Neurobiology of Stress 18 (2022) 100446

Contents lists available at ScienceDirect

Neurobiology of Stress

journal homepage: www.elsevier.com/locate/ynstr

Pyridostigmine bromide elicits progressive and chronic impairments in the cholinergic anti-inflammatory pathway in the prefrontal cortex and hippocampus of male rats

H.E. Burzynski [a,*], V.A. Macht [a,1], J.L. Woodruff [a], J.N. Crawford [a], J.M. Erichsen [a], G.G. Piroli [a], C.A. Grillo [a,b], J.R. Fadel [a,b], L.P. Reagan [a,b]

[a] University of South Carolina School of Medicine, Department of Pharmacology, Physiology, and Neuroscience, Columbia, SC, 29208, USA
[b] Columbia VA Health Care System, Columbia, SC, 29208, USA

ARTICLE INFO

Keywords:
Acetylcholine
Stress
Cytokine
Neuroinflammation
Gulf War Illness
α7 nicotinic acetylcholine receptor

ABSTRACT

Gulf War Illness (GWI) is a multi-symptom illness that continues to affect over 250,000 American Gulf War veterans. The causes of GWI remain equivocal; however, prophylactic use of the acetylcholinesterase inhibitor pyridostigmine bromide (PB), and the stress of combat have been identified as two potential causative factors. Both PB and stress alter acetylcholine (ACh), which mediates both cognition and anti-inflammatory responses. As inflammation has been proposed to contribute to the cognitive deficits and immune dysregulation in GWI, the goal of this study was to determine the long-term effects of PB and stress on the cholinergic anti-inflammatory pathway in the central nervous system and periphery. We used our previously established rat model of GWI and *in vivo* microdialysis to assess cholinergic neurochemistry in the prefrontal cortex (PFC) and hippocampus following a mild immune challenge (lipopolysaccharide; LPS). We then examined LPS-induced changes in inflammatory markers in PFC and hippocampal homogenates. We found that PB treatment produces a long-lasting potentiation of the cholinergic response to LPS in both the PFC and hippocampus. Interestingly, this prolonged effect of PB treatment enhancing cholinergic responses to LPS was accompanied by paradoxical increases in the release of pro-inflammatory cytokines in these brain regions. Collectively, these findings provide evidence that neuroinflammation resulting from dysregulation of the cholinergic anti-inflammatory pathway is a mechanistic mediator in the progression of the neurochemical and neurocognitive deficits in GWI and more broadly suggest that dysregulation of this pathway may contribute to neuroinflammatory processes in stress-related neurological disorders.

1. Introduction

Soldiers who served in the 1990–1991 Gulf War (GW) returned with an unexplained, multi-symptom illness that affected several physiological systems, including the central nervous system (CNS), as well as the immune, endocrine, cardiovascular, and gastrointestinal systems. After returning, their health complaints included memory impairments, attentional deficits, chronic pain, poor cardiovascular health, respiratory problems, and gastrointestinal disturbances (Kang et al., 2009; Li et al., 2011). Thirty years later, the causes of this constellation of symptoms, now termed Gulf War Illness (GWI), are still unknown and roughly 250,000 American veterans remain without a cure (Pope et al., 2005). While the origins of this unique symptomology are still unknown, the prophylactic use of the reversible acetylcholinesterase inhibitor pyridostigmine bromide (PB), and the stress of combat have been identified as two potential causative factors (Haley et al., 1997; Steele et al., 2012; White et al., 2016). Due to concerns of chemical warfare during the GW, soldiers were advised to ingest 30 mg PB tablets every 8

Abbreviations: GWI, Gulf War Illness; PB, Pyridostigmine Bromide; RRS, Repeated Restraint Stress; ACh, Acetylcholine; AChE, Acetylcholinesterase; α7 nAChR, α7 Nicotinic Acetylcholine Receptor.

* Corresponding author. Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, 6439 Garners Ferry Road, D4, Columbia, SC, 29208, USA.
E-mail address: hannah.burzynski@uscmed.sc.edu (H.E. Burzynski).
[1] Present address: Bowles Center for Alcohol Studies, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA 27599.

https://doi.org/10.1016/j.ynstr.2022.100446
Received 21 December 2021; Received in revised form 18 March 2022; Accepted 8 April 2022
Available online 15 April 2022

hours to provide continuous pharmacological protection against exposure to irreversible cholinesterase inhibitors (i.e., chemical nerve agents) (Gordon et al., 1978; von Bredow et al., 1991; Steele, 2000). Though PB was prescribed to every GW soldier and many GWI symptoms are consistent with cholinergic toxicity, the incidence of GWI is more prevalent in ground forces 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 (Parihar et al., 2013; Hattiangady et al., 2014; Macht et al., 2018).

Both PB and stress alter levels of acetylcholine (ACh), an essential neurotransmitter for both the central and peripheral nervous systems. The cholinergic system is extremely sensitive, and disrupting its regulation can have deleterious effects on immune responses and cognition. Specifically, the cholinergic anti-inflammatory pathway uses ACh, mediated by the vagus nerve, to suppress the release of pro-inflammatory cytokines from immune cells by binding to α7 nicotinic ACh receptors (α7 nAChRs) on the surface of macrophages (periphery), astrocytes (CNS) and microglia (CNS) (Wu et al., 2021). Importantly, several preclinical and clinical studies have found a dysregulation of the immune response in GWI, suggesting that the cholinergic anti-inflammatory pathway is impaired (Broderick et al., 2013; Johnson et al., 2016; Georgopoulos et al., 2017; Macht and Reagan, 2018; Macht et al., 2019). Both ACh and inflammation are key components of cognitive function and synaptic plasticity, and it is well accepted that impairing cholinergic neurotransmission results in both memory and attentional deficits, impairments that are routinely seen in GWI patients (Hubbard et al., 2014; Tillman et al., 2017). Similarly, neuroinflammation has been proposed to be a critical mechanistic mediator in the initiation and progression of cognitive decline (Sparkman and Johnson, 2008; Ransohoff, 2016).

In view of these observations, our previous studies investigated whether PB and stress interact to impair central and peripheral cholinergic function in a rat model of GWI (Macht et al., 2018, 2019, 2020). We previously reported that 10 days following a 14-day treatment paradigm of PB and repeated restraint stress (RRS), PB treatment alone or in combination with stress elicited dysregulation of peripheral immune responses to a modest immune challenge, namely a 30 μg/kg dose of lipopolysaccharide (LPS) (Macht et al., 2019). Additionally, this study found that a history of RRS blunted the cholinergic response to LPS in the prefrontal cortex (PFC) but a history of PB treatment alone blunted the cholinergic response to LPS in the hippocampus 10 days after the cessation of treatment (Macht et al., 2019). While the results from this study indicated that both PB and stress elicit immediate changes in peripheral and central cholinergic pathways, they did not address the enduring effects of acetylcholinesterase inhibition and stress as they relate to the progressive nature of GWI. Indeed, one of the most devastating aspects of GWI is that its symptoms, especially those in the CNS, have become progressively worse in aging veterans. Unfortunately, long-term preclinical studies evaluating a potential progressive dysregulation of the cholinergic anti-inflammatory pathway remain limited. Accordingly, the goal of this study was to use our previously established rat model of GWI, consisting of PB treatment, alone and in combination with RRS, to determine the long-term (approximately 3 months after the cessation of treatment) effects on the cholinergic anti-inflammatory pathway in the CNS. Collectively, the results of the current study suggest that enduring dysregulation of the cholinergic anti-inflammatory pathway results in an enhanced proinflammatory cytokine response to an LPS challenge in the hippocampus and PFC of PB-treated rats, thereby providing a potential mechanism for the progressive neurological deficits observed in veterans with GWI.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats (Envigo, 200–225g) were individually housed with irradiated Sani-Chips wood bedding (P.J. Murphy Forest Products Corp.) at the University of South Carolina School of Medicine animal facility. Rooms were temperature controlled (22°C) and kept on a 12/12hr light-dark cycle, with lights on at 7:00 a.m. Animals were given *ad libitum* access to food and water, and nylabones were provided for enrichment. Rats were approximately 7 weeks old upon arrival and treatment began after a one-week acclimation period. Only males were used in this study as over 90% of Gulf War veterans are male (Nettleman, 2015). All procedures were performed in accordance with all guidelines and regulations of the Dorn VA Animal Care and Use Committee.

2.2. GWI paradigm

The experimental design of our 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 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 body weight). This dose has been described previously to mimic the dose taken by soldiers and effectively decrease plasma cholinesterase activity (Marino et al., 1998). 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 a.m.-4:00 p.m.) for 10 consecutive days. NSC rats were housed in a separate room, were handled each day, and then returned to their home cage. Early (day 24 post treatment, approximately 11–12 weeks old) and delayed cohorts (day 100 post treatment, approximately 6 months old) were subjected to an immune challenge of 30 μg/kg LPS followed by assessment of immune or neurochemical endpoints. See Fig. 1 for experimental timeline.

2.3. Stereotaxic surgery

One hundred days after the treatment paradigm, rats in cohort #1 were anesthetized with isoflurane and underwent stereotaxic surgery to place guide cannulae into the PFC and ipsilateral dorsal hippocampus, as previously described (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, + 3.2 mm; L, ± 0.5 mm; DV, - 2.5 mm for the PFC, and AP, - 5.5 mm; L, ± 4.0 mm; DV, - 3.8 mm at a 10° angle for the hippocampus. Coordinates were based on Paxinos and Watson rat brain atlas (Paxinos and 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.

2.4. In vivo microdialysis

Each rat in cohort #1 underwent a session of microdialysis as previously described (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. This habituation period ensured rats received a full week of recovery before microdialysis began. On the day of the 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 $CaCl_2H_2O$; 0.183 mM $MgCl_26H_2O$; 5 mM D-glucose) with 100 nM neostigmine at a rate of 2 μL/min. The first 3 h (8:00 a.m.-11:00 a.m.) of collection were discarded to allow for recovery

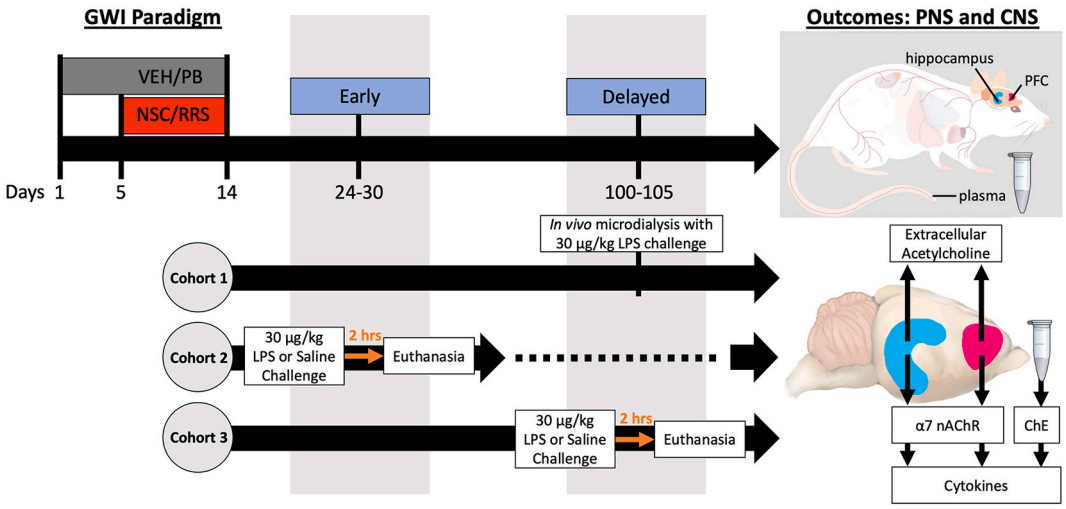

Fig. 1. Experimental Timeline. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control (NSC), repeated restraint stress (RRS)). Cohort #1 underwent *in vivo* microdialysis 100 days after the treatment paradigm to assess the cholinergic response of the hippocampus and PFC to i.p. LPS at a delayed timepoint. Separate cohorts were used to assess immune responses at both the early (days 24–30, cohort #2) and delayed (days 100–105, cohort #3) timepoints in tissue homogenates and plasma 2 hours following i.p. saline or LPS administration.

from probe insertion. Collections were then taken at 15-min intervals with the first four collections serving as baseline measurements. Rats were injected intraperitoneally (i.p.) with 30 µg/kg of LPS at the start of the 5th collection, and collections continued for 3 hours post injection. This dose of LPS was based on prior dose-response studies (Macht et al., 2019). Samples were immediately frozen and stored at −80 °C at the end of each collection.

2.5. Transcardial perfusion

Following microdialysis, rats in cohort #1 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 as shown previously (Macht et al., 2019).

2.6. High performance liquid chromatography

ACh concentration in dialysate samples was measured as previously described (Fadel et al., 2005; Calva et al., 2018; Macht et al., 2019). Briefly, dialysate samples were thawed individually and 20 µL was 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 current of +450 mV oxidized the hydrogen peroxide at the platinum electrochemical detector. The potential 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.

2.7. Plasma endocrine measures

Separate cohorts of rats (cohorts #2 and #3) underwent the GWI paradigm explained above. One day (cohort #2) or 90 days (cohort #3) after the treatment paradigm rats were exposed to 3 habitual i.p. saline injections (0.1 mL/kg body weight) 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 or 30 µg/kg LPS. This dose and time course would allow for more direct comparisons between LPS-induced changes in cholinergic neurochemistry and brain cytokine levels. Tail blood collected on day 14 (cohort #2, no stimulus) or day 90 (cohort #3, 2 hours following i.p. saline injection) was used to assess plasma cholinesterase activity and leptin levels. Plasma cholinesterase activity was measured with a colorimetric assay (Abcam, ab#138871) according to manufacturer's instructions. Enzyme-linked immunosorbent assay (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.).

2.8. 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 1 mm × 1 mm 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 (137 mM NaCl, 20 mM Tris-HCl, 10% Glycerol, 1% Tergitol-type NP40). The ratio of lysis buffer to tissue was 50 µL buffer per punch for PFC and 5 µL buffer per 1 mg tissue for hippocampus. 0.5 mm 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×*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 used neat, 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. 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.).

2.9. 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).

2.10. Statistical analysis

Plasma measures for cholinesterase, leptin and CINC-3 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). For *in vivo* microdialysis, data were assessed as a 2 × 2 × 16 and the additional factor of *time*. Comparison of basal levels of ACh prior to LPS administration was assessed by a 2 x 2 ANOVA. ACh efflux between early and delayed cohorts was assessed at each collection by unpaired *t*-test. 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 $\alpha = 0.05$. Following a significant interaction, *post-hoc* follow-up analyses were analyzed with a Bonferroni correction. Bonferroni corrections were applied for every post-hoc, performed automatically with SPSS software as 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).

3. Results

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

At the completion of the 14-day GWI paradigm, plasma cholinesterase activity exhibited the expected decrease in PB-treated rats, irrespective of stress conditions [$F(1,36) = 125.6$ ($p < 0.0001$] (Fig. 2, Panel A). In agreement with our previous observations, plasma cholinesterase activity was significantly elevated in PB-treated rats at the delayed timepoint, 90 days following completion of the GWI paradigm [$F(1, 28) = 12.26$, $p = 0.002$] (Fig. 2, Panel B) (Macht et al., 2018, 2019, 2020). Based on previous clinical studies (Johnson et al., 2016), we also measured plasma leptin levels in our 4 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 [$F(1,33) = 33.41$, $p < 0.0001$] (Fig. 2, 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 [$F(1,32) = 5.093$, $p = 0.031$] (Fig. 2, 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).

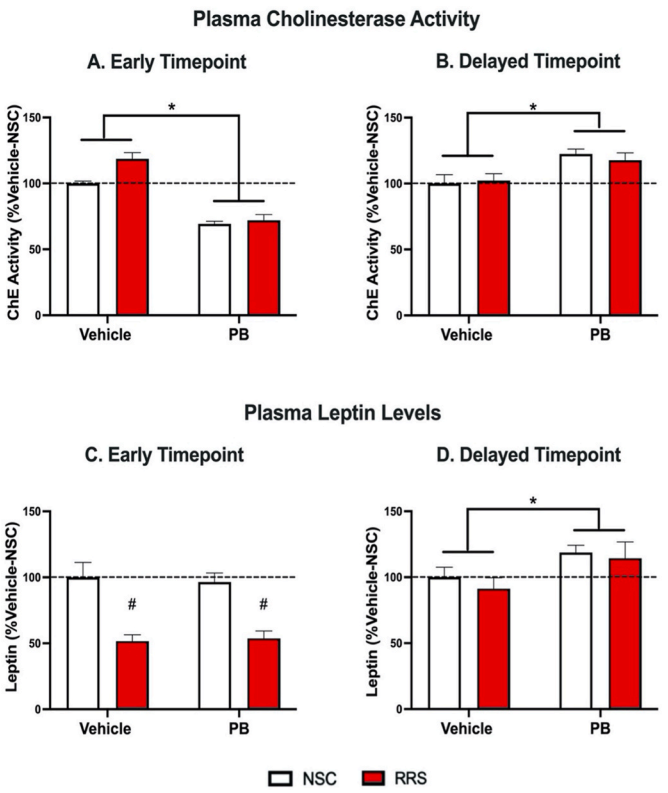

Fig. 2. Plasma measures of saline-treated early and delayed cohorts. A history of PB treatment suppresses plasma cholinesterase activity at the early timepoint (Panel A) but significantly increases plasma cholinesterase activity at the delayed timepoint (Panel B) compared to vehicle-treated groups. At the early timepoint, a history of repeated restraint stress (RRS) elicits significantly lower plasma leptin levels compared to non-stressed controls (NSC; 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 non-stressed controls + SEM, $n = 8$–10. [*: Significant effect of PB, $p < 0.05$. #: Significant effect of RRS, $p < 0.001$].

Our previous studies demonstrated that peripheral responses to an immune challenge, namely LPS administration (30 μg/kg), 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, we measured LPS-induced changes in plasma inflammatory markers in a delayed cohort of rats (cohort #3). In the delayed cohort, LPS-induced increases in TNF-α levels were significantly greater in PB-treated rats compared to vehicle-treated rats on days 100–105 [$F(1,25) = 5.221$, $p = 0.031$] (Fig. 3, 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 [$F(1,27) = 6.807$, $p = 0.015$] (Fig. 3, Panel B). Collectively, these results illustrate that PB administration elicits long lasting impairments in the peripheral responses of the cholinergic anti-inflammatory pathway to a modest immune challenge.

3.2. Prior history of PB treatment enhances LPS-induced release of acetylcholine: hippocampus

To determine the long-lasting effects of PB and stress on the cholinergic anti-inflammatory pathway in the CNS, we examined the

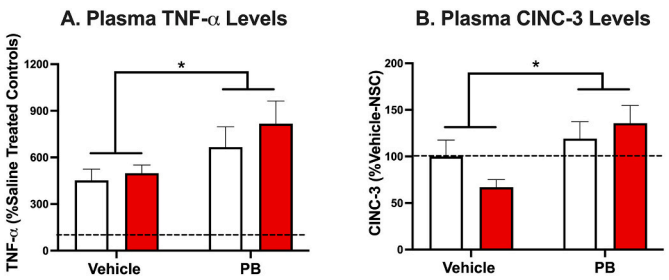

Fig. 3. LPS-induced inflammatory markers in plasma of delayed cohort. Panel A: A history of PB treatment elicits an increase in plasma TNF-α levels in response to LPS compared to vehicle-treated groups in the delayed cohort. Panel B: A history of PB treatment elicits an increase in plasma CINC-3 levels in response to LPS compared to vehicle-treated groups in the delayed cohort. 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 non-stressed controls (NSC) + SEM as CINC-3 was not detected in saline treated animals, n = 6–8. [*: Significant effect of PB, $p < 0.05$].

effects of LPS administration on ACh release in the hippocampus of Vehicle-NSC rats, PB-NSC rats, Vehicle-RRS rats, and PB-RRS rats by *in vivo* microdialysis. While basal levels of ACh were not affected by a prior history of PB or stress (Fig. 4 inset), prior exposure to PB and RRS interacted over time to significantly influence the cholinergic response to LPS in the hippocampus (Fig. 4) [F (15, 390) = 1.744, p = 0.041]. Bonferroni corrected *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 treatment 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, 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.

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 of the delayed cohort to LPS was compared to our previous neurochemical findings in an early cohort (Macht et al., 2019). These comparisons revealed that Vehicle-NSC rats (Fig. 5, Panel A) and Vehicle-RRS rats (Fig. 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 (Fig. 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 (Fig. 5, Panel D). Such results illustrate that an enhanced cholinergic response to an immune challenge is a long-lasting consequence of PB exposure in the rat hippocampus.

3.3. Prior history of PB treatment alters LPS-induced release of acetylcholine: prefrontal cortex

In the same cohort from the delayed timepoint (cohort #1), we also examined the effects of LPS administration on cholinergic neurochemistry in the PFC. 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 (Fig. 6 inset) [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 [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; Fig. 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).

We also compared LPS-induced changes in cholinergic neurochemistry in the PFC between the early and delayed cohorts. Similar to observations in the hippocampus, LPS-induced changes in ACh levels did not differ in Vehicle-NSC rats (Fig. 7, Panel A) or Vehicle-RRS rats (Fig. 7, 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; Fig. 7, 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; Fig. 7, Panel D).

3.4. A prior history of PB treatment elicits potentiated cholinergic responses to an LPS challenge: hippocampus

Similar to basal plasma cytokine levels at the early timepoint, neither a history of PB treatment nor stress elicited significant changes in hippocampal cytokines in saline-treated rats at the early timepoint (cohort #2), see Supplementary Table 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] (Fig. 8, 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 (Fig. 8, Panel C).

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, see Supplementary Table 1. 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] (Fig. 8, Panel D), IL-12: [F (1, 28) = 5.270, p = 0.029] (Fig. 8, Panel F) and GM-CSF: [F (1, 26) = 10.82, p = 0.003] (Fig. 8, Panel H) responses to LPS compared to vehicle-treated rats, increases that were not observed at the early timepoint (Fig. 8, Panels C, E and G).

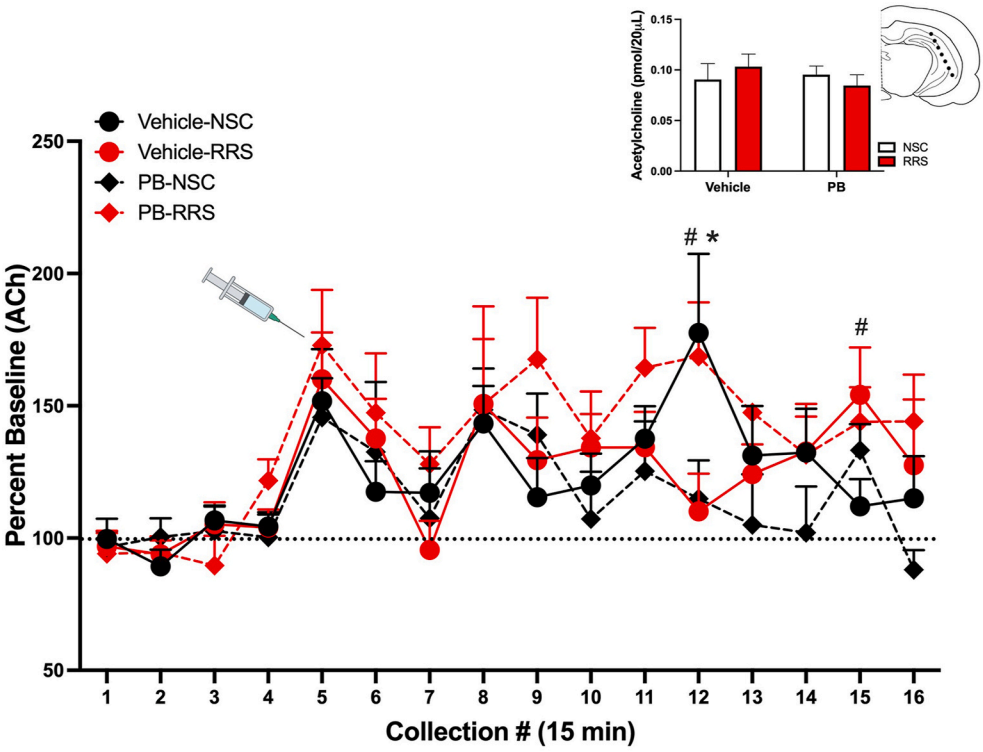

Fig. 4. Hippocampal cholinergic response to LPS of delayed cohort. Prior to LPS administration, there was no effect of a history of PB treatment or repeated restraint stress (RRS) on basal ACh levels in the hippocampus (inset). A history of RRS in vehicle-treated rats suppressed the cholinergic response to LPS at collection 12, but then increased this response at collection 15 compared to vehicle non-stressed controls (NSC). A history of PB treatment suppresses the cholinergic response to LPS at collection 12 compared to Vehicle-NSC 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, $p < 0.05$].

3.5. A prior history of PB treatment alters cholinergic responses to an LPS challenge: prefrontal cortex

We also assessed the early and delayed effects of PB and RRS on the central cholinergic anti-inflammatory pathway 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; see Supplementary Table 2. 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, $p = 0.037$] (Fig. 9, Panel A).

Similar to the early timepoint, saline administration did not elicit any differences in PFC cytokine levels between any group at the delayed timepoint; see Supplementary Table 2. We next examined PFC cytokines of LPS-treated rats compared to saline-treated rats from each respective group. Similar to observations in the hippocampus in which PB treatment did not affect the IL-12 or GM-CSF response to LPS at the early timepoint (Fig. 9, Panels C and E), a history of PB treatment significantly enhanced IL-12 [F (1, 25) = 5.324, $p = 0.030$] (Fig. 9, Panel D) and GM-CSF [F(1, 25) = 9.514, $p = 0.004$] (Fig. 9, Panel F) responses to LPS relative to vehicle-treated rats at the delayed timepoint. However, the IL-1β response to LPS in the PFC was not affected by PB or stress at the delayed timepoint (Fig. 9, Panel B).

3.6. PB and stress do not affect α7 nAChR expression in hippocampus or prefrontal cortex

Since previous studies have identified the α7 subunit of the nAChR as an important component of the cholinergic 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 PFC and hippocampus (Fig. 10). Fluorescence intensity analysis revealed that PB treatment elicited a non-significant decrease in α7 levels in the hippocampus of the early cohort compared to vehicle-treated rats ($p = 0.053$; Fig. 10, Panel A). However, α7 expression did not differ between groups in the hippocampus at the delayed timepoint (Fig. 10, Panel B) or the PFC at the early (Fig. 10, Panel C) or delayed (Fig. 10, Panel D) timepoints.

4. Discussion

The clinical and preclinical literature support the concept that dysregulation of immune function is a key component of the neurological complications of GWI (Broderick et al., 2013; Johnson et al., 2016; Macht and Reagan, 2018; Macht et al., 2019). However, the mechanistic basis for the neurological complications, as well as the basis for the progressive nature of GWI neuropathology, are unknown. Central and peripheral cholinergic systems provide critical anti-inflammatory feedback to regulate immune responsivity to challenges (Hoover, 2017). However, results from the current study indicate that PB disrupts the central and peripheral cholinergic systems over time, as evidenced by greater peripheral cholinesterase activity and exaggerated cholinergic responses to an innate immune challenge within the PFC and hippocampus. Coupled with exaggerated peripheral and central cytokine responses to this same innate immune challenge, these findings suggest that PB disrupts the efficacy of cholinergic anti-inflammatory feedback. As the cholinergic response to LPS in the CNS becomes progressively more exaggerated over time, these findings provide mechanistic insight into the role of ACh and neuroinflammation as major components of the progressive nature of GWI pathophysiology. For example, in response to a peripheral injection of a modest dose of LPS (30 μg/kg), PB-treated rats exhibit potentiated increases in plasma TNF-α and CINC-3, illustrating a sensitized response to an immune challenge nearly 4 months after PB administration. These results demonstrate that PB administration elicits long-lasting impairments in the activity of the peripheral cholinergic anti-inflammatory pathway in response to an immune challenge, which is consistent with the immune dysregulation observed in veterans with GWI.

Beyond these peripheral observations, our study also provides

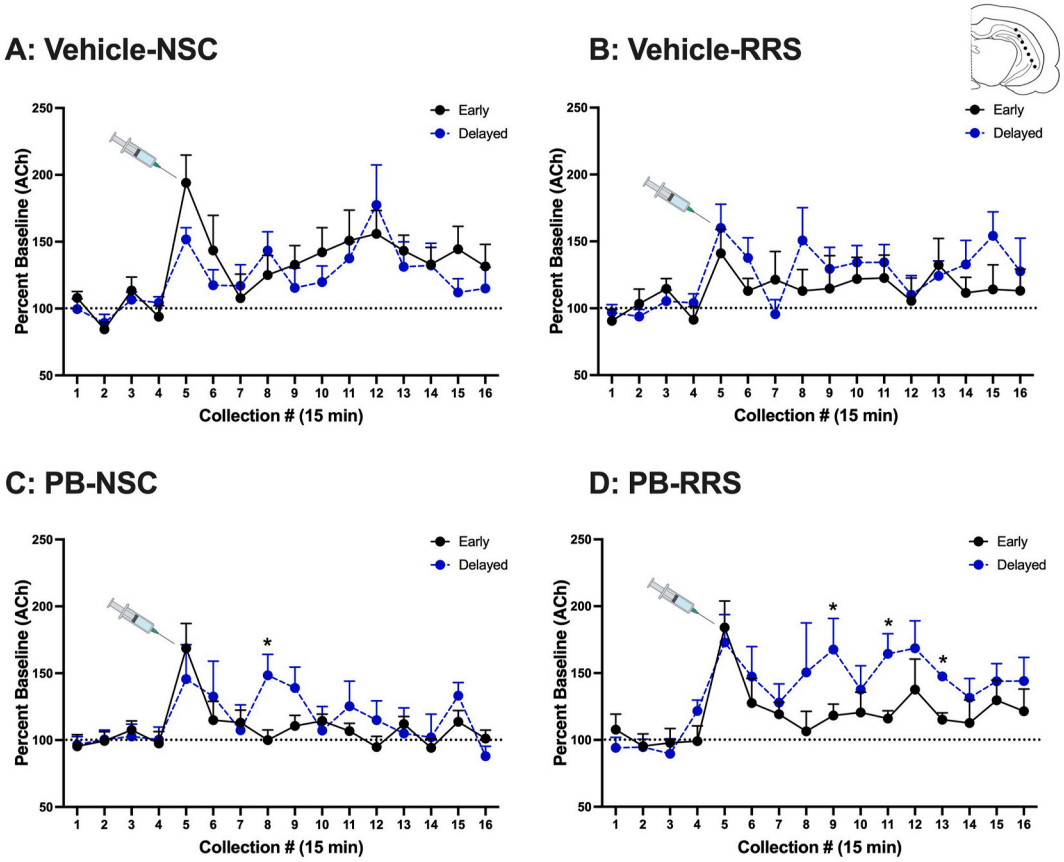

Fig. 5. Comparison of the hippocampal cholinergic response to LPS in early and delayed cohorts. There were no significant differences in the cholinergic response to LPS between early and delayed cohorts of vehicle-treated non-stressed controls (Vehicle-NSC; Panel A) or vehicle-treated rats subjected to repeated restraint stress (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, $p < 0.05$].

insight into how impairments in the brain cholinergic anti-inflammatory pathway contributes to the progressive neurological complications of GWI. For example, while LPS-induced increases in hippocampal and PFC ACh levels were similar in vehicle-treated rats irrespective of stress conditions, PB-treated rats from the delayed cohorts exhibit potentiated responses to LPS administration compared to the early PB-treated cohorts. Such observations would predict that the cholinergic response of the anti-inflammatory pathway is sensitized long after the administration of PB and as such PB-treated rats should exhibit reductions in neuroinflammation following LPS administration. However contrary to this prediction, delayed cohort PB-treated rats exhibit exacerbated increases in neuroinflammation in the hippocampus and PFC compared to early cohort PB-treated rats. For example, PB-treated rats exhibit potentiated increases in the pro-inflammatory cytokines IL-1β, IL-12 and GM-CSF compared to vehicle-treated rats. The GM-CSF findings are particularly interesting as intrahippocampal administration of this cytokine elicits behavioral deficits and activation of microglia in the rat hippocampus (Zhu et al., 2014). Similarly, GM-CSF induces microglia proliferation and the combination of GM-CSF and LPS induces neuroinflammation and increases in oxidative stress in hippocampal organotypic cultures (Dikmen et al., 2020). These results suggest that microglia activation and potentiated neuroinflammatory responses may represent an enduring consequence of PB administration, which provides a potential mechanism for the progressive neurological deficits associated with GWI.

The increases in plasma leptin levels in PB-treated rats at the delayed timepoint are also interesting because: 1) elevations in plasma leptin likely reflects leptin resistance in the CNS (Levin and Dunn-Meynell, 2002); and 2) increases in plasma leptin levels are also observed in veterans with GWI (Johnson et al., 2016). 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 neuroinflammation and central leptin resistance may be contributing to the cognitive deficits observed in GWI veterans. As such, evaluation of CNS leptin signaling/leptin resistance represents an interesting and important future direction for our studies.

4.1. Role of cholinergic anti-inflammatory circuit in GWI

Through activation of the vagus nerve, the cholinergic anti-inflammatory pathway is proposed to reduce pro-inflammatory cytokine expression and release [for review see (Hoover, 2017)]. This circuit is known to modulate neurobehavioral responses during an immune threat (Rosas-Ballina et al., 2011; Olofsson et al., 2012). Indeed, the release of brain cytokines leads to sickness behavior, including deficits in cognitive and motor function and many of these symptoms parallel GWI. Importantly, veterans suffering from GWI are reported to exhibit a blunted vagal component of the autonomic nervous system (Haley et al., 2004, 2013). Beyond autonomic function, exaggerated responses to

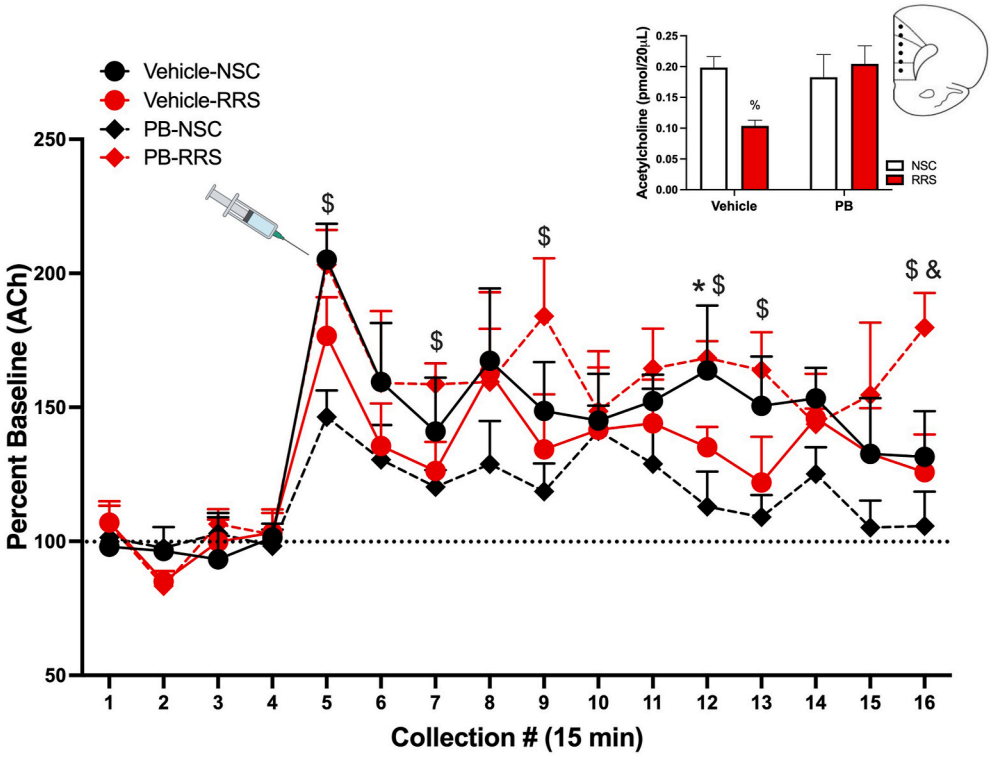

Fig. 6. PFC cholinergic response to LPS of delayed cohort. Prior to LPS administration, there was a significant cross-over effect of PB and repeated restraint stress (RRS) on basal ACh levels in the PFC (inset). Rats with a history of PB treatment exhibited an attenuated cholinergic response to LPS at collection 12 compared to vehicle non-stressed controls (Vehicle-NSC). 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 than 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, $p < 0.05$. %: Significant cross-over effect of PB and RRS, $p < 0.05$].

immune challenges in aging have been associated with neurodegenerative diseases such as Alzheimer's disease and severe detriments in cognitive functions after infection or stress (Buchanan et al., 2008; Sparkman and Johnson, 2008). Such observations are also relevant to GWI since one of the more insidious aspects of GWI is that it is a chronic disorder with progressive cognitive impairments. Indeed, the number of GW veterans diagnosed with GWI continues to rise in the post-deployment period (Blanchard et al., 2006; Kang et al., 2009; Li et al., 2011; Dursa et al., 2016). Several additional key clinical observations provide insight into the potential mechanisms that contribute to GWI pathology. Specifically, GWI veterans exhibit exaggerated immune responses to physiological stressors [e.g., exercise (Whistler et al., 2009; Broderick et al., 2011, 2013), which when combined with other studies [see (James et al., 2016, 2017; Georgopoulos et al., 2017) support the concept that neuroinflammation is a key component in the etiology and progression of GWI pathology. While these clinical studies are supportive of a role for neuroinflammation in GWI, preclinical studies have not examined the consequences of immune stressors in animal models of GWI. Indeed, examination of these dysregulated responses to immune challenges post PB treatment may provide a mechanistic basis for the increasing rates of GWI in the post-deployment period. In this regard, we have previously described how the relationships between stress, immune responses and neurochemistry shift across development in the PFC (Macht and Reagan, 2018). During this aging transition, stress has a heightened impact on immune function, which could further exacerbate the degeneration of cholinergic processes. While we consider our delayed cohorts young adult rats (approximately 6 months old), we recognize that performing studies in older rats (middle-aged and aged) represents an interesting and important future direction for our studies as GW veterans continue to age. In addition, we understand that the cholinergic alterations reported in these studies may differ in female rats, especially in the RRS groups as sex differences in stress reactivity and responses are well established (Galea et al., 1997). Expanding our GWI paradigm to include female rats is another interesting and important direction of these studies.

As α7 nAChRs expressed on microglia and astrocytes are proposed to be critical regulators of the cholinergic anti-inflammatory pathway (Wu et al., 2021), we predicted that α7 nAChR levels would be reduced in PB-treated rats at the delayed timepoint. Such observations would be consistent with the inability of elevated ACh levels to suppress LPS-induced elevations of pro-inflammatory cytokines in the hippocampus and PFC of PB-treated rats at the delayed timepoint. However, we 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). As this study examined the effects of PB at the neuromuscular junction, 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.

4.2. Potential consequences in the disruption of the central cholinergic anti-inflammatory pathway: cognitive deficits

Animal models highlight the importance of examining the delayed consequences of exposure to GWI-related chemicals in combination with stress. In this regard, deficits in water maze performance were seen more than 52 days after a PB + stress paradigm, suggesting long-term dysfunction in learning processes (Lamproglou et al., 2009). Other investigators have reported similar observations in the water maze (Parihar et al., 2013) and in other learning and memory tasks (Hattiangady et al., 2014). However, other studies that have examined long-term behavioral changes in GWI animals have reported no deficits in these same learning and memory tasks (Abdullah et al., 2012; Parihar et al., 2013). Additionally, a recent study from our group did not detect differences in contextual fear conditioning 3 months post PB + stress treatment (Macht et al., 2018). Other studies reported behavioral deficits in the water maze and the Barnes maze approximately 4 months post treatment (Abdullah et al., 2011; Zakirova et al., 2015b) but these same

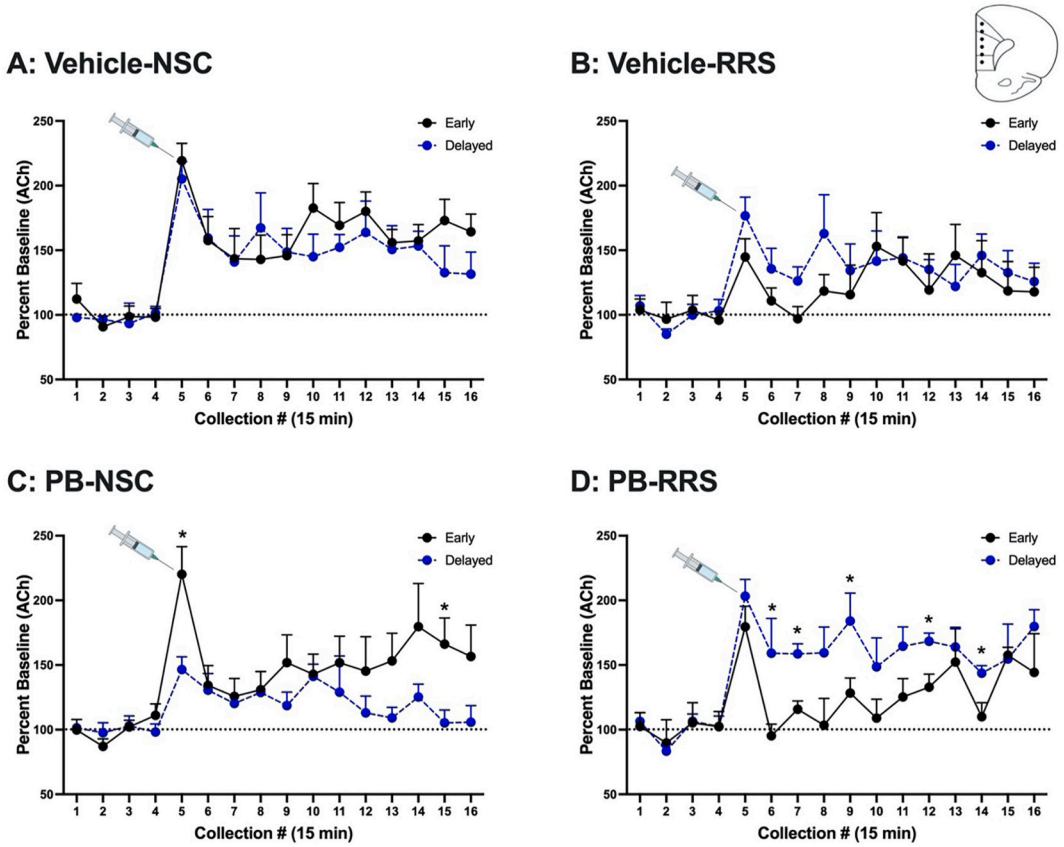

Fig. 7. Comparison of the PFC cholinergic response to LPS in early and delayed cohorts. There were no significant differences in the cholinergic response to LPS between early and delayed cohorts of vehicle-treated non-stressed controls (Vehicle-NSC; Panel A) or vehicle-treated rats subjected to repeated restraint stress (Vehicle-RRS; Panel B). Panel C: 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 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 6, 7, 9, 12 and 14 compared to the PB-RRS early cohort. All data are expressed as mean + SEM, $n = 6–10$. [*: Significant difference between early and delayed cohorts, $p < 0.05$].

investigators reported that behavioral performance in these same tests was unaffected in GWI mice compared to control mice 13 months after treatment (Zakirova et al., 2015a). The take home message from these studies is that the delayed effects of exposure to GWI-related chemicals with or without stress in rodent models has yielded equivocal results in a variety of learning and memory tasks that are considered to be hippocampal-dependent or PFC-dependent. When placed in the *context* of the current findings, one possible explanation for these disparate results is that behavioral impairments may be more consistently observed only under conditions in which GWI rodents experience an immune challenge prior to the learning and memory tasks, an approach that may be highly relevant to veterans with GWI. It is also interesting to speculate that repeated normal life exposures to bacteria or viruses may elicit exacerbated CNS immune responses in individuals with impairments in the cholinergic anti-inflammatory pathway and that the cumulative effects of these intensified neuroinflammatory responses leads to accelerated neuroplasticity deficits.

In summary, the results of the current study suggest that impairments in the activity of the cholinergic anti-inflammatory pathway may be responsible for exacerbated inflammatory responses observed in the hippocampus and PFC of PB-treated rats and suggest that neuroinflammation is a mechanistic mediator of the progressive neurological deficits observed in veterans with GWI. From a *broader perspective*, these results support the concept that dysregulation of the cholinergic anti-inflammatory pathway is a contributing factor in the neuroinflammatory mechanisms responsible for neuroplasticity deficits observed in metabolic disorders (Grillo et al., 2019), neuropsychiatric disorders (Hersey et al., 2021) and aging (Macht and Reagan, 2018).

4.3. Context and broader perspectives: reflections on the influence of Dr. Bruce S. McEwen

The design, data and interpretation of the current findings reflects the seismic influence of Dr. Bruce McEwen on our science. As aspiring postdoctoral fellows (GGP, CAG and LPR), we joined the Margaret and Milliken Hatch Laboratory of Neuroendocrinology to work with the world's leader in the field of stress neurobiology. Beyond learning how to make 10x SSC for our hybridization reactions, we now have a much greater appreciation of how Bruce influenced the maturation and evolution of our science. We appreciate how to integrate our findings from the bench to the context of the clinical setting and more broadly to the societal outlook. Bruce always took the "30,000-foot view" and he helped us to recognize the importance of the "Big Picture Perspective" and context of our results. Such a mindset emphasizes the critical importance of integrating analyses from the molecular and synaptic level to the connections of individual brain regions to the rest of the CNS (i.e., neuronal circuitry) to the cross-talk between the periphery and brain and ultimately to how this integration affects the overall health of an individual. This mindset is reflected in our studies that have examined the importance of recognizing the bidirectional relationship of peripheral-CNS interactions, as well as how stress effects are exacerbated in the presence of pre-existing imbalances in homeostasis, namely the concept of allostatic load (McEwen and Stellar, 1993). In a seminal publication with Eliot Stellar, Bruce expanded the concept of allostasis coined by Sterling and Eyer to integrate the impact of stress upon the individual. This landmark publication further highlights Bruce's approach to science: an encyclopedic memory of all the literature

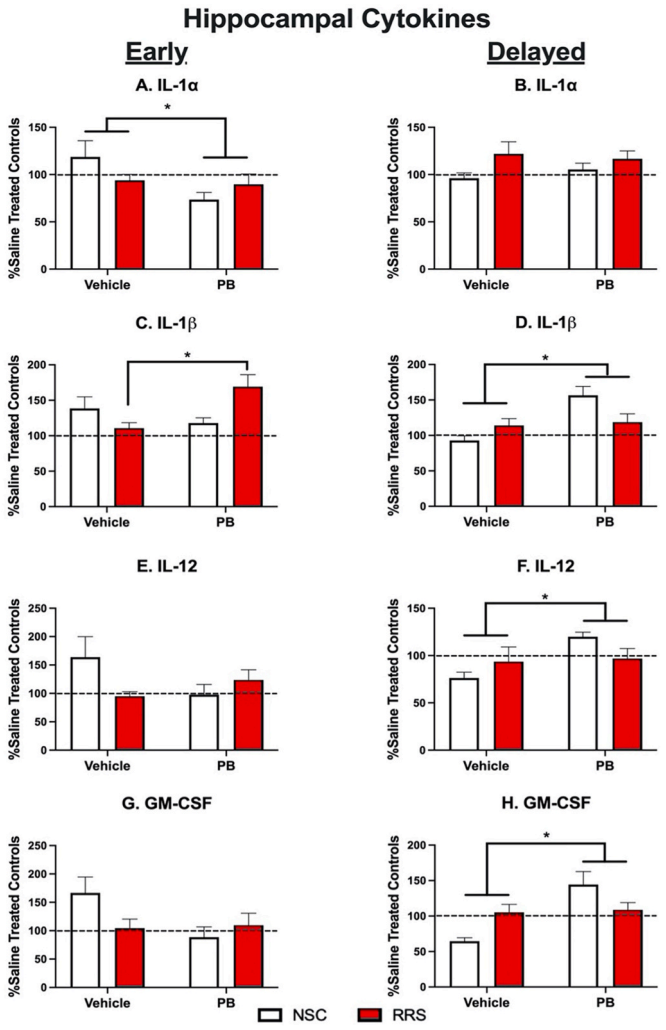

Fig. 8. Effects of PB on the hippocampal cytokine response to LPS. At the early timepoint, PB treatment suppresses the IL-1α response to LPS in the hippocampus compared to vehicle-treated controls. Only the combination of PB and repeated restraint stress (RRS) elicits an increase in IL-1β levels compared to Vehicle-RRS animals at the early timepoint. At the delayed timepoint, a history of PB treatment elicits an increased IL-1β, IL-12 and GM-CSF response 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 Supplementary Table 1 for complete list of hippocampal cytokines measured. [*: Significant effect of PB treatment compared to vehicle treatment].

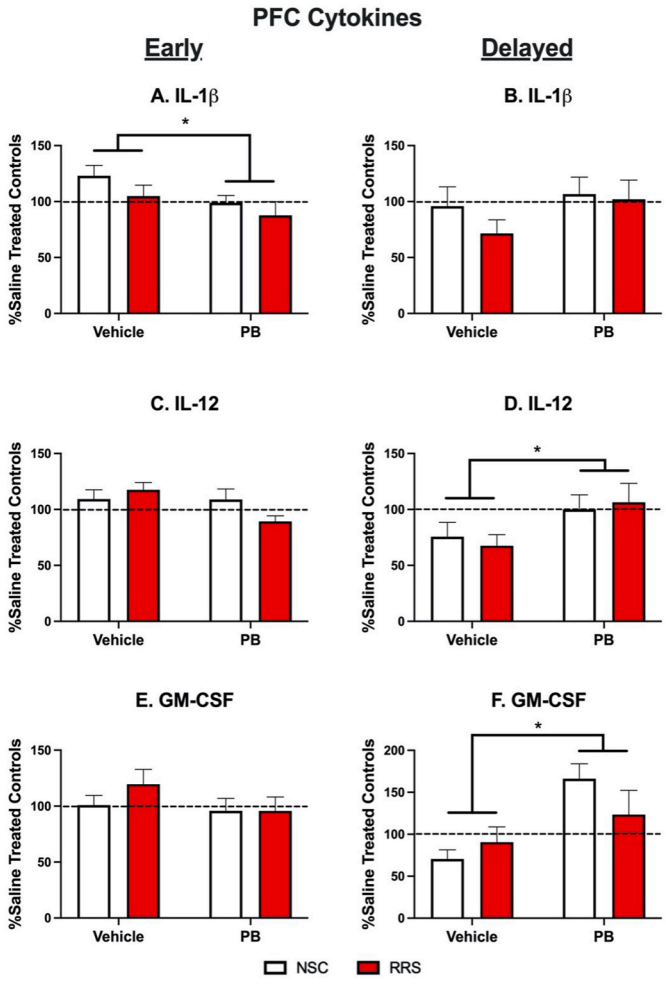

Fig. 9. Effects of PB on the PFC cytokine response to LPS. At the early timepoint, PB treatment suppresses the IL-1β response to LPS in the PFC compared to vehicle-treated controls. At the delayed timepoint, a history of PB treatment elicits an increased IL-12 and GM-CSF response to LPS in the PFC compared 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 Supplementary Table 2 for complete list of PFC cytokines measured. [*: Significant effect of PB treatment compared to vehicle treatment].

(remember those cabinets in the office filled with thousands of reprints?) and through promoting the work of colleagues. In this way, Bruce did not view science as competition, but rather as an opportunity for collaboration. Indeed, appreciation of the contribution of colleagues to the literature, integration of studies, consolidation of views from nucleic acids to whole body physiology to population-based analyses that promote opportunities for collaboration were the qualities that made Bruce a great scientist and he instilled the importance of taking these approaches in our work.

But Bruce's influence on us was far greater from a personal perspective. Bruce welcomed us into the family of "McEwenites" with open arms and a generous heart without pretense. We all can vividly remember the context of our first "Big Mac Attack" and the gentle way he influenced our science and our life decisions. We were always excited to introduce our trainees to their scientific grandfather, and our lives were constantly enriched by his continued support and enthusiasm for our successes long after we left 1230 York Avenue. He truly was our adopted father and we miss him every day. Even with his passing, Bruce continues to influence our science and our lives and we honor his legacy by passing down these values and virtues to his grandchildren.

Funding and disclosure

Financial support for these studies was provided by the Department of Veterans Affairs grant numbers I21 BX002085 and IO1 BX001804 (LPR), the National Institute of Health grant numbers R01AG050518 (JRF) and F31AG066501 (JME) and the National Science Foundation grant number IOS-1656626 (CAG). Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the U.S. Department of Veterans Affairs, the United States Government or the other stated funding agencies. The authors declare no competing financial interests.

CRediT authorship contribution statement

H.E. Burzynski: Investigation, Conceptualization, Formal analysis, Writing – review & editing. **V.A. Macht:** Investigation, Conceptualization, Formal analysis, Writing – review & editing. **J.L. Woodruff:**

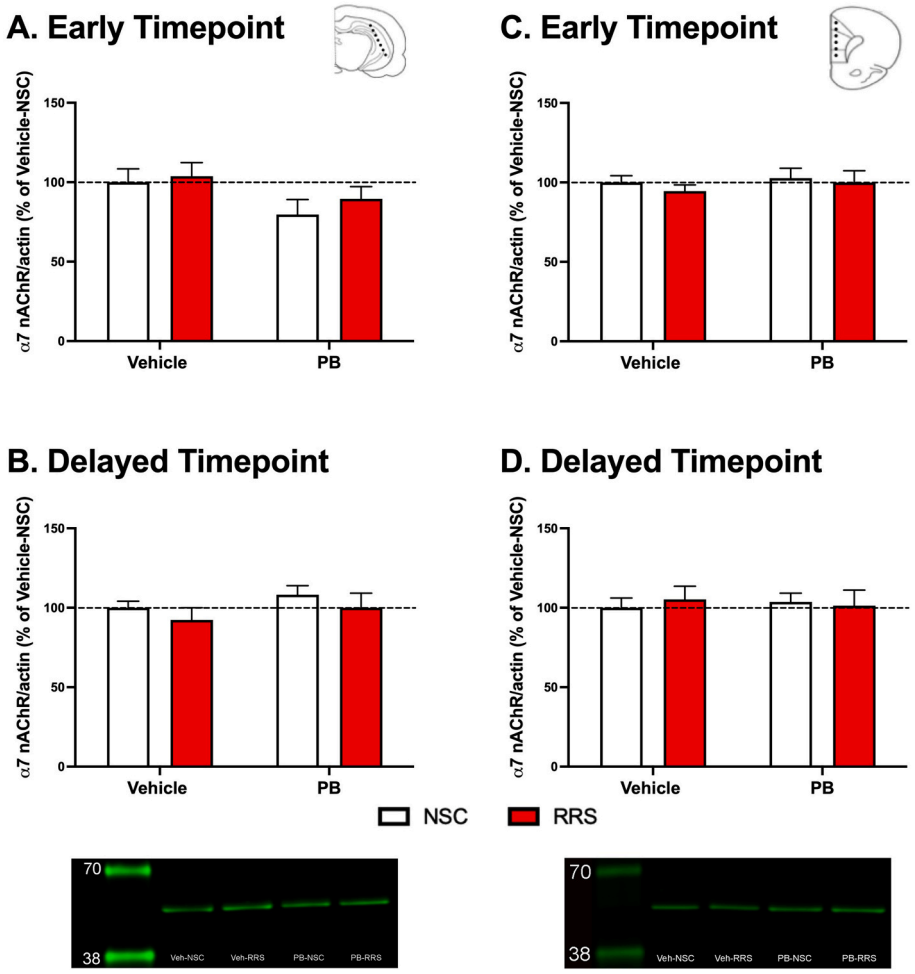

Fig. 10. Expression of α7 nAChRs in hippocampal and PFC homogenates of saline-treated early and delayed cohorts. There were no significant differences in α7 nAChR expression between groups at the early or delayed timepoint in either region. However, there was a nonsignificant decrease ($p = 0.0536$) in hippocampal α7 nAChR expression in PB-treated animals in the early cohort. All data are expressed as mean percent change from vehicle non-stressed controls (Vehicle-NSC) + SEM, $n = 7$-8.

Investigation. **J.N. Crawford:** Investigation. **J.M. Erichsen:** Investigation. **G.G. Piroli:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **C.A. Grillo:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **J.R. Fadel:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **L.P. Reagan:** Conceptualization, Formal analysis, Writing – review & editing, Supervision, Funding acquisition, is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Declaration of competing interest

The authors declare no competing financial interests.

Acknowledgments

Supported by the Department of Veterans Affairs grant numbers I21 BX002085 and IO1 BX001804 (LPR), the National Science Foundation grant number IOS-1656626 (CAG) and the National Institutes of Health grant number R01AG050518 (JRF) and F31AG066501 (JME) .

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2022.100446.

References

Abdullah, L., Crynen, G., Reed, J., Bishop, A., Phillips, J., Ferguson, S., Mouzon, B., Mullan, M., Mathura, V., Mullan, M., Ait-Ghezala, G., Crawford, F., 2011. Proteomic CNS profile of delayed cognitive impairment in mice exposed to Gulf War agents. NeuroMolecular Med. 13, 275–288.

Abdullah, L., Evans, J.E., Bishop, A., Reed, J.M., Crynen, G., Phillips, J., Pelot, R., Mullan, M.A., Ferro, A., Mullan, C.M., Mullan, M.J., Ait-Ghezala, G., Crawford, F.C., 2012. Lipidomic profiling of phosphocholine-containing brain lipids in mice with sensorimotor deficits and anxiety-like features after exposure to Gulf War agents. NeuroMolecular Med. 14, 349–361.

Blanchard, M.S., Eisen, S.A., Alpern, R., Karlinsky, J., Toomey, R., Reda, D.J., Murphy, F. M., Jackson, L.W., Kang, H.K., 2006. Chronic multisymptom illness complex in Gulf War I veterans 10 years later. Am. J. Epidemiol. 163, 66–75.

Broderick, G., Ben Hamo, R., Vashishtha, S., Efroni, S., Nathanson, L., Barnes, Z., Fletcher, M.A., Klimas, N., 2013. Altered immune pathway activity under exercise challenge in Gulf War Illness: an exploratory analysis. Brain Behav. Immun. 28, 159–169.

Broderick, G., Kreitz, A., Fuite, J., Fletcher, M.A., Vernon, S.D., Klimas, N., 2011. A pilot study of immune network remodeling under challenge in Gulf War Illness. Brain Behav. Immun. 25, 302–313.

Buchanan, J.B., Sparkman, N.L., Chen, J., Johnson, R.W., 2008. Cognitive and neuroinflammatory consequences of mild repeated stress are exacerbated in aged mice. Psychoneuroendocrinology 33, 755–765.
Calva, C.B., Fayyaz, H., Fadel, J.R., 2018. Increased acetylcholine and glutamate efflux in the prefrontal cortex following intranasal orexin-A (hypocretin-1). J. Neurochem. 145 (3), 232–244.
Dikmen, H.O., Hemmerich, M., Lewen, A., Hollnagel, J.O., Chausse, B., Kann, O., 2020. GM-CSF induces noninflammatory proliferation of microglia and disturbs electrical neuronal network rhythms in situ. J. Neuroinflammation 17, 235.
Dursa, E.K., Barth, S.K., Schneiderman, A.I., Bossarte, R.M., 2016. Physical and mental health status of Gulf war and Gulf era veterans: results from a large population-based epidemiological study. J. Occup. Environ. Med. 58, 41–46.
Erichsen, J.M., Fadel, J.R., Reagan, L.P., 2022. Peripheral versus central insulin and leptin resistance: role in metabolic disorders, cognition, and neuropsychiatric diseases. Neuropharmacology 203, 108877.
Fadel, J., Pasumarthi, R., Reznikov, L., 2005. Stimulation of cortical acetylcholine release by orexin A. Neuroscience 130, 541–547.
Galea, L.A., McEwen, B.S., Tanapat, P., Deak, T., Spencer, R.L., Dhabhar, F.S., 1997. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience 81, 689–697.
Georgopoulos, A.P., James, L.M., Carpenter, A.F., Engdahl, B.E., Leuthold, A.C., Lewis, S. M., 2017. Gulf War illness (GWI) as a neuroimmune disease. Exp. Brain Res. 235, 3217–3225.
Gordon, J.J., Leadbeater, L., Maidment, M.P., 1978. The protection of animals against organophosphate poisoning by pretreatment with a carbamate. Toxicol. Appl. Pharmacol. 43, 207–216.
Grillo, C.A., Woodruff, J.L., Macht, V.A., Reagan, L.P., 2019. Insulin resistance and hippocampal dysfunction: disentangling peripheral and brain causes from consequences. Exp. Neurol. 318, 71–77.
Haley, R.W., Charuvastra, E., Shell, W.E., Buhner, D.M., Marshall, W.W., Biggs, M.M., Hopkins, S.C., Wolfe, G.I., Vernino, S., 2013. Cholinergic autonomic dysfunction in veterans with Gulf War illness: confirmation in a population-based sample. JAMA Neurol. 70, 191–200.
Haley, R.W., Kurt, T.L., Hom, J., 1997. Is there a Gulf War Syndrome? Searching for syndromes by factor analysis of symptoms. J. Am. Med. Soc. 277, 215–222.
Haley, R.W., Vongpatanasin, W., Wolfe, G.I., Bryan, W.W., Armitage, R., Hoffmann, R.F., Petty, F., Callahan, T.S., Charuvastra, E., Shell, W.E., Marshall, W.W., Victor, R.G., 2004. Blunted circadian variation in autonomic regulation of sinus node function in veterans with Gulf War syndrome. Am. J. Med. 117, 469–478.
Hattiangady, B., Mishra, V., Kodali, M., Shuai, B., Rao, X., Shetty, A.K., 2014. Object location and object recognition memory impairments, motivation deficits and depression in a model of Gulf War illness. Front. Behav. Neurosci. 8, 78.
Hersey, M., Hashemi, P., Reagan, L.P., 2021. Integrating the monoamine and cytokine hypotheses of depression: is histamine the missing link? Eur. J. Neurosci. In press.
Hoover, D.B., 2017. Cholinergic modulation of the immune system presents new approaches for treating inflammation. Pharmacol. Ther. 179, 1–16.
Hubbard, N.A., Hutchison, J.L., Motes, M.A., Shokri-Kojori, E., Bennett, I.J., Brigante, R. M., Haley, R.W., Rypma, B., 2014. Central executive dysfunction and deferred prefrontal processing in veterans with Gulf war illness. Clin. Psychol. Sci. 2, 319–327.
James, L.M., Christova, P., Engdahl, B.E., Lewis, S.M., Carpenter, A.F., Georgopoulos, A. P., 2017. Human leukocyte antigen (HLA) and Gulf war illness (GWI): HLA-DRB1*13:02 spares subcortical atrophy in Gulf war veterans. EBioMedicine 26, 126–131.
James, L.M., Engdahl, B.E., Leuthold, A.C., Georgopoulos, A.P., 2016. Brain correlates of human leukocyte antigen (HLA) protection in Gulf war illness (GWI). EBioMedicine 13, 72–79.
Johnson, G.J., Slater, B.C., Leis, L.A., Rector, T.S., Bach, R.R., 2016. Blood biomarkers of chronic inflammation in Gulf war illness. PLoS One 11, e0157855.
Kang, H.K., Li, B., Mahan, C.M., Eisen, S.A., Engel, C.C., 2009. Health of US veterans of 1991 Gulf War: a follow-up survey in 10 years. J. Occup. Environ. Med. 51, 401–410.
Lamproglou, I., Barbier, L., Diserbo, M., Fauvelle, F., Fauquette, W., Amourette, C., 2009. Repeated stress in combination with pyridostigmine Part I: long-term behavioural consequences. Behav. Brain Res. 197, 301–310.
Levin, B.E., Dunn-Meynell, A.A., 2002. Reduced central leptin sensitivity in rats with diet-induced obesity. Am. J. Physiol. Regul. Integr. Comp. Physiol. 283, R941–R948.
Li, B., Mahan, C.M., Kang, H.K., Eisen, S.A., Engel, C.C., 2011. Longitudinal health study of US 1991 Gulf War veterans: changes in health status at 10-year follow-up. Am. J. Epidemiol. 174, 761–768.
Macht, V.A., Reagan, L.P., 2018. Chronic stress from adolescence to aging in the prefrontal cortex: a neuroimmune perspective. Front. Neuroendocrinol. 49, 31–42.
Macht, V.A., Woodruff, J.L., Burzynski, H.E., Grillo, C.A., Reagan, L.P., Fadel, J.R., 2020. Interactions between pyridostigmine bromide and stress on glutamatergic neurochemistry: insights from a rat model of Gulf War Illness. Neurobiol. Stress 12, 100210.
Macht, V.A., Woodruff, J.L., Grillo, C.A., Wood, C.S., Wilson, M.A., Reagan, L.P., 2018. Pathophysiology in a model of Gulf war illness: contributions of pyridostigmine bromide and stress. Psychoneuroendocrinology 96, 195–202.
Macht, V.A., Woodruff, J.L., Maissy, E.S., Grillo, C.A., Wilson, M.A., Fadel, J.R., Reagan, L.P., 2019. Pyridostigmine bromide and stress interact to impact immune function, cholinergic neurochemistry and behavior in a rat model of Gulf War Illness. Brain Behav. Immun. 80, 384–393.
Marino, M.T., Schuster, B.G., Brueckner, R.P., Lin, E., Kaminskis, A., Lasseter, K.C., 1998. Population pharmacokinetics and pharmacodynamics of pyridostigmine bromide for prophylaxis against nerve agents in humans. J. Clin. Pharmacol. 38, 227–235.
McEwen, B.S., Stellar, E., 1993. Stress and the individual. Mechanisms leading to disease. Arch. Intern. Med. 153, 2093–2101.
Nettleman, M., 2015. Gulf war illness: challenges persist. Trans. Am. Clin. Climatol. Assoc. 126, 237–247.
Olofsson, P.S., Rosas-Ballina, M., Levine, Y.A., Tracey, K.J., 2012. Rethinking inflammation: neural circuits in the regulation of immunity. Immunol. Rev. 248, 188–204.
Parihar, V.K., Hattiangady, B., Shuai, B., Shetty, A.K., 2013. Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. Neuropsychopharmacology 38, 2348–2362.
Pascuzzo, G.J., Akaike, A., Maleque, M.A., Shaw, K.P., Aronstam, R.S., Rickett, D.L., Albuquerque, E.X., 1984. The nature of the interactions of pyridostigmine with the nicotinic acetylcholine receptor-ionic channel complex. I. Agonist, desensitizing, and binding properties. Mol. Pharmacol. 25, 92–101.
Paxinos, G., Watson, C., 1998. The Rat Brain in Sterotaxic Coordinates. Academic Press.
Pope, C., Karanth, S., Liu, J., 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. Environ. Toxicol. Pharmacol. 19, 433–446.
Ransohoff, R.M., 2016. How neuroinflammation contributes to neurodegeneration. Science 353, 777–783.
Rosas-Ballina, M., Olofsson, P.S., Ochani, M., Valdes-Ferrer, S.I., Levine, Y.A., Reardon, C., Tusche, M.W., Pavlov, V.A., Andersson, U., Chavan, S., Mak, T.W., Tracey, K.J., 2011. Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science 334, 98–101.
Sparkman, N.L., Johnson, R.W., 2008. Neuroinflammation associated with aging sensitizes the brain to the effects of infection or stress. Neuroimmunomodulation 15, 323–330.
Steele, L., 2000. Prevalence and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. Am. J. Epidemiol. 152, 992–1002.
Steele, L., Sastre, A., Gerkovich, M.M., Cook, M.R., 2012. Complex factors in the etiology of Gulf War illness: wartime exposures and risk factors in veteran subgroups. Environ. Health Perspect. 120, 112–118.
Tamashiro, K.L., Nguyen, M.M., Ostrander, M.M., Gardner, S.R., Ma, L.Y., Woods, S.C., Sakai, R.R., 2007. Social stress and recovery: implications for body weight and body composition. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R1864–R1874.
Tillman, G.D., Calley, C.S., Buhl, V.I., Chiang, H.S., Haley, R.W., Hart Jr., J., Kraut, M.A., 2017. Electrophysiological correlates of semantic memory retrieval in Gulf War Syndrome 2 patients. J. Neurol. Sci. 373, 66–72.
von Bredow, J.D., Adams, N.L., Groff, W.A., Vick, J.A., 1991. Effectiveness of oral pyridostigmine pretreatment and cholinolytic-oxime therapy against soman intoxication in nonhuman primates. Fundam. Appl.Toxicol. 17, 761–770.
Whistler, T., Fletcher, M.A., Lonergan, W., Zeng, X.R., Lin, J.M., Laperriere, A., Vernon, S.D., Klimas, N.G., 2009. Impaired immune function in Gulf war illness. BMC Med. Genom. 2, 12.
White, R.F., Steele, L., O'Callaghan, J.P., Sullivan, K., Binns, J.H., Golomb, B.A., Bloom, F.E., Bunker, J.A., Crawford, F., Graves, J.C., Hardie, A., Klimas, N., Knox, M., Meggs, W.J., Melling, J., Philbert, M.A., Grashow, R., 2016. Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: effects of toxicant exposures during deployment. Cortex 74, 449–475.
Wu, Y.J., Wang, L., Ji, C.F., Gu, S.F., Yin, Q., Zuo, J., 2021. The role of α7nAChR-mediated cholinergic anti-inflammatory pathway in immune cells. Inflammation 44, 821–834.
Zakirova, Z., Crynen, G., Hassan, S., Abdullah, L., Horne, L., Mathura, V., Crawford, F., Ait-Ghezala, G., 2015a. A chronic longitudinal characterization of neurobehavioral and neuropathological cognitive impairment in a mouse model of Gulf war agent exposure. Front. Integr. Neurosci. 9, 71.
Zakirova, Z., Tweed, M., Crynen, G., Reed, J., Abdullah, L., Nissanka, N., Mullan, M., Mullan, M.J., Mathura, V., Crawford, F., Ait-Ghezala, G., 2015b. Gulf War agent exposure causes impairment of long-term memory formation and neuropathological changes in a mouse model of Gulf War Illness. PLoS One 10, e0119579.
Zhu, F., Liu, Y., Zhao, J., Zheng, Y., 2014. Minocycline alleviates behavioral deficits and inhibits microglial activation induced by intrahippocampal administration of Granulocyte-Macrophage Colony-Stimulating Factor in adult rats. Neuroscience 266, 275–281.