB treatment did not affect the IL-12 or GM-CSF response to LPS in the PFC at the early timepoint (Figure 4.4, Panels C and E). However, a history of PB treatment significantly enhanced IL-12 \([F(1, 25) = 5.324, p = 0.030]\) (Figure 4.4, Panel D) and GM-CSF \([F(1, 25) = 9.514, p = 0.004]\) (Figure 4.4, Panel F) responses to LPS relative to vehicle-treated rats at the delayed timepoint. Unlike the hippocampus, the IL-1β response to LPS in the PFC was not affected by PB or stress at the delayed timepoint (Figure 4.4, Panel B).

**Table 4.4 Cytokine levels in the PFC of Vehicle-NSC, Vehicle-RRS, PB-NSC and PB-RRS rats in the delayed cohort 2 hours following i.p. saline or LPS injections.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Saline</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle-NSC</td>
<td>Vehicle-RRS</td>
</tr>
<tr>
<td>IL-1β</td>
<td>13.54 ± 1.53</td>
<td>18.19 ± 2.23</td>
</tr>
<tr>
<td>IL-12</td>
<td>11.23 ± 1.48</td>
<td>13.56 ± 1.26</td>
</tr>
<tr>
<td>TNF-α</td>
<td>13.14 ± 1.63</td>
<td>15.70 ± 3.41</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>7.66 ± 1.40</td>
<td>7.48 ± 0.85</td>
</tr>
<tr>
<td>IL-1β</td>
<td>12.99 ± 2.34</td>
<td>14.44 ± 0.48</td>
</tr>
<tr>
<td>IL-12</td>
<td>8.49 ± 1.43</td>
<td>9.16 ± 1.35</td>
</tr>
<tr>
<td>TNF-α</td>
<td>8.77 ± 1.09</td>
<td>11.60 ± 2.46</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.41 ± 0.82</td>
<td>6.77 ± 1.35</td>
</tr>
</tbody>
</table>

Data expressed as mean pg of cytokine per mg of protein ± SEM, \(n = 6-9\). The following cytokines were not detected: IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, IFN-γ.
4.3.4 Neither a history of PB or stress alters ChE activity in hippocampus or PFC

Given that a history of PB treatment exacerbates the pro-inflammatory response to LPS in the hippocampus and PFC, I next assessed if the delayed cohort exhibited alterations in ChE activity in these regions. Approximately three months after treatment cessation, neither a history of PB nor stress altered hippocampal ChE activity (Figure 4.5, Panel A). Additionally, ChE activity in the PFC was not affected by PB or stress three months after treatment cessation (Figure 4.5, Panel B).
Figure 4.5 ChE activity in hippocampus and PFC 3 months after treatment cessation. In the delayed cohort, ChE activity in hippocampal homogenates did not differ between any group (Panel A). Similarly, ChE activity in PFC punches from the delayed cohort did not differ between any group (Panel B). All data are expressed as mean percent change from Vehicle-NSCs + SEM, n = 6-8.
4.3.5 PB and stress do not affect α7 nAChR expression in hippocampus or PFC

Since previous studies have identified the α7 subunit of the nAChR as an important component of the anti-inflammatory pathway in the periphery and CNS, western blot analysis was performed for α7 nAChR expression in total membrane fractions isolated from the hippocampus and PFC of our four treatment groups. The α7 subunit was identified as a single band at the expected molecular weight of 55 kDa in total membrane fractions isolated from hippocampus and PFC (Figure 4.6). Hippocampal α7 expression did not differ between groups at the early (Panel A) or delayed timepoints (Panel B). Similarly, there were no significant differences in α7 expression in the PFC at the early (Figure 4.5, Panel C) or delayed (Figure 4.5, Panel D) timepoints.
Figure 4.6 Expression of α7 nAChRs in hippocampal homogenates and PFC punches. 
α7 nAChR expression in the hippocampus and PFC of saline-treated animals did not differ between any groups at the early or delayed timepoints. All data are expressed as mean percent change from vehicle non-stressed controls (Vehicle-NSC) + SEM, n = 7-8.
4.4 Conclusions

Results from this study indicate that PB disrupts the central and peripheral immune systems over time, as evidenced by exaggerated peripheral and central cytokine responses to an innate immune challenge. Specifically, in response to a peripheral injection of a modest dose of LPS (30 µg/kg BW), PB-treated rats exhibit potentiated increases in plasma TNF-α and CINC-3 levels, illustrating a sensitized response to an immune challenge more than three months after PB administration. As PB treatment also elevates plasma ChE activity at this delayed timepoint, these enhanced pro-inflammatory responses may be due to a reduction in ACh and its anti-inflammatory actions. Within the CNS, a history of PB treatment produced exacerbated increases in neuroinflammation in the hippocampus and PFC. For example, PB-treated rats in the delayed cohort exhibit potentiated increases in the pro-inflammatory cytokines IL-1β, IL-12 and GM-CSF compared to vehicle-treated rats when challenged with LPS. Unlike the periphery, PB treatment did not affect ChE activity in the hippocampus or PFC in the delayed cohort, suggesting a dysregulation of the central cholinergic anti-inflammatory pathway. Interestingly, this immune dysregulation was only evident at the delayed timepoint, suggesting that peripheral and central immune dysfunction may be a delayed consequence of PB treatment. Furthermore, this neuroinflammation is not seen in PB-treated rats that receive saline injections, highlighting the latent phenotype of GWI that emerges when a physiological stressor is present.

As α7 nAChRs expressed on microglia and astrocytes are proposed to be critical regulators of the anti-inflammatory pathway in the CNS (Wu et al., 2021), I predicted that α7 levels would be reduced in PB-treated rats at the delayed timepoint. However, I did not
observe statistically significant changes in α7 expression levels in the hippocampus or PFC in any of our treatment groups at either the early or delayed timepoints. Alternatively, a long-lasting consequence of PB treatment may include pharmacodynamic or functional changes in α7 receptors. For example, previous studies have reported that PB has weak agonist activity at nAChRs and elicits desensitization of nAChRs (Pascuzzo et al., 1984). However, this study did not measure receptor expression and only examined the effects of PB at the neuromuscular junction (Pascuzzo et al., 1984). Thus, an important future direction would be to examine the short term and long-lasting effects of PB administration on the electrophysiological properties of α7 nAChRs in the hippocampus and PFC of PB-treated rats.

Collectively, these results demonstrate that PB administration elicits long-lasting impairments in the activity of the peripheral cholinergic anti-inflammatory pathway by increasing plasma ChE activity and reducing ACh’s anti-inflammatory actions. Conversely, the central cholinergic anti-inflammatory pathway is dysregulated by PB treatment as ChE activity is not affected, yet neuroinflammatory responses are potentiated in the hippocampus and PFC. As neuroinflammation has been implicated in various neurodegenerative diseases (Kalaria et al., 1989; Lee et al., 1999; Rozovsky et al., 1998), these findings provide mechanistic insight into the LPS-induced cognitive deficits presented in Chapter 3. Importantly, these data are consistent with the immune dysregulation observed in veterans with GWI, which are best observed following a physiological stressor (Broderick et al., 2013; Broderick et al., 2011; Whistler et al., 2009), and further emphasize the validity of this experimental model of GWI and its latent phenotype.
CHAPTER 5
MEASURING CHOLINERGIC NEUROCHEMISTRY IN AN EXPERIMENTAL MODEL OF GWI: HIPPOCAMPUS

5.1 Introduction

As a modest immune challenge caused robust pro-inflammatory responses and significant hippocampal-dependent memory deficits in our model of GWI, I hypothesized that such impairments are due to suppressed ACh neurotransmission in the hippocampus. As described in Chapter 1, hippocampal ACh efflux is an integral part of memory acquisition and consolidation and altering hippocampal ACh efflux elicits significant learning and memory impairments (Haam & Yakel, 2017). ACh is also a key regulator of the anti-inflammatory response and reduced hippocampal ACh efflux can cause rampant neuroinflammation, which will further impair hippocampal-dependent learning and memory (Pavlov et al., 2003). To determine if ACh neurotransmission is attenuated in our GWI model, hippocampal ACh efflux was assessed with in vivo microdialysis. In vivo microdialysis involves the perfusion of molecules through an implanted, semi-permeable membrane which is collected in a dialysate (Ungerstedt, 1991). For neurochemical studies, such membranes are implanted into brain regions of interest and neurotransmitter efflux can be measured in the dialysates (Chefer et al., 2009). Importantly, this technique is performed in awake, freely moving animals and the continuous collection of dialysates allows for real-time measurement of neurotransmitter efflux (Darvesh et al., 2011). While
the Reagan and Fadel labs previously assessed cholinergic neurochemistry in an early cohort of our GWI model (Macht et al., 2019), I sought to perform these studies in a delayed cohort as this timepoint is when neuroinflammatory and memory impairments emerge. Given that these impairments are induced by LPS, my *in vivo* microdialysis experiments also included this mild immune challenge. Additionally, I conducted a second session of *in vivo* microdialysis that included an immobilization stress as our model also exhibited 24-hour memory impairments in the MWM, which is inherently stressful. Including both an immune and immobilization stress challenge in these studies is highly relevant as dysregulated immune and stress responses have been observed in GWI patients and are known to impair cognition (Rayhan et al., 2013; Washington et al., 2020). Furthermore, GW veterans continue to experience daily life stressors and immune threats as they age and understanding how these responses are disrupted over time may provide insight into their progressive neurological deficits.

### 5.2 Materials and methods

This chapter describes a cohort of rats (cohort #4) that underwent the GWI treatment paradigm outlined in Chapter 2 and *in vivo* microdialysis in the hippocampus and PFC at the delayed timepoint. ACh efflux of this delayed cohort was then compared to neurochemical data from an early cohort described previously (Macht et al., 2019). This chapter will describe the data collected from the hippocampus while Chapter 6 will describe the PFC findings.

#### 5.2.1 Stereotaxic surgery

Approximately 100 days after the GWI treatment paradigm, rats in cohort #4 were anesthetized with isoflurane and underwent stereotaxic surgery to place two guide cannulae
into the PFC and dorsal hippocampus, as previously described (Burzynski et al., 2022; Macht et al., 2019). Briefly, interlocking intracerebral guide cannulae and stylets from Bioanalytical Systems Incorporated (BASi: #MD-2251) were unilaterally implanted relative to bregma: AP, + 3.2mm; L, ± 0.5mm; DV, - 2.5mm for the PFC, and AP, - 5.5mm; L, ± 4.0mm; DV, - 3.8mm at a 10° angle for the hippocampus. Coordinates were based on Paxinos and Watson rat brain atlas (Paxinos & Watson, 1998). Left and right hemispheres were counterbalanced across groups. Rats were left undisturbed for one full day following surgery for recovery, before beginning habituation to the microdialysis bowls. No differences in surgical recovery were observed in any group.

5.2.2 In vivo microdialysis

Each rat in cohort #4 underwent a session of microdialysis as previously described (Macht et al., 2020; Macht et al., 2019). Briefly, rats were habituated to the microdialysis bowls in the BASi Raturn system for 20 hours over the course of 4 days (approximately 5 hours per day). This habituation period ensured rats received a full week of recovery before microdialysis began. On the day of the first microdialysis session, BASi probes (2 mm, MD-2200) were placed into the guide cannulae and perfused with artificial cerebral spinal fluid (150 mM NaCl; 3 mM KCl; 1.7 mM CaCl₂H₂O; 0.183 mM MgCl₂6H₂O; 5 mM D-glucose) with 100nM neostigmine at a rate of 2μL/min. The first three hours (8:00 am-11:00 am) of collection were discarded to allow for recovery from probe insertion. Collections were then taken at 15-minute intervals with the first four collections serving as baseline measurements. Rats were injected with LPS (30 µg/kg BW i.p.) at the start of the 5th collection, and collections continued for 3 hours post injection. Samples were immediately frozen and stored at -80°C at the end of each collection.
Rats in cohort #4 were given one day of rest before undergoing a second microdialysis session 48 hours later. While repeated in vivo microdialysis sessions require multiple probe insertions that likely create tissue damage, it is well established that repeated in vivo microdialysis sessions separated by 48 hours do not affect basal or stimulated ACh efflux (Johnson & Bruno, 1995; Moore et al., 1995). As in the first session, the first three hours (8:00 am-11:00 am) of collection were discarded to allow for recovery from probe insertion. Collections were then taken at 15-minute intervals with the first four collections serving as baseline measurements. Rats were placed in a plexiglass restrainer at the start of the 5th collection and were removed from the restrainer at the end of the 8th collection for a total of 1-hour of immobilization. Collections continued for 1 hour after immobilization. Samples were immediately frozen and stored at -80°C at the end of each collection until analysis. It is important to note that this restrainer is different from the mesh restrainers used during the GWI treatment paradigm and this immobilization session takes place in the microdialysis bowls instead of the home cage. Therefore, this immobilization challenge is a novel stressor to all animals. See Figure 5.1 for experimental timeline.
Figure 5.1 Experimental timeline of *in vivo* microdialysis studies. Cohort #4 underwent *in vivo* microdialysis approximately 100 days after treatment cessation. The first session of microdialysis occurred one week after cannulation surgery and included an acute LPS challenge (30 µg/kg BW, i.p.). The second session of microdialysis occurred 48-hours later and included a 1-hour immobilization stress challenge. Figure made with www.biorender.com.

5.2.3 Transcardial perfusion

Following the second session of microdialysis, rats in cohort #4 were anesthetized with isoflurane and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and placed in a 30% sucrose/0.1M phosphate buffer solution at 4°C for several days and then rapidly frozen using isopentane on dry ice and stored at -80°C. A sliding microtome was used to cut 40 µm coronal sections to verify probe placement in each rat (Figure 3.2) as shown previously (Macht et al., 2019).
Figure 5.2 Probe placement validation. Probe placement is depicted for targets for the medial prefrontal cortex (Panel A) and dorsal hippocampus (Panel B). Representative examples of probe placement are depicted in the prefrontal cortex (Panel C) and hippocampus (Panel D). Samples were stained with a background cholinesterase stain to enhance contrast of the probe location.

5.2.4 High performance liquid chromatography

ACh concentration in dialysate samples was measured as previously described (Burzynski et al., 2022; Calva et al., 2018; Fadel et al., 2005; Macht et al., 2019). Briefly, dialysate samples were thawed individually and 20 μL loaded onto an Eicom AC-GEL reverse-phase analytical column, where choline and ACh 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. A dual enzymatic column AC-ENZYM II from Eicom metabolized ACh into hydrogen peroxide by acetylcholinesterase and choline oxidase. An applied potential of +450 mV oxidized the
hydrogen peroxide at the platinum electrochemical detector. The current was read with the Eicom HT-500 detector system with a detection limit of 10 fmol and a retention time of 15 minutes. Concentration of ACh in samples was interpolated against a three-point standard curve.

5.2.5 Statistical analysis

Basal levels of ACh during the first in vivo microdialysis session were assessed with a 2 x 2 ANOVA with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (NSC, RRS). In vivo microdialysis data were assessed with a 2 x 2 x 16 (LPS session) or a 2 x 2 x 12 (immobilization session) mixed ANOVA as previously described (Macht et al., 2020; Macht et al., 2019). Briefly, for between-subjects factors, this study had 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (NSC, RRS). Within-subjects repeated measures consisted of 16 or 12 levels as there were 16 (LPS) or 12 (immobilization) consecutive collections during the microdialysis sessions. Comparisons of basal levels of ACh between the LPS and immobilization microdialysis sessions were analyzed by unpaired t-test. For all analyses, statistical significance was set at $\alpha = 0.05$. Unless otherwise stated, following a significant interaction, post-hoc follow-ups were assessed with a Bonferroni-corrected simple main effects analyses. Post-hoc tests assessed all levels of drug treatment within each level of stress, and all levels of stress within each level of drug treatment across each level of time, when applicable. While efflux measurements were not corrected for probe recovery, previous studies have found these microdialysis probes to have a reliable recovery rate of 10-15% (Fadel et al., 2001) and any variations in probe recovery are accounted for by representing ACh efflux as a mean percent change from baseline.
5.3 Results

5.3.1 Prior history of PB treatment enhances LPS-induced release of ACh in hippocampus

To determine the long-lasting effects of PB and stress on cholinergic neurotransmission, I first examined the effects of LPS administration on hippocampal ACh release in our GWI model by *in vivo* microdialysis at the delayed timepoint. While basal levels of ACh were not affected by a prior history of PB or stress (Figure 5.3), prior exposure to PB and RRS interacted over time to significantly influence the cholinergic response to LPS in the hippocampus (Figure 5.4) \[F (15, 390) = 1.744, p = 0.041\]. Bonferroni correct *post-hoc* follow-up measures indicated that in Vehicle-NSC rats, LPS produced a latent increase in ACh relative to baseline measures which emerged at timepoint 12 \(p = 0.003\). Prior exposure to RRS in vehicle-treated rats suppressed this cholinergic response to LPS at collection 12, \(p = 0.021\), but then conversely increased ACh levels at collection 15 relative to Vehicle-NSC rats \(p = 0.039\). This suggests that prior stress exposure delays the reaction of the cholinergic system to innate immune challenges. Similarly, in NSC rats, prior exposure to PB suppressed the cholinergic response to LPS at collection 12 \(p = 0.035\), suggesting that a history of PB impairs the cholinergic response to a later innate immune challenge. Interestingly, within the PB-treated cohorts, rats which had a prior exposure to RRS had a trend for an exaggerated cholinergic response to LPS versus PB-NSC counterparts \(p = 0.078, 0.069, \text{ and } 0.051\) at collections 12, 13, and 16, respectively). These effects replicate previous findings (Macht et al., 2019) that PB and stress interact to differentially disrupt the latent cholinergic response to LPS in the hippocampus.
Figure 5.3 Basal hippocampal acetylcholine levels of delayed cohort. During the first session of *in vivo* microdialysis, there was no effect of PB treatment or RRS on basal ACh levels in the hippocampus approximately 100 days after treatment cessation. All data are expressed as mean ± SEM, *n* = 6-9.
Figure 5.4 Hippocampal cholinergic response to LPS of delayed cohort. A history of RRS in vehicle-treated rats suppressed the cholinergic response to LPS at collection 12, but then increased this response at collection 15 compared to Vehicle-NSC rats. Within NSCs, a history of PB treatment suppresses the cholinergic response to LPS at collection 12 compared to vehicle-treated rats. Unlike PB-NSC rats, the combination of PB and stress exaggerated the cholinergic response to LPS at collections 12, 13 and 16, although these increases did not achieve statistical significance. All data are expressed as mean ± SEM, n = 6-9. [*: Significant effect of PB in NSC rats, p < 0.05. #: Significant effect of RRS in vehicle-treated rats, p < 0.05].
Comparison of the effects of the LPS challenge in the early versus delayed cohorts could provide important insight into the progressive nature of the neurological deficits that are characteristic of GWI. The cholinergic response to LPS of the delayed cohort was compared to our previous neurochemical findings in an early cohort (Macht et al., 2019). These comparisons revealed that Vehicle-NSC rats (Figure 5.5, Panel A) and Vehicle-RRS rats (Figure 5.5, Panel B) exhibited strikingly similar increases in hippocampal ACh levels at the early and delayed timepoints. Conversely, delayed cohort PB-treated rats exhibited potentiated increases in ACh levels compared to early cohort PB-treated rats. For example, delayed cohort PB-NSC rats exhibited statistically significant increases in ACh levels at collection 8 (approximately 60 minutes following LPS administration; \( p = 0.014 \)) compared to the PB-NSC rats in the early cohort (Figure 5.5, Panel C). Similarly, delayed cohort PB-RRS rats exhibited significant increases in hippocampal ACh levels following LPS administration at collections 9, 11, and 13 (\( p = 0.046, 0.006, 0.0004 \), respectively) compared to early cohort PB-RRS rats (Figure 5.5, Panel D). These results suggest that a history of PB treatment, not RRS, is driving the long-lasting changes within the hippocampal cholinergic system in our model of GWI.
Figure 5.5 Comparison of the hippocampal cholinergic response to LPS in early and delayed cohorts. There were no significant differences in the cholinergic response to LPS between early and delayed cohorts of Vehicle-NSCs (Panel A) or vehicle-treated rats subjected to RRS (Vehicle-RRS; Panel B). Panel C: In rats with a history of PB treatment, the cholinergic response to LPS was significantly higher in the delayed cohort at collection 8 compared to the PB-NSC early cohort. Panel D: In rats with a history of PB treatment and RRS, the cholinergic response to LPS was significantly higher in the delayed cohort at collections, 9, 11 and 13 compared to the PB-RRS early cohort. All data are expressed as mean + SEM, n = 6-10. [*: Significant difference between early and delayed cohorts, p < 0.05].
5.3.2 A history of PB treatment impairs recovery of hippocampal acetylcholine from stress challenge

Two days after the in vivo microdialysis session paired with an acute LPS challenge, cohort #4 underwent a second session of in vivo microdialysis paired with a 1-hour immobilization stress and 1-hour recovery period. As observed in the LPS session, basal ACh levels did not differ between groups during this second session (Table 5.1). Additionally, within each treatment group, there were no significant differences in basal ACh levels between the first and second session, meaning prior administration of LPS did not alter basal levels of ACh prior to immobilization stress 48-hours later.

Table 5.1 Basal levels of hippocampal acetylcholine (ACh) prior to LPS administration or immobilization stress challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal ACh prior to LPS Rx (pmols/20 µL)</th>
<th>Basal ACh prior to stress Rx (pmols/20 µL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-NSC</td>
<td>0.071 ± 0.02</td>
<td>0.075 ± 0.02</td>
<td>0.896</td>
</tr>
<tr>
<td>Vehicle-RRS</td>
<td>0.108 ± 0.03</td>
<td>0.059 ± 0.01</td>
<td>0.124</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>0.099 ± 0.02</td>
<td>0.056 ± 0.02</td>
<td>0.106</td>
</tr>
<tr>
<td>PB-RRS</td>
<td>0.114 ± 0.02</td>
<td>0.078 ± 0.02</td>
<td>0.232</td>
</tr>
</tbody>
</table>

All data expressed as mean ± SEM, n = 5-9/group.

In response to immobilization stress, there was a significant interaction of PB and RRS to potentiate the hippocampal cholinergic response at this delayed timepoint (Figure 5.6, Panel A) [2 x 2 x 12 mixed ANOVA, F(11, 209) = 2.058, p = 0.025] but there were no significant main effects of PB [2 x 2 x 12 mixed ANOVA, F(11,209) = 0.849, p = 0.591] or RRS [2 x 2 x 12 mixed ANOVA, F(11,209) = 0.896, p = 0.545]. Bonferroni corrected
post-hoc follow-up measures revealed that prior exposure to PB in RRS rats significantly increased ACh levels in the post-stress period, namely at collections 11 (p = 0.018) and 12 (p = 0.027), relative to Vehicle-RRS rats. The potentiated cholinergic response observed in PB-RRS rats was also significantly greater than cholinergic responses of PB-NSC rats at collection 11 (p = 0.017).

Given the interesting differences in ACh levels during the post-stress period, I next conducted area under the curve analysis of ACh efflux during the 1-hour of immobilization stress and the 1-hour following stress. During immobilization stress, there was an increase in ACh efflux above baseline in all groups, but there were no significant differences between groups (Figure 5.6, Panel B). While all groups continued to have elevated ACh efflux in the post-stress period, a two-way ANOVA indicated that rats with a history of PB treatment exhibit potentiated hippocampal ACh levels during the post-stress period relative to vehicle-treated rats (Figure 5.6, Panel C) [F(1,19) = 11.62, p = 0.003]. There was not a significant main effect of stress history [F(1,19) = 1.925, p = 0.181] or a significant interaction of PB and RRS [F(1,19) = 2.814, p = 0.110]. These findings illustrate that PB treatment alone has lasting effects on hippocampal cholinergic responses to stress, specifically during the recovery of an acute stress challenge.
Figure 5.6 Hippocampal cholinergic responses to immobilization stress 3 months after treatment cessation. Within rats previously subjected to RRS, a history of PB treatment potentiates the cholinergic response to immobilization stress during the 1-hour recovery period (collections 11 and 12) compared to Vehicle-RRS rats (Panel A). This potentiated response in PB-RRS rats at collection 11 is also significantly greater than the responses of PB-NSC rats. Area under the curve analysis revealed that hippocampal ACh efflux during the 1-hour immobilization stress does not differ between groups (Panel B). However, prior history of PB treatment produces significant elevations in hippocampal ACh efflux in the 1-hour post-stress recovery period (Panel C). All data are expressed as mean ± SEM, n = 5-7/group. [#]: Significant effect of PB in RRS rats, p < 0.05. *: Significant effect of RRS in PB-treated rats, p <0.05. **: Significant effect of PB, p < 0.01.
Beyond area under the curve analysis, the delayed cohort’s cholinergic response to immobilization stress was also compared to our previous neurochemical findings in an early cohort (Macht et al., 2019). As in the LPS session, Vehicle-NSC rats (Figure 5.7, Panel A) and Vehicle-RRS rats (Figure 5.7, Panel B) exhibited similar cholinergic responses to the immobilization stress between the early and delayed timepoints. While responses of early and delayed cohorts of PB-NSC rats were not significantly different (Figure 5.7, Panel C), the delayed cohort’s ACh efflux continues to increase through collection 10, while the early cohort’s ACh efflux begins returning to baseline at collection 10. This sustained elevation of ACh in the post-stress recovery period may be indicative of PB’s progressive disruption of hippocampal function. Indeed, PB-RRS rats in the delayed cohort also show potentiated ACh efflux in the post-stress recovery period relative to the early cohort (Figure 5.7, Panel D) which reaches statistical significance at collection 11 ($p = 0.005$).
Figure 5.7 Comparison of the hippocampal cholinergic response to immobilization stress in early and delayed cohorts. There were no significant differences in the cholinergic response to immobilization stress between early and delayed cohorts of vehicle non-stressed controls (Vehicle-NSC; Panel A), vehicle-treated rats subjected to repeated restraint stress (Vehicle-RRS; Panel B), or PB non-stressed controls (PB-NSC; Panel C). Panel D: In rats with a history of PB treatment and RRS, the cholinergic response to immobilization stress was significantly higher in the delayed cohort at 11 compared to the PB-RRS early cohort. All data are expressed as mean + SEM, n = 6-10. [*: Significant difference between early and delayed cohorts, p < 0.01].
5.4 Conclusions

The results of the current study indicate that PB disrupts the hippocampal cholinergic system over time, as evidenced by exaggerated ACh efflux in PB-treated rats when responding to an immune or immobilization challenge three months after treatment cessation. These results are not what I hypothesized as elevations in ACh efflux should strengthen anti-inflammatory responses in the hippocampus. However, PB-treated rats in a separate delayed cohort exhibit exaggerated hippocampal inflammation when exposed to the same LPS challenge (see Chapter 4). Such conflicting results highlight a dysregulation in the cholinergic anti-inflammatory pathway within the hippocampus, which may be an enduring consequence of PB administration. The sustained elevation of hippocampal ACh in PB-treated rats after an immobilization challenge suggests that PB produces similar sensitized ACh responses when responding to stress. Importantly, the immune and immobilization sessions were not counterbalanced in this study to allow for any carryover effects of LPS to be observed. As basal ACh efflux was not changed between the two sessions, which is consistent with our previous studies measuring both hippocampal ACh (Macht et al., 2019) and glutamate (Macht et al., 2020), I do not believe that responses to the immobilization challenge 48 hours later are influenced by lasting immune responses. Collectively, these findings highlight PB’s long-lasting dysregulation of ACh and its anti-inflammatory actions within the hippocampus which likely contributes to the memory impairments observed in our GWI model (see Chapter 3). Additionally, these neurochemical findings provide further evidence of GWI’s latent phenotype as PB treatment does not affect basal levels of hippocampal ACh at the early (Macht et al., 2019) or delayed timepoint, and disruptions in ACh efflux are only seen following a stimulus.
6.1 Introduction

Along with the hippocampus, I also investigated the long-lasting effects of PB and/or stress on cholinergic neurochemistry in the PFC with *in vivo* microdialysis. The PFC is a key regulator of executive function, which is associated with goal-oriented behavior that requires attention and cognitive flexibility (Miller & Cohen, 2001). These processes are separate from habitual or automatic responses that are mediated by other brain regions (Friedman & Robbins, 2022). The PFC is an important region for GWI research as attentional deficits are a prominent symptom that continues to worsen as veterans age (Jeffrey et al., 2019). While PFC-dependent tasks have not been performed in our GWI model, a history of PB treatment elicits enhanced pro-inflammatory responses to an LPS challenge in this region (see Chapter 4) (Burzynski et al., 2022). Thus, as in the hippocampus, I hypothesized that this exacerbated neuroinflammation is due to suppressed cholinergic neurotransmission in the PFC and likely contributes to the attentional deficits observed in GWI patients.

6.2 Materials and methods

This chapter discusses neurochemical results from the same cohort described in Chapter 5 (cohort #4). As in Chapter 5, ACh efflux of this delayed cohort was then
compared to neurochemical data from an early cohort described previously (Macht et al., 2019).

6.3 Results

6.3.1 A history of PB and stress interact to potentiate LPS-induced release of ACh in PFC

As in the hippocampus, I first examined the effects of LPS administration on cholinergic neurochemistry in the PFC of a delayed cohort. Interestingly, unlike observations in the hippocampus, there was a significant cross-over effect of PB and stress in the PFC, in which PB-RRS rats exhibited increased basal ACh levels compared to Vehicle-RRS rats and PB-NSC rats (Figure 6.1) \[ F (1, 96) = 4.159, \ p = 0.044 \]. Additionally, prior exposure to PB and RRS interacted over time to significantly influence the cholinergic response to LPS in the PFC (Figure 6.2) \[ F (15, 345) = 1.72, \ p = 0.046 \]. Bonferroni post-hoc follow-up measures indicated that in Vehicle-NSC rats, LPS increased ACh levels at collections 5, 12, 13, relative to baseline \( p = 0.001, 0.04, \) and 0.03, respectively; Figure 6). There was no effect of prior stress history on ACh levels in response to an LPS challenge relative to NSC rats at any timepoint. In contrast, prior exposure to PB attenuated the levels of ACh at collection 12 within NSC-rats, indicating that PB alone inhibits the cholinergic response to an innate immune challenge \( p = 0.01 \). A prior stress history selectively increased ACh levels within PB-treated rats at collections 5, 7, 9, 12, 13, and 16 \( p = 0.004, 0.032, 0.013, 0.008, 0.012, \) and 0.001, respectively). PB-RRS rats exhibited a significantly greater cholinergic response to LPS than Vehicle-RRS rats at collection 16 \( p = 0.02 \), with a trend for an exaggerated cholinergic response to LPS at collections 7, 9, and 13 \( p = 0.082, 0.062, \) and 0.056, respectively).
Figure 6.1 Basal PFC acetylcholine levels of delayed cohort. Prior to LPS administration in the first *in vivo* microdialysis session, there was a significant cross-over effect of PB and repeated restraint stress (RRS) on basal ACh levels in the PFC. All data are expressed as mean ± SEM, *n* = 6-8. [%: Significant cross-over effect of PB and RRS, *p* < 0.01].
Figure 6.2 PFC cholinergic response of delayed cohort to LPS. Rats with a history of PB treatment exhibited an attenuated cholinergic response to LPS at collection 12 compared to Vehicle-NSC rats. In rats with a history of PB and RRS, the cholinergic response to LPS was significantly greater at collections 5, 7, 9, 12, 13 and 16 compared to PB-NSC rats. Rats with a history of PB and RRS also exhibited exaggerated cholinergic responses to LPS compared to Vehicle-RRS rats with a significant increase at collection 16 and non-significant increases at collections 7, 9 and 13. All data are expressed as mean ± SEM, n = 6-8. [*: Significant effect of PB in NSC rats, p < 0.05. $: Significant effect of RRS in PB-treated rats, p < 0.05. &: Significant effect of PB treatment in rats with a history of RRS, p < 0.05].
I also compared LPS-induced changes in cholinergic neurochemistry in the PFC between this delayed cohort and an early cohort described previously (Macht et al., 2019). Similar to observations in the hippocampus, LPS-induced changes in ACh levels did not differ in Vehicle-NSC rats (Figure 6.3, Panel A) or Vehicle-RRS rats (Figure 6.3, Panel B) in the early cohorts compared to the delayed cohorts. In PB-NSC rats, LPS-induced increases in ACh levels were blunted in the delayed cohort compared to the early cohort at collection 5 (the first collection after LPS administration) and at collection 15 ($p = 0.011$, 0.024, respectively; Figure 6.3, Panel C). Interestingly, RRS dramatically altered the responses of PB-treated rats in that the delayed cohort exhibited much more robust increases in ACh release in response to the LPS immune challenge compared to the early cohort. These enhanced responses were statistically significant at collections 6, 7, 9, 12 and 14 ($p = 0.035, 0.001, 0.037, 0.016, 0.025$, respectively; Figure 6.3, Panel D).
Figure 6.3 Comparison of the PFC cholinergic response to LPS in early and delayed cohorts. There were no significant differences in the cholinergic response to LPS between early and delayed cohorts of Vehicle-NSC rats (Panel A) or Vehicle-RRS rats (Panel B). In rats with a history of PB treatment, the cholinergic response to LPS was significantly lower in the delayed cohort at collection 5 and 15 compared to the PB-NSC early cohort (Panel C). In rats with a history of PB treatment and RRS, the cholinergic response to LPS was significantly higher in the delayed cohort at collections 6, 7, 9, 12 and 14 compared to the PB-RRS early cohort (Panel D). All data are expressed as mean ± SEM, \( n = 6-10 \). [*: Significant difference between early and delayed cohorts, \( p < 0.05 \).]
6.3.2 Delayed interaction of PB and stress potentiates release of ACh in PFC after immobilization stress

Two days after the *in vivo* microdialysis session paired with an acute LPS challenge, cohort #4 underwent a second session of *in vivo* microdialysis paired with a 1-hour immobilization stress and 1-hour recovery period. Unlike the LPS session, basal ACh levels did not differ between groups during this second session (Table 6.1). Additionally, within each treatment group, there were no significant differences in basal ACh levels between the first and second session, meaning prior administration of LPS did not alter basal levels of ACh prior to immobilization stress 48-hours later.

### Table 6.1 Basal levels of PFC acetylcholine (ACh) prior to LPS administration or immobilization stress challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal ACh prior to LPS Rx (pmols/20 µL)</th>
<th>Basal ACh prior to stress Rx (pmols/20 µL)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle-NSC</td>
<td>0.198 ± 0.03</td>
<td>0.176 ± 0.03</td>
<td>0.664</td>
</tr>
<tr>
<td>Vehicle-RRS</td>
<td>0.114 ± 0.02</td>
<td>0.097 ± 0.03</td>
<td>0.628</td>
</tr>
<tr>
<td>PB-NSC</td>
<td>0.153 ± 0.06</td>
<td>0.127 ± 0.03</td>
<td>0.703</td>
</tr>
<tr>
<td>PB-RRS</td>
<td>0.199 ± 0.07</td>
<td>0.098 ± 0.03</td>
<td>0.196</td>
</tr>
</tbody>
</table>

All data expressed as mean ± SEM, *n* = 5-9/group.

As in the hippocampus, there was a significant interaction of PB and RRS to potentiate the hippocampal cholinergic response to immobilization stress at this delayed timepoint (Figure 6.4, Panel A) \[F(11, 220) = 2.381, p = 0.008\] but there were no significant main effects of PB \[F(11,220) = 0.835, p = 0.605\] or RRS \[F(11,220) = 0.575, p = 0.848\]. Bonferroni corrected *post-hoc* follow-up measures revealed that prior exposure to RRS in vehicle-treated rats significantly decreased ACh levels in the post-stress period, namely at
collections 11 \((p = 0.046)\) and 12 \((p = 0.023)\). Similarly, within NSC rats, a history of PB treatment significantly attenuated ACh efflux in the PFC at collection 12 \((p = 0.044)\). Area under the curve analysis revealed that during immobilization stress, there was a significant cross over effect of PB and stress to elevate ACh efflux relative to Vehicle-RRS and PB-NSC groups (Figure 6.4, Panel B) \([F(1,20) = 5.1, \ p = 0.034]\). This interaction of PB and RRS was also observed in the 1-hour recovery period (Figure 6.4, Panel C) \([F(1,20) = 17.45, \ p = 0.0005]\).

Again, these findings were compared to the cholinergic responses observed in an early cohort previously described (Macht et al., 2019). Interestingly, unlike the LPS session, the PFC cholinergic response to immobilization stress was almost identical between early and delayed cohorts of all groups (Figure 6.5). Specifically, there were no significant differences in ACh efflux between early and delayed cohorts of Vehicle-NSC (Panel A), Vehicle-RRS (Panel B) and PB-NSC (Panel C) rats. Within PB-RRS rats (Figure Panel D), the delayed cohort only exhibited significant increases in ACh efflux at collection 11 \((p = 0.038)\) in the post-stress recovery period.
Figure 6.4 PFC cholinergic responses to immobilization stress 3 months after treatment cessation. Within vehicle-treated rats, a history of RRS attenuates ACh efflux in the 1-hour period after an immobilization challenge at collections 11 and 12 (Panel A). Relative to NSC rats, a history of PB treatment attenuated the cholinergic response at collection 12. Area under the curve analysis revealed that PB and stress interact to increase ACh efflux during immobilization stress relative to Vehicle-RRS and PB-NSC rats (Panel B). This cross-over effect of PB and stress is also observed in the post-stress recovery period (Panel C). All data are expressed as mean ± SEM, n = 5-7/group. [*: Significant effect of PB in NSC rats, p < 0.05. #: Significant effect of RRS in vehicle-treated rats, p < 0.01, %: Significant interaction of PB and RRS, p < 0.05].
Figure 6.5 Comparison of the PFC cholinergic response to immobilization stress in early and delayed cohorts. There were no significant differences in the cholinergic response to immobilization stress between early and delayed cohorts of Vehicle-NSC rats (Panel A), Vehicle-RRS rats (Panel B) or PB-NSC rats (Panel C). In rats with a history of PB treatment and RRS, the cholinergic response to immobilization stress was significantly higher in the delayed cohort at collection 11 compared to the PB-RRS early cohort (Panel D). All data are expressed as mean + SEM, n = 5-9. [*: Significant difference between early and delayed cohorts, p < 0.05].
6.4 Conclusions

The results of the current study highlight region-specific effects of PB and stress on cholinergic neurochemistry. Specifically, within the PFC, PB and stress have synergistic effects to increase ACh efflux after an immune challenge at the delayed timepoint. Conversely, relative to their early cohort counterparts, PB-NSC rats in the delayed cohort exhibit attenuated cholinergic responses to this immune challenge. These findings differ from my hippocampal studies (see Chapter 5) which found that PB treatment, irrespective of stress history, potentiate these cholinergic responses over time (Burzynski et al., 2022). While similar effects of PB are observed in the hippocampus when animals are subjected to an immobilization stress (see Chapter 5) (Burzynski et al., 2023), the PFC cholinergic responses to immobilization stress do not significantly differ between PB-treated early and delayed cohorts. These region-specific differences to an immune or immobilization challenge may be due to the PFC’s extreme susceptibility to stress (Arnsten, 2009). For example, rodents subjected to acute stressors showed impairments in PFC-dependent tasks (Murphy et al., 1996), but such mild stressors had no effect on hippocampal-dependent tasks or memory consolidation (McEwen, 2004). Similar results were seen in human studies as the Trier social stress test produced working memory impairments but improved hippocampal spatial memory (Luethi et al., 2009). Thus, the PFC’s sensitivity to stress may be masking PB and/or stress-induced alterations in cholinergic responses to an immobilization stress. Thus, these findings illustrate that the cholinergic neurochemistry of our GWI model differs between brain regions as well as between physiological stressors. To better understand how differences in PFC neurochemistry may contribute to cognitive deficits in GWI, assessing performance in PFC-dependent tasks in our model is an
important future direction to determine if PB treatment alone, or PB and stress produce similar attentional deficits.
CHAPTER 7

GENERAL DISCUSSION

The overarching goal of this project was to gain mechanistic insight into the progressive cognitive deficits observed in GWI. My neurochemical, neuroinflammatory and behavioral studies using an experimental model of GWI have identified that PB administration produces long-lasting impairments in the peripheral and central cholinergic systems. These impairments emerge when stimulated by a physiological stressor, which support the idea of a latent phenotype of GWI and emphasize the need for such stressors in future GWI studies.

7.1 PB administration produces lasting alterations in the peripheral immune system

As expected, PB administration significantly suppressed plasma ChE activity in our GWI model during the 14-day treatment paradigm (Burzynski et al., 2022; Macht et al., 2018). However, I was not expecting plasma ChE activity to increase in PB-treated rats roughly three months after treatment cessation (see Chapter 4). This rebound effect may be a compensatory measure to PB’s initial suppression of ChE activity, but future studies are needed to further elucidate this unexpected finding. Importantly, increasing plasma ChE activity likely decreases peripheral ACh which is a critical component of the anti-inflammatory response. Thus, it is unsurprising that the same delayed cohort of PB-treated rats exhibit exacerbated pro-inflammatory responses of TNF-α and CINC-3 to a low-dose LPS challenge. Mainly secreted by activated macrophages and monocytes, TNF-α is a pro-
inflammatory cytokine that initiates acute immune responses through the production of IL-6 and CRP (Jang et al., 2021). Similarly, the chemokine CINC-3, also referred to as macrophage inflammatory protein (MIP)-2, initiates acute immune responses by activating neutrophils that phagocytose damaged cells to prevent the spread of infection or injury (Bardoel et al., 2014). Interestingly, chronic inflammation resulting from sustained elevations of plasma TNF-α or neutrophils has been implicated in various diseases such as irritable bowel syndrome, rheumatoid arthritis, and psoriasis, which have also been reported in patients with GWI (Bardoel et al., 2014; Blanchard et al., 2005; Herrero-Cervera et al., 2022; Jang et al., 2021; B. Li et al., 2011; Nettleman, 2015). Moreover, my findings are consistent with clinical studies that report systemic inflammation as a key feature of GWI (Broderick et al., 2013; Broderick et al., 2011; Butterick et al., 2019; Johnson et al., 2016).

Along with peripheral implications, chronic inflammation can also compromise the integrity of the BBB and increase infiltrating cytokines and neuroinflammation (Galea, 2021), but this potential mechanism has not yet been assessed in our model. Future studies can assess BBB permeability in our GWI model by injecting or perfusing a peripheral dye and measuring the amount of dye that reaches the CNS (Nag, 2008; Takasato et al., 1984). Additionally, obesity studies have found that chronic inflammation can decrease vagal activity and in turn, its anti-inflammatory reflex (De Lartigue et al., 2011, 2012; Lee et al., 2012; Pavlov & Tracey, 2012). Similar disruptions may be occurring in GWI as clinical studies report a blunted vagal component of the autonomic nervous system in GWI patients (Haley et al., 2013; Haley et al., 2004). It is also possible that the oral administration of PB directly impaired the GI branch of the vagus nerve through cholinergic toxicity. As the
vagal gut-brain circuit is a chief component of the cholinergic anti-inflammatory response (Suarez et al., 2018; Yu et al., 2020), future studies should assess this bidirectional axis in our rodent model of GWI.

The increases in plasma leptin levels in PB-treated rats at the delayed timepoint (see Chapter 4) are also interesting because: 1) increases in plasma leptin levels are also observed in veterans with GWI (Johnson et al., 2016); and 2) elevations in plasma leptin likely reflect leptin resistance in the CNS. Leptin receptors are members of the type 1 cytokine receptor family and as such activate intracellular signaling cascades similar to those of many pro-inflammatory cytokines, including the JAK-STAT pathway. While the neuroplasticity deficits associated with leptin resistance are normally discussed in the context of obesity, insulin resistance and type 2 diabetes (Erichsen et al., 2022), it is interesting to note that neuroplasticity deficits are a shared complication of metabolic disorders and GWI. In this manner, some combination of systemic inflammation, neuroinflammation and central leptin resistance may be contributing to the cognitive deficits observed in GWI veterans. As obesity and GWI present with altered metabolic pathways (Naviaux et al., 2019; Singla et al., 2010), inflammatory adipokines and cytokines secreted from adipocytes may be contributing to the observed immune dysregulation in our GWI model (Kawai et al., 2021). As such, evaluation of CNS leptin signaling/leptin resistance represents an interesting and important future direction for these studies.

7.2 Delayed consequences of PB treatment on hippocampal function

Beyond the peripheral consequences of PB treatment and their potential contribution to the cognitive deficits of GWI, it was important to investigate how PB
administration directly affects the hippocampus to gain a clearer understanding of these progressive impairments.

7.2.1 Neurochemical impairments and memory consolidation

After assessing hippocampal-dependent learning and memory in our GWI model, I have shown that PB treatment produces memory impairments that emerge long after treatment cessation, but only when stimulated by a physiological stressor (see Chapter 3). At the delayed timepoint, PB-treated rats were capable of performing NOR with a 24-hour ITI when injected with saline but exhibited significant 24-hour memory deficits in this task when challenged with LPS. As ACh is a critical component of memory formation (Hasselmo, 1999), my in vivo microdialysis studies assessing ACh efflux in response to LPS provide insight into these lasting memory impairments (see Chapter 5). In a separate delayed cohort, I found that basal levels of ACh were not affected by PB treatment, but LPS potentiates hippocampal ACh efflux in PB-treated rats compared to their early cohort counterparts. This sustained elevation in hippocampal ACh may be impairing memory consolidation and producing the 24-hour memory deficits observed in NOR when paired with LPS. Indeed, the role of ACh in cognition is dynamic, and the optimal levels of ACh required for memory encoding and consolidation differ greatly (Haam & Yakel, 2017). Specifically, increased ACh efflux in the hippocampus is necessary for memory acquisition, but consolidation requires low levels of ACh (Hasselmo, 1999; Kametani & Kawamura, 1990; Marrosu et al., 1995). Previous in vivo microdialysis studies have shown that hippocampal ACh efflux increases when exploring both the familiar and novel objects during NOR testing and there is a significant correlation between hippocampal ACh efflux and novel object exploration time (Stanley et al., 2012). These findings are consistent with
clinical studies that have shown reducing ACh levels with intravenous administration of the muscarinic antagonist scopolamine impairs memory acquisition but does not affect retention (Ghoneim & Mewaldt, 1977; Petersen, 1977). Building upon these studies, Rogers and Kesner refined the role of ACh in learning and memory by selectively manipulating ACh efflux in the rat hippocampus. They found that intrahippocampal injections of scopolamine impaired spatial acquisition using a modified Hebb-Williams maze, but retention was not affected (Rogers & Kesner, 2003). Conversely, when the AChE inhibitor physostigmine was injected into the rat hippocampus to keep ACh levels elevated, memory consolidation was significantly impaired (Rogers & Kesner, 2003).

The possibility that sensitized hippocampal cholinergic responses are mediating the cognitive deficits observed in GWI is further supported by my studies that included a stress challenge. When subjected to MWM testing, a delayed cohort of PB-treated rats exhibit 24-hour memory impairments but do not show any deficits in 1-hour memory (see Chapter 3). As the MWM is innately stressful due to forced swimming (Harrison et al., 2009), my in vivo microdialysis studies using an immobilization stress can better elucidate these impairments. As observed in the LPS session, a delayed cohort of PB-treated rats exhibits sustained elevations in hippocampal ACh efflux following an immobilization stress, (see Chapter 5). Importantly, there was no effect of treatment history on escape latency during the training trials, meaning PB treatment does not produce acquisition impairments, which further supports Rogers and Kesner’s findings. Additionally, these findings are consistent with previous studies from the Reagan lab that did not observe any acquisition deficits in contextual fear conditioning in our GWI cohorts (Macht et al., 2018). Taken together, my neurochemical and behavioral studies suggest that over time, PB treatment potentiates the
hippocampal cholinergic response to stressors, which may explain the deficits in memory consolidation observed in our delayed model of GWI, as well as the progressive cognitive deficits observed in GWI patients.

7.2.2 Dysregulation of the hippocampal cholinergic anti-inflammatory pathway

Along with alterations in cholinergic neurochemistry, I also assessed if neuroinflammation is contributing to the cognitive impairments observed in PB-treated rats by measuring hippocampal cytokines (see Chapter 4). Interestingly, my findings highlight a dysregulation of the cholinergic anti-inflammatory response within the hippocampus of PB-treated rats. For example, a delayed cohort of PB-treated rats exhibited significant increases in pro-inflammatory cytokines (IL-1β, GM-CSF, and IL-12) in the hippocampus when challenged with low-dose LPS. This response was not seen in vehicle-treated rats challenged with LPS or in PB-treated rats that received saline injections. Furthermore, this exaggerated immune response was not seen in the hippocampus of PB-treated rats in an early cohort. As basal levels of hippocampal ACh and ChE activity are not affected by PB treatment at this delayed timepoint, one would assume that the heightened pro-inflammatory response is due to suppressed ACh efflux as ACh exerts anti-inflammatory actions (Czura et al., 2003). However, as described above, the hippocampal cholinergic response to this low-dose LPS is potentiated in a delayed cohort of PB-treated rats (see Chapter 5). This discrepancy suggests that PB diminishes the peripheral cholinergic anti-inflammatory response by increasing plasma ChE activity but may be dysregulating the central cholinergic anti-inflammatory response through a different mechanism as hippocampal ChE activity is not affected. I next assessed if PB administration reduced hippocampal α7 nAChR expression as these receptors suppress the release of pro-
inflammatory cytokines from microglia and astrocytes when activated by ACh (Egea et al., 2015; Revathikumar et al., 2016). While hippocampal α7 nAChR expression is not changed in PB-treated rats (see Chapter 4), it is possible that PB administration elicits functional deficits in these receptors and future electrophysiology studies are needed to determine if such impairments are driving the dysregulation of the hippocampal cholinergic anti-inflammatory response.

An early hypothesis regarding the underlying causes of GWI revolved around the concept that stress altered the pharmacokinetic profile of PB to allow the drug to cross the BBB and inhibit brain AChE activity. While some studies have demonstrated exposure to GW-related chemicals, including PB, inhibits brain AChE activity (Beck et al., 2003; Friedman et al., 1996; Kaufer et al., 1998; Tian et al., 2002) other studies 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). Moreover, my neurochemical studies do not support the concept that PB acts in the CNS to modulate brain ACh levels. Specifically, our group did not observe changes in basal levels of ACh in the hippocampus in PB-treated rats approximately 10 days (Macht et al., 2019) or three months (see Chapter 5) after PB administration. Additionally, the hippocampal ChE activity of PB-treated rats does not differ from vehicle-treated rats at the delayed timepoint (see Chapter 4). Taken together, these findings do not support the concept that the neurological complications of GWI result from central effects of PB on AChE activity. As the vagus nerve exerts bidirectional control of the cholinergic anti-inflammatory response, these findings provide further evidence that PB’s dysregulation of cholinergic neurochemistry may be a result of an impaired vagal gut-brain axis. Indeed, studies that selectively ablated GI vagal afferents with a targeted toxin
reported impairments in hippocampal-dependent memory which is consistent with my findings in our GWI model (Suarez et al., 2018).

While dysregulated hippocampal ACh efflux is likely contributing to the memory impairments observed in PB-treated rats, increased pro-inflammatory cytokines in this region are also known to disrupt memory consolidation. One such pro-inflammatory cytokine is IL-1β, which is elevated in the hippocampus of PB-treated rats following LPS administration at the delayed timepoint. IL-1β is thought to be a key contributor to hippocampal neuroinflammation and subsequent cognitive decline due to its dense receptor expression in this region (Farrar et al., 1987). While it has been shown that low levels of IL-1β facilitate hippocampal-dependent learning and memory, high levels of IL-1β can inhibit these processes (Lynch, 2015; Yirmiya & Goshen, 2011). For example, numerous studies have found that blocking IL-1β signaling with IL-1 receptor antagonists decreases long-term potentiation (LTP), a cellular correlate of learning and memory (Goshen et al., 2007; Schneider et al., 1998; Spulber et al., 2009). Conversely, other electrophysiological studies have demonstrated that bath application of IL-1β blocks LTP in hippocampal slices from both mice (Katsuki et al., 1990) and rats (Bellinger et al., 1993). Moreover, in vivo studies have found that intrahippocampal injections of IL-1β impair memory consolidation in contextual fear conditioning (Gonzalez et al., 2009). Similarly, intracerebroventricular injections of IL-1β produced retention deficits in the MWM but did not impair acquisition (Oitzl et al., 1993). These findings provide evidence that the exaggerated IL-1β response seen in the hippocampus of PB-treated rats after LPS administration may contribute to the impaired memory consolidation observed in PB-treated rats when challenged with LPS.
Similar to IL-1β, GM-CSF is a pro-inflammatory cytokine that has beneficial and deleterious actions within the CNS. GM-CSF has been shown to promote hippocampal-dependent learning and memory as aged, wild-type mice performed better in a radial arm water maze when treated with low-dose GM-CSF (5 µg, subcutaneous) (Ahmed et al., 2022). Additionally, GM-CSF knockout mice exhibited reduced dendritic complexity in the hippocampus and showed significant impairments in active avoidance, MWM and fear conditioning paradigms (Krieger et al., 2012). However, preclinical studies using organotypic hippocampal slice cultures have found that chronic exposure to GM-CSF induces microglia proliferation and impairs gamma oscillations (Dikmen et al., 2020). Furthermore, intrahippocampal administration of this cytokine elicits behavioral deficits and activates microglia in the rat hippocampus (Zhu et al., 2014). Interestingly, microglia that rapidly proliferate in response to GM-CSF become senescent much quicker than non-stimulated cells (Flanary & Streit, 2004). As senescence can lead to abnormal cellular actions and responses, senescent microglia are associated with excessive neuroinflammation and subsequent neurodegeneration and cognitive decline (Luo et al., 2010; Matsudaira et al., 2023). In this context, elevated levels of GM-CSF have been reported in neurodegenerative diseases including Alzheimer’s disease and vascular dementia (Tarkowski et al., 2001). Thus, along with IL-1β, the elevated levels of GM-CSF in the hippocampus of PB-treated rats after LPS administration are likely involved in the 24-hour memory impairments observed in PB-treated rats.

IL-12 is another cytokine that is elevated in the hippocampus of PB-treated rats when challenged with LPS approximately three months after treatment cessation. IL-12 is mainly produced by activated microglia when a type-1, pro-inflammatory response is
initiated (Gately et al., 1998). Exaggerated levels of IL-12 in the CNS have been reported in animal models of experimental autoimmune encephalomyelitis and individuals with multiple sclerosis (Ahmed et al., 2001; Balashov et al., 2000; Balashov et al., 1997; Issazadeh et al., 1995; Kouwenhoven et al., 2001; Smith et al., 1997; Tuohy et al., 2000). IL-12 is also thought to contribute to neurodegeneration as IL-12 knockout mice were protected against excitotoxic neurodegeneration after a kainic acid (KA)-induced hippocampal injury (Chen et al., 2004). Similarly, hippocampal neurodegeneration in this KA-injury model is reduced when IL-12 production is inhibited by the overexpression of the neuroprotective factor metallothioneine (Penkowa et al., 2005). While studies examining IL-12’s role in hippocampal-dependent learning and memory are limited, its neuroinflammatory and neurodegenerative actions likely contribute to the LPS-induced memory deficits observed in PB-treated rats. The heightened responses of multiple pro-inflammatory cytokines in the hippocampus of PB-treated rats are consistent with other animal studies that implicate neuroinflammation as a mechanistic mediator of GWI (Abdel-Rahman et al., 2004; Alhasson et al., 2017; Joshi et al., 2018; Kodali et al., 2018; Madhu et al., 2019; Miller et al., 2018; Parihar et al., 2013; Shetty et al., 2020; Shetty et al., 2017; Zuchra Zakirova et al., 2015). Furthermore, my studies also support recent clinical findings that visualized greater amounts of activated macrophages and astrocytes in the brains of GWI patients relative to healthy GW veterans and civilians with *in vivo* imaging (Alshelh et al., 2020).

Collectively, my studies suggest that PB treatment has lasting effects on the hippocampal cholinergic system, specifically as it relates to memory consolidation and are consistent with clinical observations in veterans with GWI (Blanchard et al., 2005; Fukuda
et al., 1998; Group, 1997; X. Li et al., 2011; Steele, 2000). When challenged by LPS, it is unclear whether the potentiated hippocampal ACh efflux observed in PB-treated rats is due to a hyperactive cholinergic response or is a compensatory measure to dampen the exaggerated pro-inflammatory cytokines. Regardless, the inability of ACh to suppress the pro-inflammatory response indicates an impairment in the hippocampal cholinergic anti-inflammatory pathway. While future studies are needed to determine if similar cytokine responses are seen following stress, I believe that both the exaggerated hippocampal cholinergic and cytokine responses observed in PB-treated rats are impairing memory consolidation (Figure 7.1). Importantly, these altered responses emerge long after treatment cessation, highlighting the enduring effects of PB treatment and how it may contribute to the progressive cognitive deficits seen in GWI patients.
Figure 7.1 The deleterious effects of PB administration on hippocampal function. Long after treatment cessation, PB-treated rats exhibit sensitized hippocampal responses to stressors, namely potentiated pro-inflammatory cytokines and ACh efflux. These consequences are known to impair memory consolidation and likely contribute to the stimulus-dependent memory impairments observed in PB-treated rats.

7.3 The latent phenotype of GWI

While my studies identify enduring consequences of PB administration, they also support the concept that GWI presents with a latent phenotype. Relative to vehicle-treated controls, PB-treated rats in a delayed cohort do not exhibit differences in hippocampal cytokines when injected with saline, but a low-dose LPS challenge produces exacerbated pro-inflammatory responses in these rats. Similarly, basal levels of ACh and ChE activity in the hippocampus are not altered by a history of PB treatment but these rats show potentiated ACh efflux after an LPS or immobilization stress challenge. Interestingly, elevations in both pro-inflammatory cytokines and ACh efflux in the hippocampus are
observed approximately 2 hours after the LPS or stress was initiated which likely interrupts memory consolidation. This may explain why PB-treated rats do not exhibit memory deficits at the early or delayed timepoint when injected with saline but show significant impairments in 24-hour memory after an immune (NOR) or stress (MWM) challenge at the delayed timepoint. Importantly, the stimulus-dependent cognitive impairments observed in our rodent model of GWI are consistent with clinical studies in which an exercise challenge is used to exacerbate cognitive deficits in GWI patients (Broderick et al., 2013; Broderick et al., 2011; Whistler et al., 2009). While my studies only assess the acute effects of a physiological stressor, GW veterans continue to face daily life stressors and occasional infections. The repeated potentiation of hippocampal ACh and inflammatory cytokines in response to these reoccurring stressors likely compounds over time and may explain the progressive cognitive deficits observed in GWI patients.

7.4 Potential role of stressors in behavioral deficits in GWI rodent studies

Though many laboratories have assessed hippocampal-dependent learning and memory in rodent models of GWI, the results from these studies are varied. One group observed deficits in MWM performance in a rat model of GWI as early as 16 days after treatment cessation and these impairments remained at days 52, 113 and 199 (Lamproglou et al., 2009). Another group saw similar deficits in MWM performance in their rat model of GWI approximately three months after treatment cessation, but did not observe any deficits in NOR (Parihar et al., 2013). Conversely, a different rat model of GWI exhibited significant impairments in both NOR and novel place recognition more than three months after treatment cessation (Hattiangady et al., 2014). Other studies using a mouse model of GWI saw MWM performance decline over time, as impairments were not seen at days 20-
30 post-treatment (Abdullah et al., 2012), but emerged by day 115 (Abdullah et al., 2011). Another study from this group observed similar changes in Barnes maze performance, only seeing deficits 106 days post-treatment, but not at day 14 (Zuchra Zakirova et al., 2015). Interestingly, when this group performed behavioral tests 22.5 months after treatment cessation, this mouse model of GWI did not show deficits in Barnes maze performance (Z. Zakirova et al., 2015). Such inconsistencies have made it difficult to identify the mechanisms responsible for the progressive cognitive deficits seen in GWI and hinders the development of potential therapeutics for affected individuals. One potential explanation for these disparate results is the use of various experimental models of GWI in each study, including different chemical exposures, stress paradigms and treatment durations. However, another important factor that I believe is responsible for the equivocal results in GWI behavioral studies is the absence of the administration of stressors during such assays. It is worth noting that the more consistent findings in GWI studies assessed hippocampal-dependent memory with the MWM (Abdullah et al., 2011; Lamproglou et al., 2009; Parihar et al., 2013), which further supports the concept that stressors (i.e., swimming) serve as a necessary stimulus to unmask hippocampal-dependent learning and memory deficits in GWI.

7.5 Future directions

Though my studies assessing hippocampal neurochemistry and neuroinflammation in our model of GWI provide clear evidence for the observed hippocampal-dependent memory deficits, my PFC findings are somewhat puzzling. While a history PB treatment produces similar increases in the pro-inflammatory response to LPS in the PFC and hippocampus (see Chapter 4), only rats with a history of PB and stress exhibit potentiated
ACh efflux in the PFC after LPS exposure (see Chapter 6). These data do not support the idea that PB treatment dysregulates the cholinergic anti-inflammatory response within the PFC. In order to elucidate these region-specific differences, the effects of PB and stress on PFC-dependent behaviors must first be assessed. If results show that PB treatment alone elicits attentional deficits, one could presume that these impairments are driven by inflammation. Conversely, if only PB-RRS rats exhibit attentional deficits, then dysregulated ACh efflux may be responsible. Until these behavioral assays are performed, it is difficult to make any conclusions about how PB and/or stress affect the PFC long-term.

Within both the PFC and hippocampus, further studies should also assess how alterations in ACh and its neuromodulatory actions affect other neurotransmitters, especially glutamate. ACh is known to activate glutamatergic neurons containing cholinergic receptors and facilitate learning and memory (Drever et al., 2011; Picciotto et al., 2012). This activation must be tightly controlled as elevations in glutamate can cause excitotoxicity and neurodegeneration (Dong et al., 2009). While previous studies from the Reagan and Fadel labs have measured glutamatergic responses in an early cohort of our GWI model (Macht et al., 2020), these neurochemical studies have not been performed in a delayed cohort. If potentiating ACh efflux results in elevated glutamate, this may also be contributing to the neuroinflammation and cognitive impairments observed in our GWI model at the delayed timepoint.

While fully elucidating the pathophysiology of GWI requires further studies, a critical future direction for this work is investigating potential therapeutics for affected individuals. As my data suggests that the cholinergic anti-inflammatory pathway may be impaired, a pharmacological intervention restoring this critical response may alleviate the
hippocampal neuroinflammation and cognitive impairments. Positive allosteric modulators of \( \alpha_7 \) nAChRs (\( \alpha_7 \) PAMs) are emerging as potential therapeutics for a variety of pathologies, ranging from ulcerative colitis to Alzheimer’s disease, for their anti-inflammatory and pro-cognitive actions (Rueda Ruzafa et al., 2021; Tregellas & Wylie, 2019; Yang et al., 2017). Unlike agonists that have the potential to elicit receptor desensitization, \( \alpha_7 \) PAMs are an attractive option as they only augment natural cholinergic signaling (Abdel-Magid, 2015). Additionally, the allosteric binding site creates higher binding specificity than current pharmacological treatments (Williams et al., 2011). PNU-120596 is an \( \alpha_7 \) PAM that has been shown to cross the BBB and inhibit LPS-induced neuroinflammation in rodent studies (Alzarea & Rahman, 2018; Christensen et al., 2010). Furthermore, PNU-120596 has been shown to enhance cognition and prevent the cognitive deficits associated with LPS administration, making it an excellent candidate for GWI research (Alzarea & Rahman, 2018; Callahan et al., 2013; Pandya & Yakel, 2013; Phenis et al., 2020; Potasiewicz et al., 2020).

### 7.6 Conclusions

In sum, my findings across multiple studies are consistent with many others (Broderick et al., 2013; Broderick et al., 2011; Rayhan et al., 2013; Washington et al., 2020) that suggest GWI may be characterized as having a latent phenotype, and the pathophysiology can be better understood when stressors are presented. I have shown that PB-treatment creates lasting deficits in peripheral and central cholinergic signaling that emerge long after treatment cessation, but only when stimulated with either an immune or stress challenge. I believe that including such stressors in GWI research, especially during behavioral studies, is necessary to fully elucidate the mechanistic basis of GWI. Lastly, by
reporting a dysregulation in the hippocampal cholinergic anti-inflammatory response, this work has identified a potential site of intervention that could be used in the treatment of the memory deficits observed in veterans with GWI.
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