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Pyridostigmine bromide and stress interact to impact immune function, cholinergic neurochemistry and behavior in a rat model of Gulf War Illness

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ABSTRACT

Gulf War Illness (GWI) is characterized by a constellation of symptoms that include cognitive dysfunction. While the causes for GWI remain unknown, prophylactic use of the acetylcholinesterase inhibitor pyridostigmine bromide (PB) in combination with the stress of deployment has been proposed to be among the causes of the cognitive dysfunction in GWI. Mechanistically, clinical studies suggest that altered immune function may be an underlying factor in the neurochemical and neurobehavioral complications of GWI. Accordingly, the goal of this study was to determine how responses to an immune challenge (lipopolysaccharide; LPS) or stress impacts inflammation, acetylcholine (ACh) neurochemistry and behavior in an experimental model of GWI. Rats with a history of PB treatment exhibited potentiated increases in C-reactive protein levels in response to a submaximal LPS challenge compared to control rats, indicating that prior treatment with this cholinesterase inhibitor leads to exacerbated inflammatory responses to a subsequent immune challenge. ACh responses to LPS administration were decreased in the hippocampus, but not prefrontal cortex (PFC), in rats with a prior history of PB treatment or stress exposure. Additionally, ACh release in response to acute immobilization stress was attenuated in the PFC and hippocampus in these groups. These attenuated cholinergic responses were accompanied by impairments in contextual and cue-based fear learning. The results of this study suggest that stress and LPS challenges adversely affect central ACh neurochemistry in a rodent model of GWI and support the hypothesis that dysregulated immune responses are mechanistically linked to the neurological complications of GWI.

1. Introduction

The use of pyridostigmine bromide (PB), a reversible acetylcholinesterase inhibitor, as a nerve-agent pretreatment tablet for soldiers during the 1990–1991 Gulf War was controversial as it was approved by the Food and Drug Administration in the absence of informed consent under an *Investigational New Drug* clause. The efficacy of PB as a prophylactic treatment was supported by a variety of preclinical studies which demonstrated that its administration drastically improves survival rates following exposure to irreversible acetylcholinesterase inhibitors, which are the primary component of G-series nerve agents (Gordon et al., 1978; von Bredow et al., 1991). Because PB was not thought to cross the blood-brain barrier and has a history of use in other clinical populations (e.g. myasthenia gravis), PB was not thought to cause any long-term deficits in the central nervous system (CNS). However, this view of PB was questioned after 25–42% of soldiers returning from combat began presenting with a variety of central and peripheral deficits consistent with cholinergic toxicity, including chronic headaches, sleep disturbances, chronic pain, respiratory problems and muscle fatigue, as well as cognitive impairments that include attentional deficits, and working and long-term memory impairments (Pope et al., 2005; Steele et al., 2012). These symptoms are collectively termed Gulf War Illness (GWI), and epidemiological studies have consistently correlated PB administration with symptom presentation (Haley et al., 1997; Steele et al., 2012; White et al., 2016).

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The correlation between PB and the unexpected CNS deficits in soldiers with GWI led to the hypothesis that chronic stress exposure creates a "leaky" blood-brain barrier (Friedman et al., 1996), allowing PB to cross and directly impact central cholinergic function; however, other studies failed to replicate this initial report (Amourette et al., 2009; Grauer et al., 2000; Kant et al., 2001; Song et al., 2002; Tian et al., 2002). As such, while studies have consistently found cognitive and behavioral consequences due to combinations of PB and stress, the neurochemical basis for these deficits remains unknown. In addition, while several preclinical studies of GWI have examined cholinesterase activity in the brain, no study has examined the functional responsiveness of central cholinergic systems to PB and different types of stress.

To address the hypothesis that stress and PB interact to influence central cholinergic function, the current study used a model of GWI in combination with *in vivo* microdialysis to test the cholinergic neurochemical response to both a lipopolysaccharide (LPS) challenge and an acute immobilization stress challenge. This rodent model of GWI was a 2 × 2 design with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (repeated restraint stress: RRS, non-stressed controls (NSC)). Acetylcholine levels were assessed in two brain regions: the prefrontal cortex (PFC) and the hippocampus. These brain regions were targeted due to their robust cholinergic innervation and critical contribution to cognitive processes which exhibit deficits in veterans with GWI. An LPS challenge during microdialysis was selected because 1) alterations in the immune system are emerging as a hallmark feature of GWI in both clinical and preclinical literature (Broderick et al., 2013; Smylie et al., 2013; Whistler et al., 2009; Khaiboullina et al., 2015; Peden-Adams et al., 2004); and 2) the cholinergic system is intimately linked to regulation of the immune response both peripherally and centrally (Wenk et al., 2003; Zaghloul et al., 2017). An acute immobilization stress challenge in a novel context was selected as a stress challenge. To assess whether PB and stress produce similar deficits in memory tasks which are dependent on cholinergic transmission in these brain regions, we also tested the interactive effects between PB and stress on contextual and cue-based fear conditioning.

2. Methods and materials

2.1. GWI model

The clinical and epidemiological data suggest that an interaction between PB treatment and stressful combat-related situations contribute to the development of GWI (Steele et al., 2012). For this reason, we developed an experimental model of GWI that focused on the cholinesterase inhibitor PB alone and in combination with RRS. The rationale and framework for this model was provided by earlier studies from a number of different laboratories. For example, prior studies provided the experimental framework related to the dose, duration and route of administration of PB (Abou-Donia et al., 2004; Parihar et al., 2013), and also highlighted the interaction of PB and repeated stress (Kant et al., 2001; Amourette et al., 2009; Barbier et al., 2009; Lamproglou et al., 2009; Parihar et al., 2013; Abdel-Rahman et al., 2004, 2002; Musazzi et al., 2010). Another important component of this design was that rats were exposed to PB prior to the initiation of stress to more closely mimic the treatment regimen of soldiers who were given PB prophylactically, as well as after deployment. The development of our model of GWI was based on these observations and as described in our previous study (Macht et al., 2018).

Specifically, adult male Sprague Dawley rats (250–300 g) were individually house in a temperature-controlled facility (22 °C) with *ad libitum* access to food and water. Rats were maintained on a 12/12 h light-dark cycle with lights on at 7:00 a.m. Although both males and females served in the Gulf War, the following studies only used males since the majority of soldiers and hence incidences of GWI were in men (Nettleman, 2015). All procedures were performed in accordance to all guidelines are regulations by the Dorn VA Animal Care and Use Committee. Rats were randomly assigned to one of four treatment conditions: vehicle-non-stressed controls (Veh-NSC), PB-NSC, vehicle-RRS (Veh-RRS), PB-RRS. PB was prepared daily at a concentration of 0.13 mg/mL in sterile water. Rats were gavaged daily from days 1–14 with either 1.3 mg/kg bw PB or sterile water (vehicle), per their treatment condition (Macht et al., 2018). On the fifth day, rats in the RRS condition were moved to a separate room and placed in wire mesh restrainers for 6 h/day for a total of 10 days [as described in (Reagan et al., 2004; Reznikov et al., 2008)]. Restraint began at 10:00 a.m. each morning, just after gavage. PB treatment began prior to the onset of stress as soldiers were authorized to take PB before deployment when being sent to high-risk zones. For a summary of the experimental timeline, see Fig. 1.

2.2. Stereotaxic surgery

The day following the end of the drug/stress paradigm, rats underwent stereotaxic surgery to unilaterally implant two guide cannulae into the PFC and dorsal hippocampus as described in our previous studies (Stanley et al., 2012). Interlocking intracerebral guide cannulae and stylets from Bioanalytical Systems Incorporated (BASi: MD-2251) were placed relative to bregma: AP, +3.0; L, ±0.5 mm; DV, −2.5 mm for the PFC, and AP, −5.2; L, ±3.8 mm; DV, −3.6 mm at a 10° angle for the hippocampus. Coordinates were selected based on the Paxinos and Watson rat brain atlas (1998). Left and right hemispheres were counterbalanced across rats. Rats were allowed one full day to recover from surgery undisturbed, followed by 4 days of habituation to the microdialysis bowls prior to microdialysis. As such, microdialysis did not commence until a week following the date of surgery. There were no differences in surgical recovery between any groups.

2.3. In vivo microdialysis

Each rat was habituated to the microdialysis bowls in the BASi Raturn system for a total of 20 h over the course of 4 days. There were two separate sessions of microdialysis separated by a 48 h recovery. The first session of microdialysis consisted of a 30 μg/kg LPS challenge, and the second session was an immobilization stress challenge. This LPS dose was selected based off of pilot data as it was the lowest dose to induce a significant but sub-maximal increase in acetylcholine (see supplementary Fig. 1). On the morning of microdialysis, probes from BASi (2 mm, MD-2200) were placed into each guide cannula 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. A three-hour discard period began at 9:00 a.m. to allow recovery from the probe insertion. All sessions began with four baseline collections. Samples were collected at fifteen-minute intervals and frozen at −80 °C at the end of the collection. Thirty μg/kg LPS was injected intraperitoneally at the start of the 5th collection and collections continued for an additional 3 h. Forty-eight hours later, rats were subjected to a second microdialysis session that included a one hour immobilization stress challenge that was initiated at the start of the 5th collection, as described previously (Reznikov et al., 2007). To assess stress induced changes in acetylcholine levels in the PFC and hippocampus rats were subjected to immobilization stress in a novel environment. Unlike the RRS paradigm that occurs in the vivarium, this stressor occurs in the microdialysis room and is more similar to an immobilization stress versus the RRS paradigm described above. The immobilization stress challenge lasted for 1 h, starting at the 5th collection, followed by an additional hour of collections after cessation of the restraint challenge. Following microdialysis, rats were anesthetized with isoflurane and transcardially per-

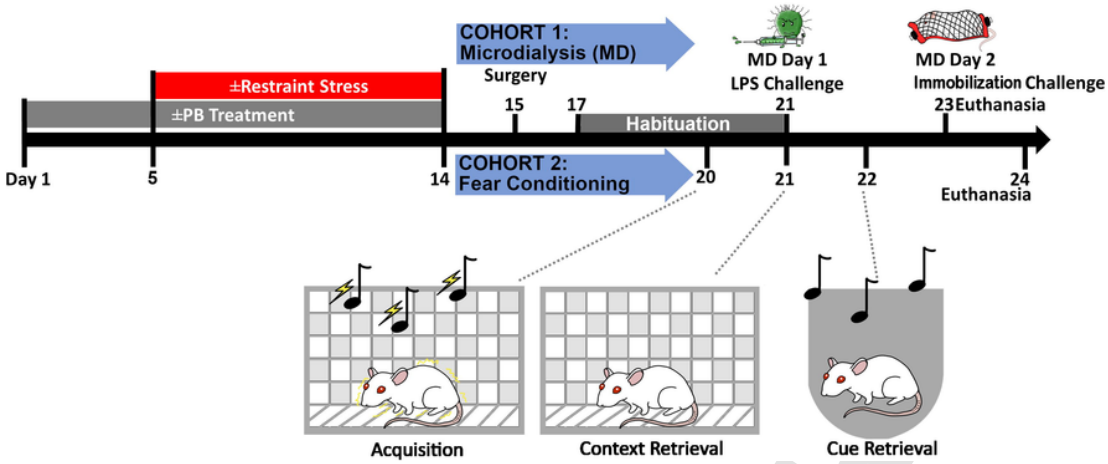

Fig. 1. Experimental Timeline. The GWI paradigm consisted of 14 days of gavage with either PB or vehicle. On day 5, rats were subdivided into groups which received restraint stress or non-stressed control conditions. Rats undergoing restraint stress were moved to a separate room so controls did not witness nor hear stress-induced vocalizations. Restraint stress was conducted from 10:00 a.m. to 4:00p.m. for a total of 10 consecutive days. Cannula surgery was conducted on day 15 of the experiment, one day following the cessation of stress/PB treatment. Rats were given 1 full day of recovery followed by 4 days of habituation before the first microdialysis session (LPS, day 21). The second microdialysis session (immobilization, day 23) was performed 48 h later. Rats were immediately euthanized following termination of the second microdialysis session. In a separate cohort of rats, fear conditioning was conducted between days 20–22, with acquisition of the fear response on day 20, contextual conditioning on day 21, and cue-conditioning tested on day 22. Rats from this cohort were sacrificed on day 24 of the paradigm.

fused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and placed in a 30% sucrose/0.1 M 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 then used to cut 40 μm sections to verify probe placement in each rat; see Fig. 2.

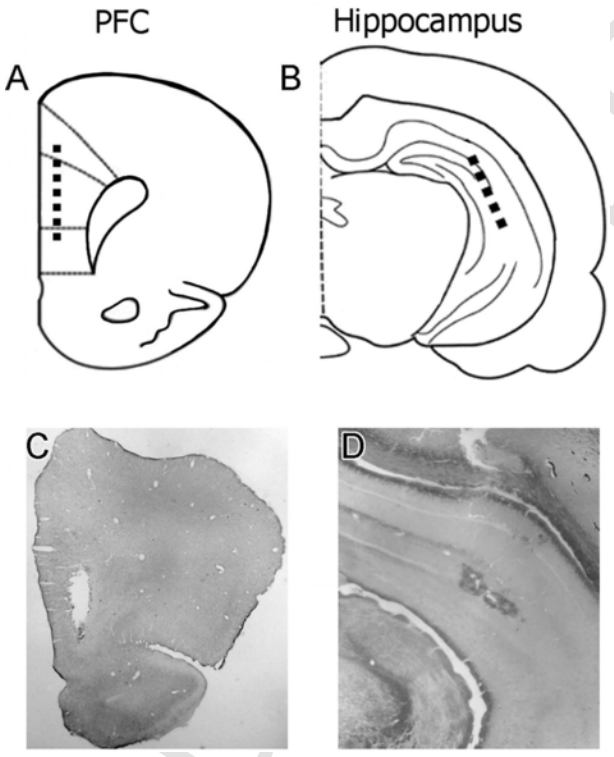

Fig. 2. Probe Placement. Probe placement is depicted for targets for the medial prefrontal cortex (Panel A) and dorsal hippocampus (Panel B). Representative examples of probe placement in prefrontal cortex (Panel C) and hippocampus (Panel D) Samples were stained with a background cholinesterase stain to enhance contrast of the probe location.

2.4. Assessment of the immune response to LPS

Tail bleeds were performed immediately after the end of the LPS microdialysis session, 3 h after the intraperitoneal injection of LPS, to assess the peripheral immune response in association with the central cholinergic response during microdialysis. Plasma was stored at −80° until analysis for cytokines and C-reactive protein (CRP). Twelve Th1/Th2 rat cytokines (i.e. IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, TNF-α) were quantified using a Bio-Plex cytokine assay from Bio-Rad (#171k1002M) according to the manufacturer's instructions (Grillo et al., 2014). Plasma samples were 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. For the current study, all data was analyzed based on raw cytokine values from rats which underwent microdialysis. A group of control rats which did not proceed through microdialysis and hence were not stimulated by LPS were included as verification of the efficacy of LPS to induce plasma cytokine changes over baseline. Of note, we previously reported that there are no differences in basal cytokine levels between any groups using this model at this time point (Macht et al., 2018). C-reactive protein (CRP) was assessed in plasma using an enzyme-linked immunosorbent assay kit from Becton Dickinson Biosciences (#557825) according to the manufacturer's instructions (Grillo et al., 2014). Samples were diluted 1:40,000 and read using a BioTek Synergy microplate reader (BioTek Instruments Inc., Winooski, VT).

2.5. High performance liquid chromatography

Twenty μL of previously frozen microdialysate sample was loaded onto an Eicom AC-GEL reverse-phase analytical column, where choline and acetylcholine were isolated from other biogenic compounds in interaction with a mobile phase consisting of 50 mM potassium bicarbonate, 300 mg/L sodium decanesulfonate, and 50 mg/mL 2Na EDTA, pH 8.4. A dual enzymatic column AC-ENZYM II from Eicom metabolized acetylcholine into hydrogen peroxide by acetylcholinesterase and choline oxidase. The hydrogen peroxide was oxidized at the platinum electrochemical detector with an applied current of +450 mV. The potential was read using the Eicom HT-500 detector system. Concentra-

tion of acetylcholine in samples was interpolated against a three-point standard curve.

2.6. Conditioned freezing

In a separate cohort of rats, conditioned freezing was performed as described previously (Grillo et al., 2011; Sharko et al., 2016). On the first day, rats were placed in a 46 × 24 × 22 cm acoustically isolated shock box (Clever Sys, Inc, Reston VA). After 3 min, rats were given three 10 s tones (2 KHz, 80 dB) that co-terminated with a 1 s, 1 mA shock with 1 min inter-stimulus intervals. Twenty-four hours later, retention of context was assessed: rats were returned to the testing box in the absence of tones or shocks for a total of 8 min. These data have been previously published in (Macht et al., 2018) and are included in this manuscript in a condensed format for illustration purposes only. For a detailed analysis of the original data, please refer to (Macht et al., 2018). Forty-eight hours after the acquisition, retention of cue-based conditioned freezing was assessed in a novel context consisting of a Plexiglas bowl with sani-chip bedding. After 3 min, to assess unconditioned freezing to a novel context, they were re-exposed to fourteen 10 s tones (2 KHz, 80 dB) at 1 min inter-stimulus intervals. Freezing was defined as the absence of movement excluding respiration, which was determined automatically by Freezescan (Clever Sys, Inc, Reston, VA). Percent of time spent freezing was binned for each 1-minute of testing in each session for analysis.

2.7. Statistical analysis

Plasma measures for cytokines were analyzed using a 2 × 2 multivariate analysis of variance (MANOVA) with 2 levels of *drug treatment* (vehicle, PB), 2 levels of *stress history* (NSC, stressed) and 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 a follow-up. We have previously reported that there are no drug or stress effects on basal cytokine levels at this time point (see Macht et al., 2018). For verification that LPS did elicit an increase in plasma cytokines, cytokines from a group of non-stimulated controls were analyzed separately in comparison to the LPS-treated rats which underwent microdialysis using a MANOVA. For *in vivo* microdialysis, data were assessed as either a 2 × 2 × 16 (LPS) or a 2 × 2 × 12 (restraint) and the additional factor of *time*. Comparison of basal levels of acetylcholine prior to LPS administration and basal levels of acetylcholine prior to immobilization stress challenge was assessed by unpaired *t*-test. Conditioned freezing was also assessed as a 2 × 2 × 8 (context) of 2 × 2 × 14 (cue) with the additional factor of *time*. For detailed statistical results of the context data, please refer to (Macht et al., 2018). Statistical significance was set at $\alpha = 0.05$ for all analyses. 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* and across each level of *time* (when applicable).

3. Results

3.1. PB dysregulates peripheral immune responses to an LPS challenge

We have previously reported that basal levels of cytokines in plasma are not modulated in Veh-RRS rats, PB-NSC rats and PB-RRS rats compared to Veh-NSC rats at the early timepoint of GWI paradigm (i.e. approximately 21 days; see timeline above) (Macht et al., 2018). However, such findings cannot assess how PB administration alone or in combination with stress impacts cytokine levels in response to an immune challenge. Accordingly, inflammatory responses were assessed in plasma isolated approximately three hours after LPS administration in Veh-NSC rats, Veh-RRS rats, PB-NSC rats and PB-RRS rats. Relative to plasma cytokines from rats under non-stimulated conditions, LPS administration significantly increased plasma levels of all measured cytokines in Veh-NSC rats, $F(1, 11) = 301.2$, $p = 0.04$. Interestingly, there was a trend for the cytokine response to LPS to be attenuated in PB-treated rats relative to vehicle-treated rats, $F(12, 13) = 0.08$. Specifically, the IL-6 response to LPS was significantly attenuated in PB-treated rats relative to vehicle-treated rats, $F(1, 28) = 7.3$, $p = 0.01$. There was also a trend for PB to attenuate the response of IL-1β and TNF-α to LPS relative to vehicle-treated rats, $p = 0.09$ and 0.05, respectively. This interaction between PB-treatment and LPS on plasma cytokines is depicted Fig. 3. For raw values of all cytokine data, see Table 1. These results are consistent with the concept that PB administration, by increasing plasma ACh levels, would enhance the cholinergic anti-inflammatory circuit (Hoover, 2017).

As an additional measure of inflammatory responses we examined plasma levels of CRP, which should be elevated under conditions of increased plasma levels of IL-6. Basal levels of CRP at Day 21 did not differ between any of the groups [$F(3,12) = 0.295$, $p = 0.829$]. Additionally, LPS treatment did not elicit significant increases in plasma CRP levels in Veh-NSC and Veh-RRS rats relative to their respective saline-treated controls (Fig. 4). However, LPS treatment elicited a significant increase in CRP levels in rats with a prior history of PB treatment relative to vehicle-treated rats given saline [$F(1,27) = 10.68$, $p = 0.002$]. These results indicate that a prior history of PB treatment elicits an exaggerated increase in plasma CRP levels to a sub-threshold dose of LPS.

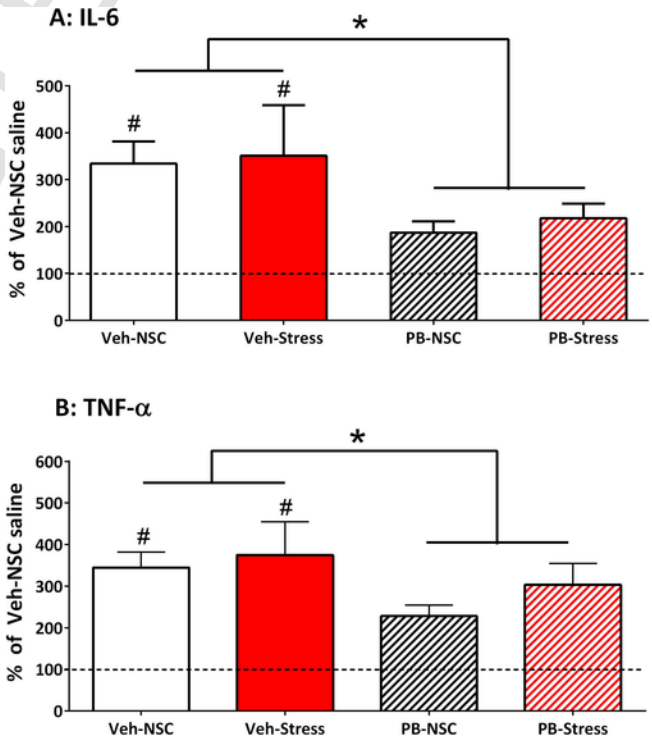

Fig. 3. Effects of PB on the Peripheral cytokine response to LPS. Panel A: Vehicle-treated rats exhibit the expected increases in plasma IL-6 levels following an i.p. injection of LPS, but LPS-induced increases in IL-6 in PB-treated rats are not significantly different from saline-treated Veh-NSC rats. Additionally, LPS-induced increased in IL-6 are significantly increased in Vehicle-treated rats compared to PB-treated rats. Panel B: LPS administration significantly increases plasma TNF-α levels in vehicle-treated rats compared to saline-treated Veh-NSC rats, increases that are not observed in rats with a prior history of PB treatment. LPS-induced increases in TNF-α are significantly different in vehicle-treated rats compared to PB-treated rats. All data are expressed as mean ± SEM, n = 5–9. [*: Significant effect of PB relative to vehicle-treated rats, $p < 0.05$; #: Significant effect of LPS, $p < 0.05$. Dashed line represents baseline values determined in saline-treated rats at Day 21 in the GWI paradigm].

Table 1
LPS-induced cytokine levels in plasma isolated from rats with a prior history of PB treatment alone or in combination with repeated restraint stress.

	Veh-NSC	Veh-RRS	PB-NSC	PB-RRS
Ra IL-1a (21)	651.0 ± 26.7	499.4 ± 118.5	581.1 ± 51.4	619.9 ± 61.0
Ra IL-1b (28)	1252.1 ± 80.4	1178.2 ± 294.7	**987.2 ± 106.3#**	**974.8 ± 145.5#**
Ra IL-2 (22)	820.7 ± 34.1	623.3 ± 180.2	747.13 ± 67.5	773.7 ± 65.5
Ra IL-4 (33)	88.4 ± 3.3	64.1 ± 1 9.7	77.8 ± 7.9	79.6 ± 6.8
Ra IL-5 (52)	575.6 ± 12.4	459.1 ± 110.8	543.0 ± 35.2	549.4 ± 28.0
Ra IL-6 (56)	2587.3 ± 368.1	2719.3 ± 549.5.	**1447.8 ± 193.1***	**1687.1 ± 240.0***
Ra IL-10 (19)	2798.8 ± 110.9	236.0 ± 74.3	2403.6 ± 216.7	2460.2 ± 181.1
Ra IL-12(p70) (78)	323.3 ± 18.0	236.0 ± 74.3	279.4 ± 34.5.	286.1 ± 35.8
Ra IL-13 (15)	204.5 ± 14.0	156.2 ± 47.6	189.4 ± 24.1	202.2 ± 23.7
Ra GM-CSF (37)	409.0 ± 26.3	293.5 ± 96.3	355.4 ± 44.4	381.4 ± 44.9
Ra IFN-g (34)	793.3 ± 26.8	627.6 ± 154.9	661.5 ± 57.6	729.7 ± 64.8
Ra TNF-a (43)	820.5 ± 89.7	893.8 ± 224.6	**543.4 ± 64.3#**	**722.9 ± 122.0#**

Data expressed as mean (pg/mL) ± SEM. **significantly different response to LPS from vehicle-treated rats; # trend for significantly different response to LPS relative to vehicle-treated rats.* 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.

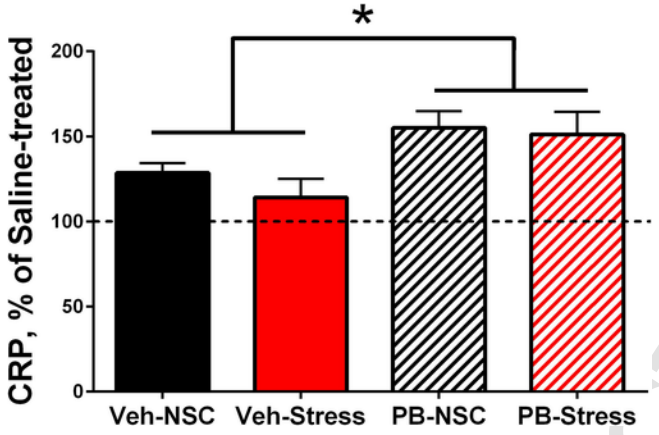

Fig. 4. Effects of PB on the Peripheral CRP Response to LPS. LPS administration elicited significant increases in plasma CRP levels in rats with a prior history of PB treatment compared to vehicle-treated rats, irrespective of stress exposure. Each bar graph represents percent change in LPS-induced increases in plasma CRP levels relative to saline-treated rats. All data are expressed as mean ± SEM, n = 5–9. [*: Significant effect of PB relative to vehicle-treated rats, $p < 0.05$. Dashed line represents baseline values determined in saline-treated rats at Day 21 in the GWI paradigm].

3.2. Repeated stress attenuates the cholinergic response to LPS in the PFC

Basal acetylcholine levels assessed on the first day of microdialysis indicate that there was no effect of either a prior history of PB or RRS on basal acetylcholine levels in the PFC, [$F(1, 25) = 0.25$, $p = 0.62$; Fig. 5, Panel A inset]. An LPS challenge significantly increased PFC acetylcholine levels over time in all rats [$F(15, 405) = 12.0$, $p < 0.001$]. There was also a main effect of RRS where a prior stress history attenuated the cholinergic response to LPS [$F(1, 27) = 6.52$, p = 0.017]. Additionally, there was a stress by time interaction [$F(15, 405) = 1.79$, p = 0.034]. While LPS increased acetylcholine relative to baseline at every collection starting at the first collection after LPS injection (collection 5), prior RRS history significantly attenuated this effect relative to NSC rats, regardless of drug treatment, at collections 5 and 6 [$p < 0.05$] (Fig. 5, Panel A).

3.3. PB mimics stress to attenuate the cholinergic response to LPS in the hippocampus

Basal acetylcholine levels assessed on the first day of microdialysis indicate that there was no effect of either a prior history of PB or RRS on basal acetylcholine levels in the hippocampus [$F(1, 27) = 2.37$, $p = 0.13$; Fig. 5, Panel B inset]. There was a main effect of time such that LPS significantly increased hippocampal acetylcholine levels over time in all rats [$F(15, 390) = 7.52$, $p < 0.001$]. There was also an interaction between prior history of PB and RRS on the cholinergic response to LPS in the hippocampus [$F(1, 26) = 6.22$, $p = 0.019$]. In vehicle-treated rats, a prior stress history attenuated the cholinergic response to LPS relative to NSC rats at collection times 11 through 14 [$p = 0.031$]. In NSC rats, PB attenuated the cholinergic response relative to vehicle-treated rats at collections 11–14 [$p < 0.05$] (Fig. 5, Panel B).

3.4. PB Attenuates the cholinergic response to immobilization stress challenge in the PFC

Acute immobilization stress increased acetylcholine efflux in the PFC and there is an interaction between PB and RRS as well as stress and time on acetylcholine levels [$F(1, 23) = 4.53$, $p = 0.04$ and $F(11, 253) = 4.81$, $p < 0.001$], respectively. In rats with a prior history of restraint stress the cholinergic response to an immobilization stress challenge was decreased at all collections after baseline [$p < 0.05$], perhaps suggesting habituation to the effects of stress. In rats with no prior restraint stress history, there is an overall effect for PB to decrease the cholinergic response to immobilization stress [$p = 0.02$]. When examined across time, statistical analysis revealed that in NSC rats, PB decreases the cholinergic response to immobilization stress specifically at collections 9 and 10 [$p < 0.05$; (Fig. 6, Panel A)]. As shown in Table 2, basal levels of acetylcholine in PFC prior to immobilization stress did not differ from basal acetylcholine levels prior to LPS. These results indicate that LPS administration did not have any 'carry-over' effect on basal acetylcholine levels as measured 48 h later, and also that the prior microdialysis session did not significantly alter basal levels.

3.5. PB and RRS interact to interfere with habituation to an immobilization stress challenge in the hippocampus

One hour acute immobilization stress increased acetylcholine levels in hippocampus and there is an interaction between PB, RRS, and time on acetylcholine levels induced by immobilization [$F(11, 231) = 2.11$, $p = 0.02$]. Specifically, RRS decreased acetylcholine levels in response to immobilization stress at collections 8 and 11 in vehicle-treated rats [$p < 0.05$]. There was also a trend for rats with a prior restraint stress history to exhibit decreased acetylcholine levels at collections 5 and 6 [$p = 0.09$ and 0.06, respectively] at the initiation of immobilization stress. However, rats with a prior history of PB in conjunction to RRS failed to show this attenuated stress effect during immobilization

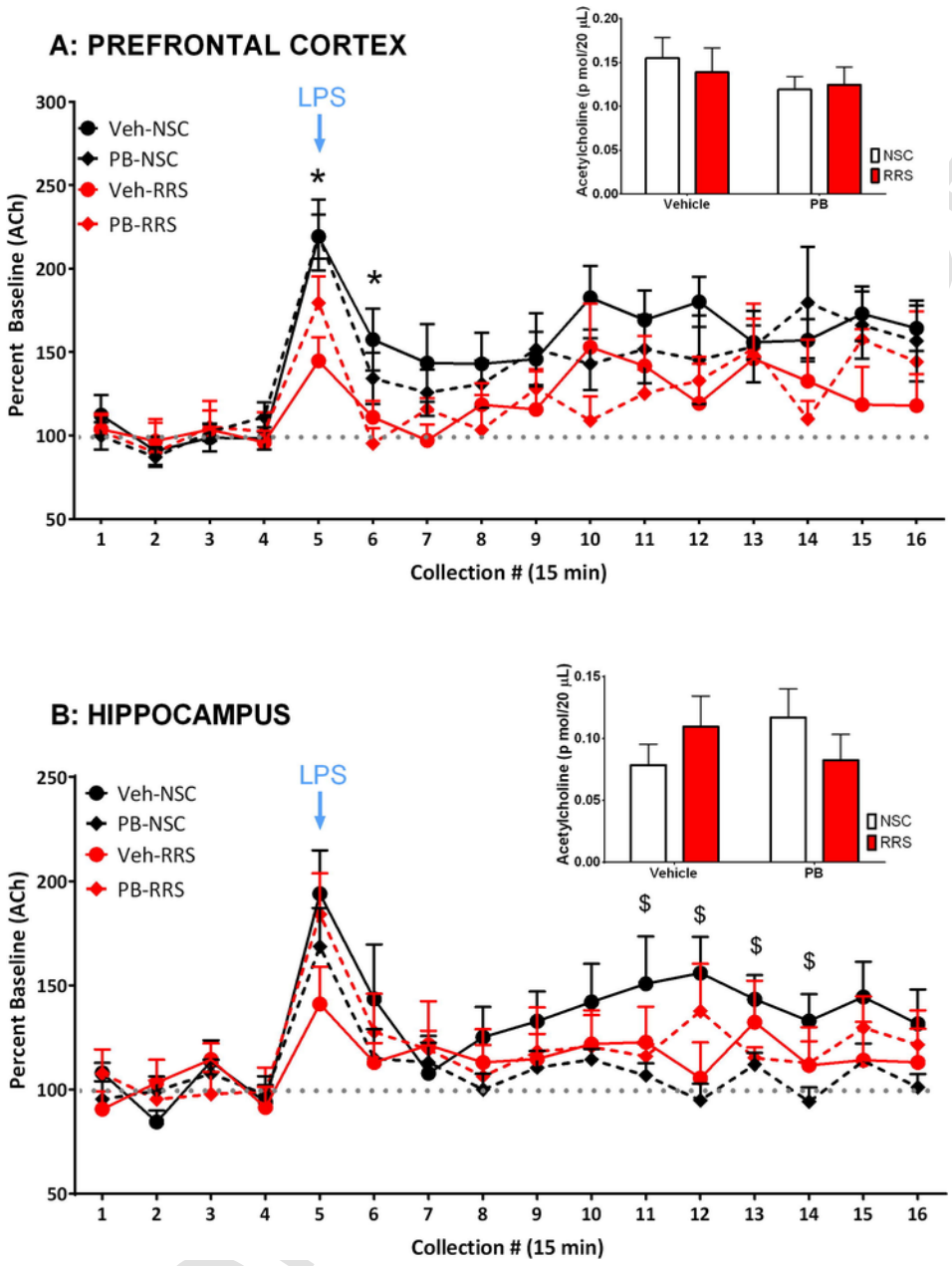

Fig. 5. Cholinergic Response to LPS. On the first day of microdialysis, prior to the LPS challenge, there was no effect of either PB or stress history on basal acetylcholine levels in the PFC (Panel A Inset) or hippocampus (Panel B Inset). Panel A. In rats without a prior restraint stress history, LPS increased acetylcholine levels over time with peak increases at collection 5 and a secondary, gradual increase starting an hour after the injection. In rats with a prior restraint stress history, the cholinergic response to LPS was significantly blunted relative to vehicle-NSC rats at collections 5 and 6. Panel B. Although LPS increased acetylcholine levels in the hippocampus in vehicle-NSC rats, either a prior history of PB or a prior history of stress attenuated this effect between collections 11–14. Rats which received PB but had no prior restraint stress history had a similar, attenuated cholinergic response to LPS as did rats with a prior restraint stress history. All data are expressed as mean ± SEM, n = 6–10. [*: Significant effect of repeated restraint stress in PB and vehicle-treated rats relative to vehicle-NSC rats, $p < 0.05$; $: Significant effect of PB in NSC rats, $p < 0.05$].

stress, exhibiting elevated levels relative to vehicle-treated counterparts at collection 5 [$p = 0.01$], and a trend to increase acetylcholine at collection 8 [$p = 0.08$] (Fig. 6, Panel B). Basal levels of acetylcholine in hippocampus prior to LPS treatment did not differ from basal acetylcholine levels prior to immobilization stress; see Table 2. These results indicate that LPS administration did not have a 'carry-over' effect on basal hippocampal acetylcholine levels as measured 48 h later.

3.6. PB and stress-induced deficits in cue fear conditioning

We previously reported that a history of stress significantly decreased contextually-conditioned freezing in rats, and that this effect is primarily driven by freezing in the PB-RRS rats (Macht et al., 2018). These data have been reformatted for the current manuscript and is presented here for comparison purposes only. For a more extensive representation of the data, please refer to (Macht et al., 2018). Freezing during acquisition of conditioned responses was similar in the four treatment groups (Fig. 7, Panel A). However, when returned to the context where shocks were administered, during the first half of the text (minutes 1–4), both Veh-RRS and PB-RRS rats exhibited significant decreases in the time spent freezing compared to NCS groups (Fig. 7, Panel B). Additionally, there was a specific RRS × PB interaction in contextually-conditioned freezing behavior in minutes 5–8 (second half of the test), with PB-RRS rats showing the lowest levels of freezing

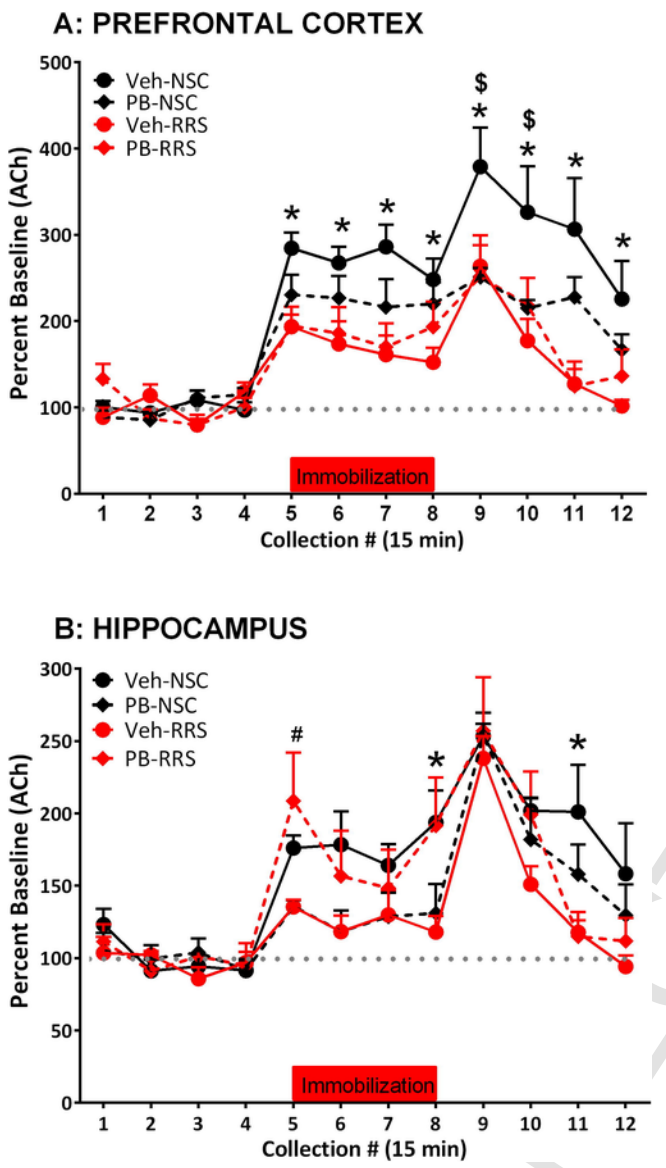

Fig. 6. Cholinergic Response to Immobilization Stress. Panel A. Either a prior history of restraint stress or PB attenuated the cholinergic response to an acute immobilization stress challenge. While a prior history of restraint stress decreased the cholinergic response to both immobilization and the release from immobilization, a prior history of PB in the absence of stress selectively decreased the cholinergic response to the release from restraint. This suggests that PB may influence cholinergic processing of the removal of a stressful stimulus. Panel B. Immobilization stress increased acetylcholine levels in the hippocampus in vehicle-NSC rats. Similar to results in the PFC, vehicle-treated rats with a prior history of restraint stress exhibit an attenuated cholinergic response to both the immobilization stress, as well as following relase immobilization stress within the hippocampus. However, PB blocks the habituation of the cholinergic response in repeated restraint stress conditions. All data are expressed as mean ± SEM, n = 6–9. [*: Significant effect of repeated restraint stress within vehicle-treated conditions, $p < 0.05$; $: Significant effect of PB in NSC rats, $p < 0.05$; #: Significant effect of PB within the repeated restraint stress condition, $p < 0.05$].

(Fig. 7, Panel C). Thus, repeated restraint stress produced deficits in contextual fear recall, and this was exacerbated by PB exposure in our model of GWI.

In the current study, we also report deficits in cue-conditioned freezing induced by a prior history of RRS or PB treatment (Fig. 7, Panel D). During the first three minutes, prior to cue presentation, there was no difference between groups in unconditioned freezing in response to the novel chamber [$p > 0.05$]. However, there is a significant interaction between RRS history, drug history, and time during

Table 2
Basal levels of ACh prior to LPS administration or immobilization stress challenge.

Group	Basal ACh prior to LPS Rx	Basal ACh prior to stress RX	P value
Prefrontal Cortex (pmols/20 ul)			
Veh-NSC	0.155 ± 0.02	0.095 ± 0.03	0.12
Veh-RRS	0.139 ± 0.03	0.081 ± 0.02	0.12
PB-NSC	0.1193 ± 0.02	0.136 ± 0.02	0.43
PB-RRS	0.125 ± 0.02	0.092 ± 0.02	0.28
Hippocampus (pmols/20 ul)			
Veh-NSC	0.078 ± 0.01	0.066 ± 0.01	0.60
Veh-RRS	0.109 ± 0.02	0.136 ± 0.03	0.50
PB-NSC	0.117 ± 0.02	0.135 ± 0.02	0.57
PB-RRS	0.082 ± 0.02	0.057 ± 0.02	0.40

conditioned cue-induced freezing [$F(16, 768) = 2.1$, $p = 0.001$]. Specifically RRS history decreased freezing in response to the 5th-8th tone presentation (minutes 8–11) in vehicle-treated rats [$p < 0.05$]. PB mimicked the effect of RRS and also decreased freezing behavior at the 5th and 6th tone (minutes 8–9) [$p < 0.05$]. As such, PB mimicked the effect of repeated restraint stress to produce deficits of cue-conditioned freezing (Fig. 7, Panel D).

4. Discussion

The mechanisms through which PB and stress interact to influence the CNShas remained a major point of controversy in the assessment of the underlying pathophysiology of GWI. The current study illustrates that PB treatment fundamentally alters inflammatory responses to subsequent immune challenges, which is consistent with the concept that inflammation is a core feature of GWI. Concommitant with these dysregulated immune responses is reflected in stimulus-evoked ACh release in the hippocampus and PFC of rats with a prior history of stress and PB treatment. These PB and stress-induced deficits in PFC and hippocampal cholinergic function parallel deficits in retention of contextual (Macht et al., 2018) and cue-based fear conditioning, suggesting that immune-mediated shifts in cholinergic function could be a potential mechanism underlying memory deficits in GWI.

4.1. PB and stress interact to produce brain-region specific deficits in cholinergic function and behavior

Altered cholinergic function following a history of PB and stress could have important consequences for the processing of stressful stimuli as the central cholinergic system is mechanistically linked to a variety of factors in the stress-response including processing of sensory stimuli (Inglis and Fibiger, 1995; Parikh et al., 2007), cortical processing of anxiety-responses to stress (Hart et al., 1999), Pavlovian fear-learning during acquisition, retrieval, and extinction (Wilson and Fadel, 2017) and cue-associated conditioning for both rewarding and non-rewarding stimuli (Acquas et al., 1998; Pirch et al., 1992). Our finding demonstrate that both hippocampus and PFC show an increase in acetylcholine efflux in response to an acute stress challenge, as well as an immune challenge. Further, a prior history of repeated restraint stress decreased the acetylcholine response to a novel stress challenge in both hippocampus and PFC, and these responses to LPS and novel stress were altered by prior PB exposure. These observations suggest that PB impairs cortical processing of novel stressful stimuli, which provides insight into the mechanisms underlying some of the executive functioning deficits in soldiers with GWI (Hubbard et al., 2014). In support of this, our study found that PB-NSC rats exhibit deficits in cholinergic processing to an immobilization stress challenge as well as deficits in retention of cue-based fear memories. Lesion studies have demonstrated that retention of contextual fear conditioning is depen-

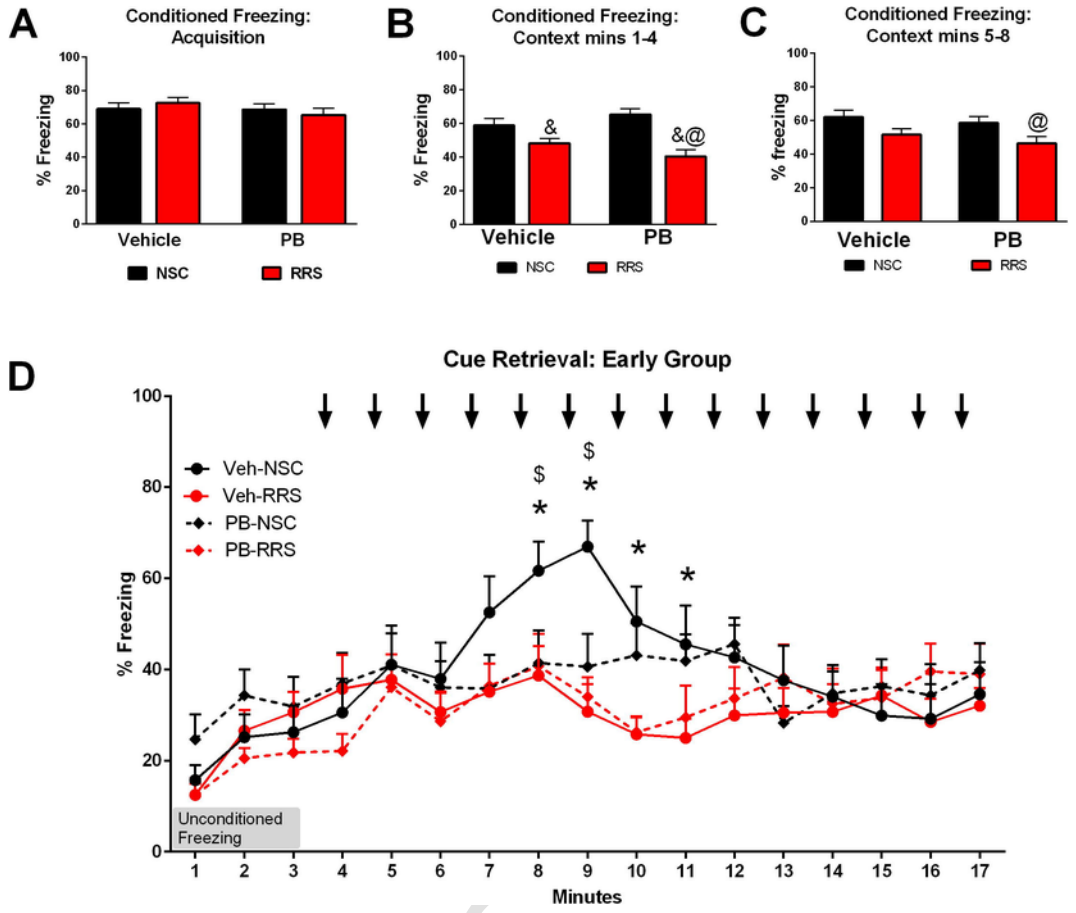

Fig. 7. Effects of PB and Stress on Retention of Context and Cued Fear Conditioning. Panel A. The acquisition of freezing behavior in response to the shocks was not altered in any of the treatment groups. Panel B. Veh-RRS and PB-RRS rats exhibited significant decreases in the time spent freezing during minutes 1 through 4 in the context. Panel C. Analysis of context freezing during minutes 5–8 min revealed a specific stress × PB interaction. The data in Panels A, B and C have been reformatted and are presented here for illustration purposes only. For a more detailed overview of the contextual freezing data, please refer to (Macht et al., 2018). Panel D. Neither a history of PB nor stress increased unconditioned freezing 48 h following acquisition of the fear response. Unconditioned freezing was evaluated as the percent time freezing during the first three minutes in the novel context, prior to the presentation of the first cue (tone). Either a history of PB or restraint stress decreased retention to the cue following conditioned freezing. This is divergent from effects on contextual fear conditioning, which demonstrate that PB exacerbates the effects of stress on retention of context (see Macht et al., 2018). Because each task relies on different brain regions, this suggests that PB and stress produce brain-region specific effects on memory-related tasks which parallel brain-region specific effects in cholinergic processing of stressful stimuli. All data are expressed as mean ± SEM, n = 13–14. [&: significantly different from Veh-NSC rats, $p < 0.05$. @: signicantly different from Veh-NSC rats, $p < 0.05$. *: Significant effect of repeated restraint stress relative to vehicle-NSC rats, $p < 0.05$. $: Significant effect of PB in NSC rats, $p < 0.05$].

dent on the dorsal hippocampus (Phillips and LeDoux, 1992), whereas retention of cue-based fear conditioning is preferentially dependent on the prefrontal cortex when the delay in retrieval is more than 24 h (Do-Monte et al., 2015). Although we assessed ACh neurochemistry and behavior in different cohorts of rats, our findings suggest that deficits in the cholinergic response in the PFC during stressful challenges may contribute to the cognitive deficits in PFC-dependent tasks. Consistent with this hypothesis, diminished prefrontal cholinergic transmission in animal models is associated with cognitive, including attentional, impairments (Parikh et al., 2007; Dalley et al., 2004).

In contrast, PB and stress interact synergistically to influence cholinergic processing of stressful stimuli in the hippocampus. This deficit in the habituation of the hippocampal cholinergic response to a novel stress challenge in PB-treated rats is mimicked by an exaggerated deficit in contextual-fear conditioning seen in the PB-stressed group (Macht et al., 2018). Activation of dorsal hippocampal cholinergic systems is integral to the consolidation of contextual fear conditioning but not cue-based fear conditioning (Wallenstein and Vago, 2001). Collectively, these data demonstrate that: 1) PB produces specific brain-region effects on the response of cholinergic systems; and 2) deficits in cholinergic function may underlie impairments in PFC and hippocampal-based conditioned memory tasks in our model of GWI.

While one possible explanation for these deficits in cholinergic neurochemical processing of inflammatory and stressful stimuli could be associated with a general deficit in responsiveness of environmental stimuli, it is important to note that PB and RRS rats acquire freezing behavior in response to shock-tone pairings at a level similar to vehicle-treated controls (Macht et al., 2018) and show similar levels of unconditioned freezing in a novel environment. As such, these results cannot be explained by a universal deficit in the behavioral response to environmental stimuli but rather a specific interaction between stress and inflammation on the central cholinergic system. In addition, variations of cholinergic processing of various stressful stimuli are unlikely to be explained by any compensatory changes in cholinesterase activity, as we have previously reported that at the time at which microdialysis occurred, there are no differences in peripheral cholinesterase activity in any group (Macht et al., 2018).

4.2. PB administration primes peripheral inflammatory responses to an immune challenge

Decreases in plasma AChE activity, as is observed following PB administration, would be expected to increase plasma ACh levels and thereby increase the effectiveness of the cholinergic anti-inflammatory

pathway in the periphery (Hoover, 2017). In support of this concept, our data demonstrate that LPS-induced increases in pro-inflammatory cytokines are suppressed in PB-treated rats compared to vehicle-treated rats. However, LPS-induced increases in plasma CRP levels are selectively observed in rats with a prior history of PB treatment despite the attenuated levels of IL-6 and TNF-α. CRP is pattern recognition molecule which is regulated by IL-6, and its expression is closely linked to the acute inflammatory response where it facilitates phagocytosis by macrophages (Black et al., 2004). As such, the current observations illustrate that a prior history of PB treatment predisposes individuals to exhibit an exacerbated inflammatory response to an immune stimulus that would otherwise not evoke an increase in CRP levels. More simply, PB enhances downstream immune responses to a sub-threshold dose of LPS. This aligns with clinical findings suggesting that elevations in CRP, among other inflammatory factors, is a significant predictor and potential biomarker of GWI (Johnson et al., 2016; Khaiboullina et al., 2015).

Potential interactions between the prior LPS challenge and subsequent immobilization challenge during microdialysis is an important caveat of the current study. Numerous studies have demonstrated that both stress and cytokines can activate common neuronal pathways (for a review see (Dunn, 2006) and that stress can prime a later immune response (Johnson et al., 2002). The cross-over between stress and inflammation is thought to occur primarily through microglia and IL-1 β (Murray and Lynch, 1998)). For example, hippocampal acetylcholine levels are significantly decreased for at least three hours by an intraperitoneal injection of 50 μg/kg IL-1β and transiently decreased for approximately one hour by an intraperitoneal injection of 20 μg/kg IL-1β (Rada et al., 1991). In this study, 7.5 μg/kg IL-1β was insufficient to elicit changes in hippocampal acetylcholine. In our current study, LPS did elicit increases in IL-1β, but these changes were in the picogram range which is below the threshold for the previous study, suggesting that even if LPS-induced plasma IL-1β in PB-treated rats persisted throughout the stress paradigm, these levels would be insufficient to elicit changes in the cholinergic response. Therefore, while it is possible that the prior LPS treatment primes the neurochemical response to the later immobilization challenge, contributing to the divergent effects between PB and vehicle-treated groups, this is unlikely to fully explain the robust stress-induced results of the current study. Future studies on GWI will also need to examine the brain-response to LPS to see if brain changes are either divergent or exaggerated relative to peripheral levels.

Beyond alterations in peripheral immune responses, inflammation also is proposed to contribute to the neurological complications of GWI (Georgopoulos et al., 2017). Prior studies have established that LPS administration or exposure to acute stressors increase neuroinflammation, including increased expression of pro-inflammatory cytokines such as IL-1β (Johnson et al., 2002; Nguyen et al., 1998). Interestingly, other studies have shown that centrally administered IL-1 β decreases hippocampal ACh levels (Taepavarapruk and Song, 2010) and the time course of this IL-1 β-induced suppression of hippocampal ACh levels is strikingly similar to the time course of LPS or stress-induced suppression of ACh levels described in the current study. Taken together, it is interesting to speculate that LPS treatment or exposure to a novel stressor elicits greater increases in IL-1β release in the hippocampus and PFC of rats with a prior history of PB exposure compared to vehicle controls. Under such conditions, PB-treated rats would exhibit attenuated cholinergic responsiveness, which would represent a potential neurochemical deficit that underlies the learning and memory impairments observed in our studies (Macht et al., 2018) and in previous preclinical GWI studies (Abdullah et al., 2011; Zakirova et al., 2015; Parihar et al., 2013; Hattiangady et al., 2014).

5. Conclusions

The current study is the first to use *in vivo* measurements to demonstrate that PB changes the response of the central cholinergic system to both stress and immune challenges. This demonstrates that regardless of whether PB crossed the blood-brain barrier, PB compromises the function of central cholinergic systems in a brain-region and stimulus-specific manner. Deficits in the ability of the cholinergic system to adequately respond to stressful and inflammatory stimuli may directly impact cognitive function, contributing to cognitive deficits evidenced in veterans with GWI. However, future studies will need to further elucidate the potential interaction between acetylcholine, inflammation, and cognitive dysfunction in GWI. The inability of the peripheral cholinergic system to adequately inhibit inflammation could make the brain vulnerable to secondary insults, contributing to the progressive nature of GWI in veterans, suggesting that treatment strategies may need to target mechanisms of negative feedback to inflammation in this population. Central cholinergic function is also implicated in age-related cognitive impairment, and as such it is possible that veterans with GWI could exhibit accelerated cognitive decline with age. As interactions between cholinergic and immune systems also change with age, examining potential cascading consequences of PB and stress in aging models in rodents is an important future direction for the field.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2019.04.015.

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