Psychoneuroendocrinology 96 (2018) 195–202

Contents lists available at ScienceDirect

Psychoneuroendocrinology

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

Pathophysiology in a model of Gulf War Illness: Contributions of pyridostigmine bromide and stress

V.A. Macht[a,b], J.L. Woodruff[a], C.A. Grillo[a], C.S. Wood[a], M.A. Wilson[a,b,c], L.P. Reagan[a,c,*]

[a] University of South Carolina School of Medicine, Department of Pharmacology, Physiology, and Neuroscience, Columbia, SC, USA
[b] University of South Carolina, Department of Psychology, Columbia, SC, USA
[c] Wm. Jennings Bryan Dorn VA Medical Center, Columbia, SC, USA

ARTICLE INFO

Keywords:
Cytokines
Restraint
Cognition
Acetylcholinesterase
Corticosterone
Fear conditioning

ABSTRACT

During the Gulf War, prophylactic treatment with pyridostigmine bromide (PB) along with the stress of deployment may have caused unexpected alterations in neural and immune function, resulting in a host of cognitive deficits which have become clinically termed Gulf War Illness (GWI). In order to test this interaction between PB and stress, the following study used a rodent model of GWI to examine how combinations of repeated restraint stress and PB induced alterations of peripheral cholinesterase (ChE) activity, corticosterone (CORT) levels, and cytokines on the last day of treatment, and then 10 days and three months post-treatment. Results indicate that PB decreases ChE activity acutely but sensitizes it by three months post-treatment selectively in rats subjected to stress. Similarly, while stress increased CORT levels acutely, rats in the PB/stressed condition continued to exhibit elevations in CORT at the delayed time point, indicating that PB and stress interact to progressively disrupt homeostasis in several peripheral measures. Because memory deficits are also common in clinical populations with GWI, we examined the effects of PB and stress on contextual fear conditioning. PB exacerbates stress-induced impairments in contextual fear conditioning ten days post-treatment, but protects against stress-induced augmentation of contextual fear conditioning at three months post-treatment. Collectively, these results provide critical insight as to how PB and stress may interact to contribute to the pathophysiological progression of GWI.

1. Introduction

The Gulf War was unusual in its use of pyridostigmine bromide (PB) as prophylactic treatment against toxicity from nerve gas agents. However, this prophylactic treatment along with the stress of deployment may have caused unexpected alterations in neural and immune function, resulting in a host of cognitive deficits which are a component of symptoms now clinically termed Gulf War Illness (GWI). Although GWI is considered a unique diagnosis for veterans deployed in the Gulf War, symptomology of GWI parallels symptoms of other conditions in civilian populations, including chronic fatigue syndrome (CFS), major depressive disorder (MDD), post-traumatic stress disorder (PTSD), and fibromyalgia. However, epidemiological studies have consistently demonstrated that the underlying physiology driving these symptoms is unique in GWI. For example, although both CFS and GWI exhibit altered immune functions, the cytokine profiles in both populations are distinct (Johnson et al., 2016; Khaiboullina et al., 2015; Parkitny et al., 2015; Smylie et al., 2013).

Endocrine profiles are also altered in veterans with GWI. Veterans with GWI exhibit exaggerated cortisol suppression in response to a low dose of dexamethasone relative to non-deployed veterans when PTSD, smoking, weight, and MDD are controlled for (Golier et al., 2006). Veterans with GWI also exhibit significant elevations in their cortisol to adrenocorticotropic hormone ratios, further emphasizing dysregulation of the hypothalamic-pituitary-adrenal axis in this population (Golier et al., 2007). Predictive computational models of GWI have suggested that 1) GWI is characterized by disruption of homeostatic states which consists of hypercortisolism and a shift towards a pro-inflammatory immune profile, and 2) persistence of clinical symptoms across the decades is perpetuated by disruption of these homeostatic systems (Craddock et al., 2014). One of the primary hypotheses of the computational model of GWI is that under normal physiological conditions, stimuli such as stress and drug exposure will make systems adapt to the event and then return to prior basal levels. However, when a disruption

* Corresponding author at: Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, 6439 Garners Ferry Road, D40, Columbia, SC, 29208, USA.
E-mail addresses: lawrence.reagan@uscmed.sc.edu, lawrence.reagan@va.gov (L.P. Reagan).

https://doi.org/10.1016/j.psyneuen.2018.07.015
Received 18 April 2018; Received in revised form 29 May 2018; Accepted 8 July 2018

is of significant duration and magnitude, the system assumes a new basal state and in some cases this new basal state can be maladaptive. In the case of GWI, a shift in the basal state of physiological parameters may underlie a variety of clinical symptoms evidenced in this population.

In view of these observations, the aim of the current study was to assess whether PB combined with stress caused shifts in physiological systems that are consistent with changes observed in veterans with GWI. We hypothesized that PB and stress would interact to disrupt homeostasis of a variety of physiological parameters – immune, endocrine, and behavioral. In addition, we hypothesized that the effects of PB and stress on these parameters would evolve over time. We therefore examined the effect of PB and stress on each of these measures at three separate time points: on the last day of treatment, 10 days following the cessation of treatment, and three months following the cessation of treatment. Thus, this study expands upon data from epidemiological and computational models by providing a mechanistic basis for the role of PB and stress in the pathophysiology of GWI. In addition, we examined the effects of PB and stress on a hippocampal-dependent task, contextual fear conditioning, to determine whether PB can accelerate stress-induced decline in hippocampal function. This hypothesis was based on several convergent findings in preclinical literature which suggest that stress-induced disruption of hippocampal structure and function is accelerated when combined with another condition which disrupts neuronal homeostasis (Grillo et al., 2005; Magarinos and McEwen, 2000; Reagan et al., 2008).

2. Material and methods

2.1. Animal housing and GWI paradigm

Adult male Sprague Dawley rats were individually housed at the University of South Carolina School of Medicine's animal facility in a temperature controlled facility (22 °C) with 12/12 h light-dark cycle with lights on at 7:00 a.m. and *ad libitum* access to food and water. Although both males and females have reported GWI, this study used males since the majority of soldiers and hence incidences of GWI were in men (Nettleman, 2015). All rats were randomly assigned to the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control, repeated restraint stress). Rats were gavaged in the morning with either 1.3 mg/kg PB or water (vehicle) from days 1–14 of treatment as this dose produces similar decreases in cholinesterase activity in rats as the dose-regimen used in soldiers (Marino et al., 1998). Drug treatment began prior to the onset of restraint stress as PB was administered to soldiers prior to and during deployment. On day 5 of treatment, rats began either repeated restraint stress (stressed) or non-stressed control conditions (NSC) (Fig. 1). Stressed rats were housed separately from NSC rats and placed in wire mesh restrainers immediately after gavage, at approximately 10 a.m. on treatment days 5–14. Restraint lasted six hours as this duration increases hippocampal vulnerability in a manner not observed with shorter daily stress durations (McLaughlin et al., 2007; Wilson et al., 2015). Rats were then further subdivided into either early or delayed conditions. Rats in the early condition underwent conditioned freezing on Days 20–21. Rats in the delayed condition went through conditioned freezing on days 100 and 101. All procedures for these experiments are in accord to all guidelines and regulations by the Dorn VA Medical Center Animal Care and Use Committee.

2.2. Plasma collection

Tail bleeds were performed on all animals 30 min following drug treatment and the start of restraint on day 14. Trunk blood was collected on ice in EDTA-treated tubes at the time of euthanasia for both the early (day 24) and delayed (day 104) time points (Fig. 1). Rats were anesthetized with isoflurane and then transcardially perfused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. All blood was collected on ice and then spun down for 15 min at 13,000 × *g*. Plasma supernatant was removed from each vial and frozen at −80 °C for further analysis. As such, plasma cholinesterase activity, corticosterone levels, and cytokines levels were assessed from plasma from tail bleeds (day 14) and trunk blood (days 24, 104).

2.3. Contextually-conditioned freezing

The conditioned freezing paradigm was performed as described previously (Grillo et al., 2011; Sharko et al., 2017). Rats were placed in a 46 × 24 × 22 cm acoustically isolated shock box and exposed to the testing box for 3 min to assess unconditioned freezing in the novel context. Rats were then given three 10 sec tones (2 KHz, 80 dB) that co-terminated with a 1 second, 1 mA shock with 1 minute inter-stimulus intervals. Twenty-four hours following acquisition of the fear response, rats were returned to the testing box in the absence of tones or shocks for a total of 8 min. The chamber was wiped clean with 5% ammonium hydroxide in between each rat's test period. All tests were recorded and analyzed using FreezeScan (Clever Systems, Inc). Freezing was defined as the absence of movement excluding respiration. FreezeScan is an automated software program which calculated freezing based on 300 ms frames. Percent of the time spent freezing over each 1 min was then binned. Accuracy of automated software was verified by a blind observer. Sample sizes were n = 13–14 per group.

2.4. Plasma cholinesterase analysis

A cholinesterase (ChE) activity assay collectively measuring

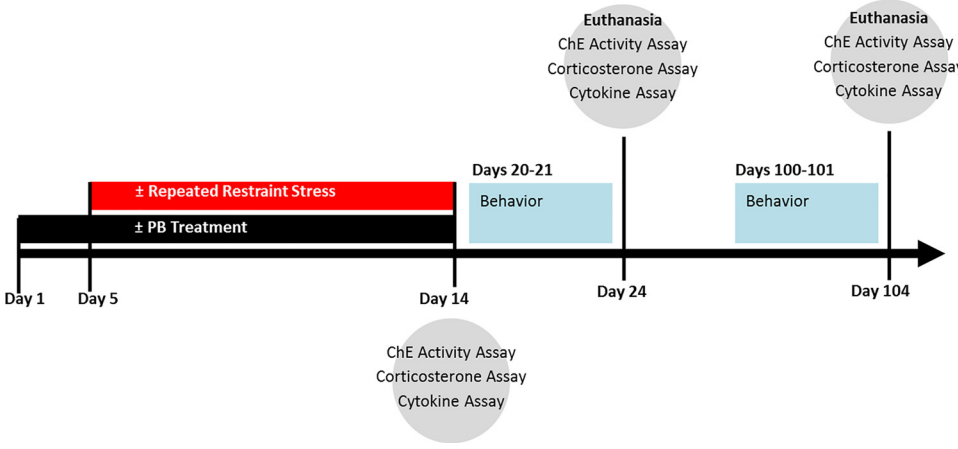

Fig. 1. Experimental Timeline. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control, repeated restraint stress). Thirty minutes following gavage on day 14, tail bleeds were performed to assess plasma levels of ChE activity, CORT, and levels of 12 different cytokines. Rats were then further subdivided into either early or delayed conditions. Rats in the early condition underwent conditioned freezing on Days 20-21. Rats in the delayed condition went through conditioned freezing on days 100 and 101. Plasma from the trunk blood isolated following euthanasia at the early and delayed time points was used for assessment of ChE activity, CORT and cytokines.

acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity was performed on plasma using the Abcam acetylcholinesterase assay kit (#ab138871) according to the manufacturer's instructions. Fifty μL of standards (mU/mL) and samples(diluted 1:100) were added to the 96-well plate in duplicates, followed by 50 μL of acetylthiocholine and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) reaction mixture. All samples were run in duplicate. The plate was read using a microplate reader at 410 nm absorbance. ChE activity from samples was interpolated from the linear standard curve. The mean cholinesterase activity across all assays for vehicle-NSC rats was 43.03 ± 2.30 mU/mL. Sample sizes were n = 5–9 per group.

2.5. Plasma corticosterone analysis

Plasma corticosterone (CORT) was assessed using a CORT ELISA kit from Enzo-Life Sciences (#ADI-900-097) according to the manufacturer's instructions and as described in our previous studies (Grillo et al., 2015). Samples were diluted 1:40 with steroid displacement reagent and kept on ice. All samples were run in duplicate. Standards ranged 20,000–32 pg/mL CORT and prepared using standard diluent. The plate was read using a microplate reader at 405 nm absorbance. CORT levels were interpolated from standards using a 4-parameter logistic curve. Sample sizes were n = 7–10 per group.

2.6. Plasma cytokine analysis

A cytokine assay kit from Bio-Rad (#171k1002M) was used according to the manufacturer's instructions to quantify 12cytokines in plasma as described previously (Grillo et al., 2014). The panel includes the following Th1/Th2 rat cytokines: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α. Eight four-fold dilution standards were prepared using the provided diluent and reconstituted standard, and samples were thawed and diluted 1:4 with diluent. All samples were run in duplicate. The plate was read on a plate reader using high photomultiplier voltage. Results were interpreted from a logarithmic standard curve. Sample sizes were n = 6–14 per group.

2.7. Statistical analyses

All data unless otherwise specified was analyzed using a 2 × 2 between-groups analysis of variance (ANOVA) with significance set at α = 0.05. For analysis of plasma ChE activity and plasma cytokine levels, data are expressed as a percentage of the vehicle-NSC rats due to assay to assay variability. Raw values for vehicle-NSC rats are presented in the Supplementary Tables. The between groups factors were drug treatment (i.e. vehicle, PB) and stress (i.e. NSC, repeated restraint stress). Day 14, early (day 24; 10 days post-treatment) and delayed (day 104; 3 months post-treatment) groups were analyzed separately as determined *a priori*. This type of analysis was selected as separate cohorts were used for early and delayed time points. Following significant interactions, simple effects follow-up tests were performed. All post-hoc tests used Bonferroni corrections for family-wise error. Contextually conditioned freezing was analyzed using a repeated measures ANOVA with the addition of the factor *time* in conjunction to the factors of *treatment* and *stress*. Following significant interactions, simple effects follow-up tests were performed with Bonferroni corrections for family-wise error.

3. Results

3.1. PB and stress acutely decrease contextually-conditioned freezing

Approximately one week after the cessation of drug and stress exposure, contextually-conditioned freezing was assessed (Fig. 2). During the first day (acquisition) of the contextually-conditioned freezing paradigm, neither stress nor PB treatment altered unconditioned

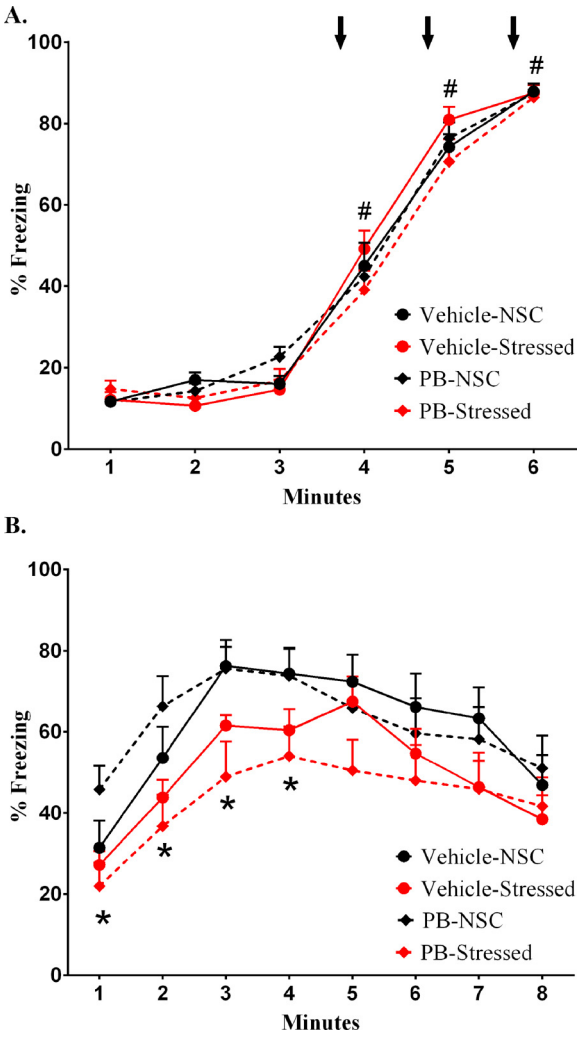

Fig. 2. Conditioned Freezing One Week Post Treatment. Panel A. There was no effect of any factor on acquisition of freezing behavior in response to three shocks. This suggests that neither stress nor PB impacts learning of fear memory at this time point. **Panel B**. Stressed rats froze less in the context where the shocks had occurred relative to NSC rats. Further analysis indicates that this effect is driven by the condition in which rats received PB and stress, suggesting that PB may exacerbate the effects of stress on consolidation and recall of fear memories. Values are expressed as group mean percent freezing ± SEM at each time point. [**Panel A**: #: significant effect of time on freezing behavior relative to previous time point, $p < 0.05$; arrows (↓): 1 s shock paired with a 10 s tone. **Panel B**: *: stressed rats significantly different from NSC rats, $p < 0.05$].

freezing (first 3 minutes) or the acquisition of freezing behavior during tone-shock pairings, $p > 0.05$. Freezing increased with the three tone-shock pairings, as expected ($F(5, 240) = 490.0$, $p < 0.0001$), but there was no difference between groups, $p > 0.05$. This suggests that all rats appropriately acquired the fear response. Twenty-four hours following acquisition of the fear response, rats were returned to the testing box in the absence of tones or shocks for a total of 8 minutes. Contextual freezing was significantly impacted by time ($F(7, 336) = 25.79$, $p < 0.001$). Freezing behavior increased incrementally over the first 3 minutes ($p < 0.05$). There was also a main effect of stress such that stress decreased contextually-conditioned freezing ($F(1, 48) = 7.34$, $p < 0.01$). Subsequent analyses determined that this effect is driven by PB-stressed rats which froze significantly less than the vehicle-NSC rats, $p < 0.05$.

At the delayed time point, neither stress nor PB treatment altered unconditioned freezing (first 3 minutes) or the acquisition of freezing during tone-shock pairings, demonstrating that all rats exhibited similar

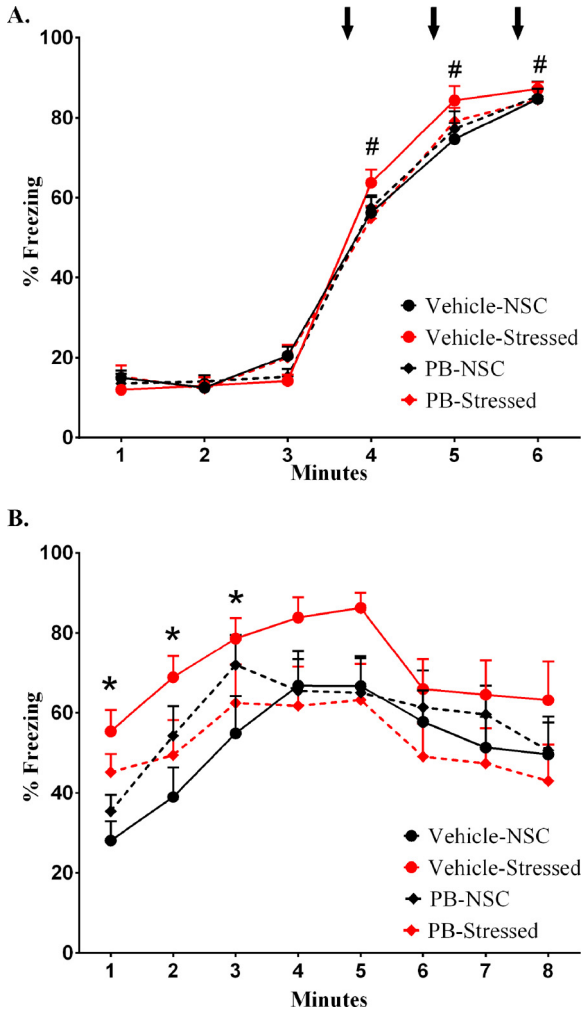

Fig. 3. Conditioned Freezing Three Months Post Treatment. **Panel A**. There was no effect of any factor on acquisition of freezing behavior in response to three shocks. This suggests that neither stress nor PB impacts learning of fear memory at this time point. **Panel B**. Rats with a prior stress history but no drug treatment froze more in the context where the shocks occurred, suggesting that a prior stress history sensitizes the fear response. Values are expressed as group mean percent freezing ± SEM at each time point. [**Panel A**: #: significant effect of time on freezing behavior relative to previous time point, $p < 0.05$; arrows (↓): 1 s shock paired with a 10 s tone. **Panel B**: *: vehicle-stressed rats significantly different from vehicle-NSC rats, $p < 0.05$].

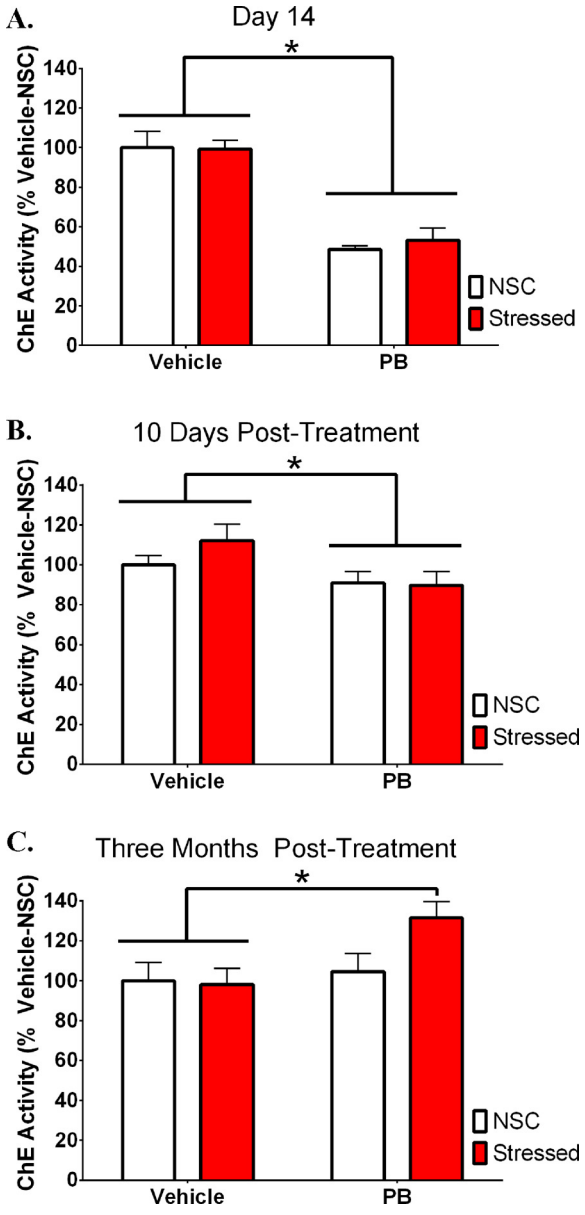

Fig. 4. Plasma Cholinesterase Activity. **Panel A**. PB treatment decreased plasma ChE activity by approximately 50% on the last day of drug treatment, which is proportional to pharmacological target values of soldiers. **Panel B.** PB treatment continued to decrease plasma ChE activity by approximately 15% ten days following the cessation of treatment. This suggests that while PB has a persistent effect, ChE activity following PB-treatment has begun to normalize. **Panel C**. Three months following the cessation of treatment, ChE activity is elevated by approximately 28% selectively in rats which were exposed to restraint stress in conjunction with PB. This suggests that PB and stress interact to produce long-term disruption in the metabolism of peripheral acetylcholine. All data are expressed as a percentage of vehicle-NSC rats. Values are expressed as mean ± SEM at each time point. [*: significant difference between designated groups, $p < 0.05$].

acquisition of the conditioned fear response (Fig. 3). Freezing increased with the three 3 tone-shock pairings ($F(5, 240) = 641.7$, $p < 0.0001$), but there was no difference between groups ($p > 0.05$). These data indicate that all rats showed similar acquisition of the conditioned fear response. Twenty-four hours later, rats were returned to the testing box in the absence of tones or shocks for a total of 8 minutes. Results indicate that time significantly affected contextual freezing within all groups ($F(7, 366) = 15.42$, $p < 0.0001$). Specifically, freezing increased incrementally from minutes 1–3 after which freezing behavior stabilized ($p < 0.05$). There was also a trend for a stress × time interaction ($F(7, 350) = 1.81$, $p = 0.08$), and a stress × drug interaction ($F(1, 50) = 3.82$, $p = 0.056$). Specifically, prior history of stress increased freezing during the first 3 minutes in vehicle-treated but not PB-treated rats, relative to vehicle-NSC rats ($p < 0.05$, see Fig. 3).

3.2. PB and stress interact to increase cholinesterase activity over time

PB decreased plasma ChE activity by approximately 50% relative to vehicle-treated controls on the last day of drug treatment (day 14), $F(1, 23) = 69.40$, $p < 0.001$ (Fig. 4). This reduction in ChE activity following PB administration aligns with the targeted ChE activity in soldiers who were administered PB, illustrating that the dose of PB administered accurately achieves the target plasma ChE activity in soldiers. Following euthanasia for the early cohort (10 days following the cessation of treatment), rats treated with PB continued to exhibit a significant, 15% decrease in plasma ChE activity relative to vehicle-treated controls ($F(1, 32) = 5.82$, $p = 0.02$). Conversely, for the delayed cohort (approximately three months following the cessation of PB

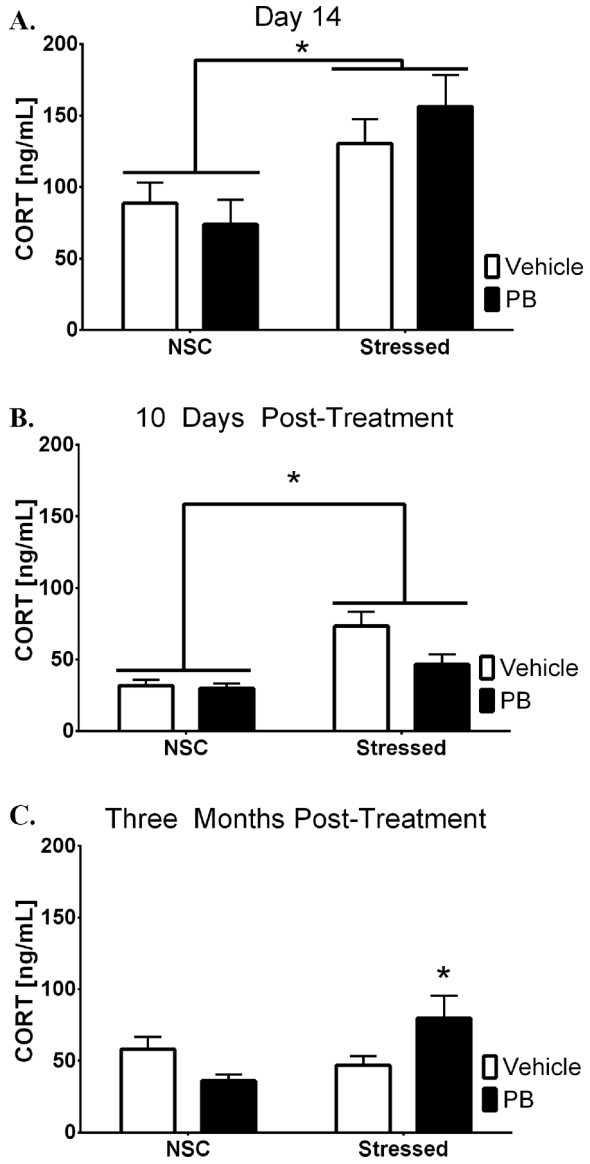

Fig. 5. Plasma Corticosterone. Panel A. During the first thirty minutes of restraint stress on the last day of the restraint stress paradigm, stressed rats have significantly higher levels of CORT than non-stressed rats. **Panel B**. Rats with a prior stress history have elevated CORT. This is ten days after the end of the restraint stress paradigm and 72 h following context-retrieval of conditioned freezing. **Panel C**. Three months following the cessation of treatment, CORT is elevated selectively in rats which were exposed to restraint stress in conjunction with PB relative to PB-NSC and vehicle-stressed counterparts. This is also 72 h following context-retrieval of conditioned freezing. Collectively, these data suggest that the effects of PB and stress on CORT change over time. Values are expressed as mean ± SEM at each time point. [*: significant difference between designated groups, p < 0.05].

treatment), there was a significant effect of PB on ChE activity ($F(1, 32) = 4.88$, $p = 0.04$). Specifically, PB-stressed rats exhibited significant increases in plasma ChE activity relative to PB-NSC and vehicle-treated controls ($p < 0.05$).

3.3. Stress and PB interact to increase plasma corticosterone over time

CORT was significantly elevated 30 min into restraint on the last day of treatment (day 14) in both vehicle and PB treated rats undergoing restraint stress relative to non-stressed controls ($F(1, 33) = 12.38$, $p < 0.01$; Fig. 5 **Panel A**). Interestingly, CORT remained above baseline in rats exposed to restraint stress regardless of drug treatment ten days following the cessation of the stress paradigm (early group; $F(1, 30) = 16.21$, $p < 0.01$; Fig. 5 **Panel B**). Three months following the cessation of treatment, there was an interaction between stress and drug treatment on plasma CORT levels ($F(1, 29) = 7.165$, $p = 0.01$; Fig. 5 **Panel C**). CORT was significantly elevated in rats with prior history of PB and repeated restraint stress relative to PB-NSC and vehicle-stressed rats, $p < 0.05$.

Table 1
Cytokine Levels Over Time

Cytokine	Vehicle-NSC	Vehicle-Stressed	PB-NSC	PB-Stressed
Last Day of PB/Stress Exposure (Day 14)				
IL-1α	100.0 ± 13.5	196.4 ± 24.3*	218.2 ± 39.7*	88.1 ± 19.6
IL-1β	100.0 ± 15.2	207.4 ± 34.8*	249.1 ± 49.0*	72.8 ± 14.9
IL-2	100.0 ± 15.3	163.6 ± 23.2*	173.9 ± 32.0*	63.3 ± 14.2
IL-4	100.0 ± 18.4	143.9 ± 22.6	125.8 ± 29.5	96.7 ± 3.3
IL-5	100.0 ± 15.4	192.0 ± 20.7*	209.3 ± 32.1*	83.4 ± 15.7
IL-6	100.0 ± 9.5	293.8 ± 85.4	493.8 ± 134.2*	124.5 ± 35.8
IL-10	100.0 ± 14.2	202.9 ± 38.3*	267.1 ± 59.8*	80.2 ± 17.0
IL-12	100.0 ± 21.3	238.9 ± 49.1*	284.0 ± 69.6*	72.3 ± 19.6
IL-13	100.0 ± 23.5	356.7 ± 82.5*	487.9 ± 122.6*	131.9 ± 40.8
TNF-α	100.0 ± 16.0	203.6 ± 49.9	265.6 ± 67.5*	91.3 ± 15.1
IFN-γ	100.0 ± 19.6	249.6 ± 48.9*	346.7 ± 84.5*	120.9 ± 33.3
GM-CSF	100.0 ± 22.2	261.8 ± 45.2*	303.6 ± 72.3*	87.3 ± 23.5
One Week Post-Treatment (Day 24)				
IL-1α	100.0 ± 7.9	99.3 ± 14.7	100.3 ± 8.0	94.2 ± 5.2
IL-1β	100.0 ± 10.1	94.0 ± 17.6	97.8 ± 10.2	94.9 ± 8.3
IL-2	100.0 ± 8.0	94.1 ± 13.7	93.8 ± 6.0	95.6 ± 5.1
IL-4	100.0 ± 12.2	94.3 ± 21.4	96.9 ± 9.9	91.9 ± 8.5
IL-5	100.0 ± 5.7	91.7 ± 11.5	95.3 ± 4.9	91.7 ± 4.9
IL-6	100.0 ± 11.4	101.6 ± 18.8	105.4 ± 10.5	91.0 ± 8.6
IL-10	100.0 ± 7.1	95.5 ± 12.0	94.4 ± 8.4	95.5 ± 5.3
IL-12	100.0 ± 9.9	94.1 ± 18.3	95.4 ± 9.6	91.5 ± 6.4
IL-13	100.0 ± 8.7	92.1 ± 17.0	95.8 ± 9.3	91.2 ± 7.3
TNF-α	100.0 ± 12.9	99.0 ± 20.3	104.3 ± 12.6	95.3 ± 9.9
IFN-γ	100.0 ± 12.6	99.0 ± 20.6	99.1 ± 11.8	84.1 ± 11.3
GM-CSF	100.0 ± 12.4	91.6 ± 18.3	92.6 ± 9.3	94.2 ± 6.8
Three Months Post-Treatment (Day 104)				
IL-1α	100.0 ± 14.3	85.9 ± 10.3	61.8 ± 9.2*	95.8 ± 14.6
IL-1β	100.0 ± 17.5	83.5 ± 10.8	60.0 ± 10.7	82.7 ± 11.8
IL-2	100.0 ± 13.6	79.7 ± 2.7	60.5 ± 10.2*	80.5 ± 8.4
IL-4	100.0 ± 16.1	77.3 ± 5.2	58.2 ± 11.5*	84.3 ± 11.0
IL-5	100.0 ± 8.0	90.9 ± 4.7	79.2 ± 6.5	95.1 ± 8.1
IL-6	100.0 ± 14.0	87.8 ± 10.0	56.5 ± 10.0*	96.0 ± 14.2
IL-10	100.0 ± 12.9	76.3 ± 3.7#	70.0 ± 8.6#	97.7 ± 14.5
IL-12	100.0 ± 16.4	79.6 ± 9.5	60.1 ± 11.5*	89.2 ± 13.4
IL-13	100.0 ± 16.0	83.9 ± 10.5	61.8 ± 10.2	81.6 ± 11.3
TNF-α	100.0 ± 17.5	75.0 ± 10.3	55.2 ± 10.1*	68.4 ± 7.0*
IFN-γ	100.0 ± 15.3	88.1 ± 11.5	59.0 ± 9.8*	99.0 ± 16.3
GM-CSF	100.0 ± 14.3	78.3 ± 9.9	63.4 ± 11.2	85.3 ± 15.4

* Values are significantly different from Vehicle-NSC.
\# Significant cross-over interaction.

3.4. PB produces inverse effects on plasma cytokines over time

On day 14, approximately 30 min into the restraint stress session, there was a significant interaction between PB and stress on levels of interleukin **(IL)-1α** [$F(1, 30) = 5.51$, $p = 0.03$], **IL-1β** [$F(1, 30) = 6.92$, $p = 0.01$], **IL-2** [$F(1, 30) = 4.37$, $p = 0.04$], **IL-5** [$F(1, 30) = 6.23$, $p = 0.02$], **IL-6** [$F(1, 30) = 4.92$, $p = 0.03$], **IL-10** [$F(1, 30) = 5.75$, $p = 0.02$], **IL-12** [$F(1, 30) = 5.41$, $p = 0.03$], **IL-13** [$F(1, 30) = 6.19$, $p = 0.02$], **GM-CSF** [$F(1, 30) = 6.57$, $p = 0.02$], **IFN-γ** [$F(1, 30) = 4.69$, $p = 0.04$], and **TNF-α** [$F(1, 30) = 5.33$, $p = 0.03$] (Table 1). The only cytokine not impacted by either stress or drug treatment was IL-4, $p > 0.05$. Follow-up ANOVAs revealed that relative to vehicle-NSC rats, both PB-NSC and vehicle-stressed rats exhibit increased plasma levels of IL-1α, IL-1β, IL-2, IL-5, IL-10, IL-12, IL-13, GM-CSF, and IFN-γ, $p < 0.05$. However, only PB-NSC rats exhibited significant elevations in IL-6 and TNF-α, $p < 0.05$.

Ten days following the cessation of treatment, there was no effect of either PB or stress on any plasma cytokine ($p > 0.05$; Table 1). This suggests that shortly after the cessation of PB and stress exposure, effects on plasma cytokines have normalized at basal conditions. However, three months following the cessation of PB and stress exposure, a different picture emerges (Table 1). At this delayed time point, PB and stress interact to affect plasma levels of **IL-1α** [$F(1, 22) = 5.09$, $p = 0.03$], **IL-2** [$F(1, 22) = 4.47$, $p = 0.046$], **IL-4** [$F(1, 22) = 4.42$, $p = 0.047$], **IL-6** [$F(1, 22) = 5.70$, $p = 0.03$], and **IL-10** [$F(1, 22) = 4.88$, $p = 0.04$],], **IL-12** [$F(1, 22) = 4.34$, $p = 0.049$], and **IFN-γ** [$F(1, 22) = 4.87$, $p = 0.04$]. Specifically, PB-NSC rats exhibit a decrease in IL-1 α, IL-2, IL-4, IL-6, IL-12, and IFN- γ relative to vehicle-NSC rats, $p < 0.05$. There is a crossover interaction between PB and stress on levels of IL-10, where PB-NSC and vehicle-stressed rats exhibit a decrease in these cytokines relative to vehicle-NSC and PB-stressed rats, $p < 0.05$. In addition, there is a main effect of PB on TNF-α such that a prior history of PB regardless of stress history decreases plasma levels of **TNF-α** [$F(1, 22) = 5.40$, $p = 0.03$].

4. Discussion

These data demonstrate that PB and stress interact over time to affect peripheral endocrine, cholinergic, and immune systems, as well as contextually conditioned freezing. This study supports two important conclusions: 1) time is a crucial factor to consider when regarding physiological and behavioral effects in response to stress and PB; and 2) the peripheral physiological effects and behavioral effects do not always progress in parallel. By identifying the progression of these effects over time in a rodent model of Gulf War Illness, these data provide critical insight as to how stress and PB may have interacted to produce some of the well-characterized physiological and behavioral symptoms in veterans with GWI.

4.1. PB disrupts homeostasis of cholinergic systems in a model of GWI

The acute effects of PB and stress on plasma ChE and plasma CORT are consistent with clinical and preclinical literature. As a ChE inhibitor, PB decreases ChE activity when measured on the last day of drug treatment (Fig. 4, **Panel A**). Similarly, rats undergoing restraint stress exhibit an increase in adrenal release of CORT levels relative to non-stressed rats (Fig. 5, **Panel A**). In contrast, there was a selective interaction between PB and stress on both CORT and ChE activity at the delayed time point (Fig. 4, **Panel C**; Fig. 5, **Panel C**). Only rats which received PB and were concurrently exposed to repeated restraint stress demonstrated elevations in CORT and ChE relative to controls three months following treatment-cessation. This provides important evidence that stress and PB interact to disrupt homeostasis of physiological systems and that these physiological changes emerge slowly over time.

Although to our knowledge no human clinical study has directly assessed plasma AChE levels in GWI, BChE levels have been examined. Even though BChE is not the primary enzyme which metabolizes acetylcholine, individuals with a genotype for low-functioning BChE are at a significantly greater risk for developing GWI if they were administered PB (Steele et al., 2015). However, there was no difference in BChE activity between veterans with GWI and veteran controls when collapsed across genotype. While our results determined that prior exposure to stress and PB increase ChE activity over time, which does not differentiate between these two enzymes, it is possible that this effect is driven by changes in AChE and not BChE. As AChE is the primary enzyme which metabolizes acetylcholine, changes in AChE activity would impact acetylcholine turnover in both the parasympathetic nervous system and the neuromuscular junction (Macintosh, 1941). Further studies will need to be performed to see if an increase in plasma ChE activity corresponds to changes in acetylcholine levels in the periphery, although it is tempting to speculate that increases in ChE activity are associated with decreases in basal acetylcholine levels. Decreased acetylcholine levels paralleled by increased acetylcholine metabolism could explain some of the symptoms in GWI such as muscular fatigue and respiratory dysfunction.

4.2. PB and stress interact to increase corticosterone in a model of GWI

Our study indicates that PB and stress interact to cause similar perturbations in glucocorticoid levels to those evidenced in human clinical populations (Golier et al., 2007, 2006). These elevations of glucocorticoids could contribute to some of the neurological changes in our model of GWI which may in turn contribute to the cognitive dysfunction – particularly learning and memory impairments. Glucocorticoids easily cross the blood-brain barrier, and as the hippocampus robustly expresses glucocorticoid receptors (McEwen et al., 1979), chronic elevations of cortisol/CORT have profound effects on this brain region. Elevations of cortisol in other diseases such as recurrent major depression (Bremner et al., 2000) and Cushing's syndrome (Starkman et al., 1992) are associated with decreases in hippocampal volume and function on learning and memory tests. Veterans with suspected exposure to low-doses of Sarin following the weapons demolition at Khamisiyah also have significantly reduced gray matter and hippocampal volume relative to non-exposed veteran peers (Chao et al., 2010). Animal models exposed to a combination of PB as well as the insecticides *N,N*-Diethyl-meta-toluamide (DEET) and permethrin in combination with mild chronic stress over the course of 4 weeks also exhibit reduced hippocampal volume along with decreases in neurogenesis and inflammation (Parihar et al., 2013). Our results provide evidence that PB in the absence of other chemical exposures is sufficient to contribute to endocrine disruption, but future studies will need to verify if PB in combination with stress is also sufficient to produce chronic decreases in hippocampal volume.

4.3. PB suppresses the immune system in a model of GWI

In the search for biomarkers for GWI, shifts in immune activation have been the most extensively investigated. One study suggested that the most important cytokines for distinguishing GWI from diseases with similar symptoms are IL-7, IL-4, TNF-α, IL-13, and IL-17F (Khaiboullina et al., 2015); in this study, IL-4 and TNF- α were down-regulated in GWI relative to controls. In our study, PB decreased both IL-4 and TNF-α at the delayed time point (Table 1), suggesting that PB may contribute to shifts in cytokine profiles evidenced in veterans. Under normal regulatory conditions, increases in IL-4 expression can provide feedback to attenuate the TNF-α response, but our model suggests that both IL-4 and TNF-α are suppressed several months after PB treatment. This could suggest that PB dysregulates homeostasis of the immune system by potentially disrupting negative-feedback signals, rendering it unable to respond appropriately to threats. Clinical data support this hypothesis as veterans with GWI exhibit an impaired coordination of endocrine and immune signaling following a mild exercise challenge, in part evidenced through increased stimulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Broderick et al., 2011; Broderick et al., 2011, 2013). Other studies have confirmed these findings, indicating that male veterans with GWI can be differentiated from healthy males based on their cytokine profiles at rest and following recovery from an exercise challenge, but not during the peak of the challenge (Smylie et al., 2013). This suggests that it is the ability of the immune system to recover from the challenge, not the peak immune response, which is impaired in veterans with GWI. Although the cytokine profiles in the current study were under non-stimulated conditions, future studies should examine whether PB and stress produce similar deficits in the Th1:Th2 coordinated response to a state of challenge.

Reduced levels of IL-4 and TNF-α may also contribute to some of the clinical symptoms which characterize GWI. Low levels of IL-4 are significantly correlated to chronic pain in some clinical studies (Üçeyler et al., 2006), and preclinical studies have shown that administration of

IL-4 decreases hyperalgesia evoked by painful stimuli (Cunha et al., 1999). However, understanding the intricacies of cytokine-cytokine interactions, coordination of appropriate immune responses, and associated physiological symptoms is extremely complex as these systems act in concert, not in isolation. Further clinical and preclinical studies are necessary to elaborate on these emerging findings.

4.4. PB exacerbates stress-induced short-term deficits in contextual fear conditioning

Although veterans with GWI exhibit a host of memory problems which have persisted over several decades, our model demonstrated only transient deficits in contextual fear conditioning following PB and stress. One week following the cessation of treatment, rats with a prior history of restraint stress exhibited deficits in contextual-fear conditioning, and this effect was exacerbated by PB, suggesting that PB and stress interact to impair fear memory (Fig. 2, **Panel B**). The hippocampus is critically involved in contextual fear conditioning, and rats with hippocampal lesions show severe deficits in the acquisition (Phillips and LeDoux, 1992) as well as the recall of contextual fear memory (Kim and Jung, 2006). One possible mechanism contributing to the stress-induced deficits in contextual fear conditioning is that elevations in CORT at 1-week post-treatment contributed to this memory deficit. Alternatively, fear conditioning may have prompted the persistent increase in CORT evidenced following euthanasia, thereby contributing to impairments in consolidation and retrieval of the contextual fear memory.

CORT levels were also increased selectively in the PB-stressed rats at three months post-treatment (Fig. 3, **Panel B**). Either 1) CORT differentially contributes to fear conditioning at these time points; or 2) by the delayed time point the homeostatic balance between multiple systems have been sufficiently disrupted to complicate these interactions. However, veterans returning from the Gulf War did not return to stress-free conditions. Therefore, an important consideration is whether intermittent recurring stressors during that three-month post-treatment stretch would have exacerbated effects in PB conditions.

Impairments in fear conditioning following combinations of PB and stress one week after the end of treatment have important clinical implications, particularly regarding PTSD rates in Gulf War veterans. PTSD is undoubtedly a prevalent condition in veterans from the Gulf War with higher rates in deployed versus non-deployed veterans (Ikin et al., 2004; Perconte et al., 1993). However, when Gulf War veterans are compared with other actively deployed veterans, prevalence of PTSD is reduced, suggesting that stress from active deployment may be a greater risk factor for PTSD than specific Gulf War conditions (Ikin et al., 2004). Another important consideration is that the prevalence of PTSD in Gulf War veterans changes over time. Immediately following the Gulf War, several studies suggested low rates of PTSD with estimates between 2% and 6% (Perconte et al., 1993; Wolfe et al., 1999). Two years after the war, PTSD rates doubled (Wolfe et al., 1999). Similarly, in the current study, we saw an increased risk for contextual fear conditioning three months following the cessation of treatment selectively in rats which were stressed but did not receive PB. This suggests that repeated psychological stress may exert some latent effects on fear conditioning, possibly explaining in part some of the increases in prevalence in PTSD in the years following the return from war. Rates of conditioned freezing were not increased in the PB-stressed rats or PB-NSC rats relative to vehicle-NSC rats, suggesting that PB in the presence of stress may mitigate this latent effect on fear conditioning. Alternatively, these data could indicate that continued post-deployment stress is an important mediator in the progression of GWI.

5. Conclusions

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

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

Conflict of interest

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 stated funding agencies. The authors have no conflicts of interest to declare.

Acknowledgements

This work was supported by the Department of Veterans Affairs grant numbers I21 BX002085 (LPR), IO1 BX001804 (LPR), I01 BX001374 (MAW), the University of South Carolina School of Medicine Research Development Fund (LPR), and the National Science Foundation grant number IOS-1656626 (CAG).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2018.07.015.

References

Bremner, J.D., Narayan, M., Anderson, E.R., Staib, L.H., Miller, H.L., Charney, D.S., 2000. Hippocampal volume reduction in major depression. Am. J. Psychiatry 157, 115–118. https://doi.org/10.1176/ajp.157.1.115.

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

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

Chao, L.L., Rothlind, J.C., Cardenas, V.A., Meyerhoff, D.J., Weiner, M.W., 2010. Effects of low-level exposure to sarin and cyclosarin during the 1991 Gulf War on brain function and brain structure in US veterans. Neurotoxicology 31, 493–501. https://doi.org/10.1016/j.neuro.2010.05.006.

Craddock, T.J.A., Fritsch, P., Rice, M.A., Del Rosario, R.M., Miller, D.B., Fletcher, M.A., Klimas, N.G., Broderick, G., 2014. A role for homeostatic drive in the perpetuation of complex chronic illness: Gulf War illness and chronic fatigue syndrome. PLoS One 9. https://doi.org/10.1371/journal.pone.0084839.

Cunha, F.Q., Poole, S., Lorenzetti, B.B., Veiga, F.H., Ferreira, S.H., 1999. Cytokine-mediated inflammatory hyperalgesia limited by interleukin-4. Br. J. Pharmacol. 126, 45–50. https://doi.org/10.1038/sj.bjp.0702266.

Golier, J., Schmeidler, J., Legge, J., Yehuda, R., 2006. Enhanced cortisol suppression to dexamethasone associated with Gulf War deployment. Psychoneuroendocrinology 31, 1181–1189.

Golier, J., Schmeidler, J., Legge, J., Yehuda, R., 2007. Twenty-four hour plasma cortisol and adrenocorticotropic hormone in Gulf War veterans: relationships to posttraumatic stress disorder and health symptoms. Biol. Psychiatry 62, 1175–1178.

Grillo, C.A., Piroli, G.G., Wood, G.E., Reznikov, L.R., McEwen, B.S., Reagan, L.P., 2005. Immunocytochemical analysis of synaptic proteins provides new insights into diabetes-mediated plasticity in the rat hippocampus. Neuroscience 136, 477–486. https://doi.org/10.1016/j.neuroscience.2005.08.019.

Grillo, C.A., Piroli, G.G., Junor, L., Wilson, S.P., Mott, D.D., Wilson, Ma., Reagan, L.P.,

2011. Obesity/hyperleptinemic phenotype impairs structural and functional plasticity in the rat hippocampus. Physiol. Behav. 105, 138–144. https://doi.org/10.1016/j.physbeh.2011.02.028.

Grillo, C.A., Mulder, P., Macht, V.A., Kaigler, K.F., Wilson, S.P., Wilson, M.A., Reagan, L.P., 2014. Dietary restriction reverses obesity-induced anhedonia. Physiol. Behav. 128. https://doi.org/10.1016/j.physbeh.2014.01.026.

Grillo, C.A., Risher, M., Bumgardner, A.L., Hang, A., Gabriel, C., Mocaër, E., Piroli, G.G., Fadel, J.R., Reagan, L.P., 2015. Repeated restraint stress-induced atrophy of glutamatergic pyramidal neurons and decreases in glutamatergic efflux in the rat amygdala are prevented by the antidepressant agomelatine. Neuroscience 284, 430–443.

Ikin, J.F., Sim, M.R., Creamer, M.C., Forbes, A.B., McKenzie, D.P., Kelsall, H.L., Glass, D.C., McFarlane, A.C., Abramson, M.J., Ittak, P., Dwyer, T., Blizzard, L., Delaney, K.R., Horsley, K.W.A., Harrex, W.K., Schwarz, H., 2004. War-related psychological stressors and risk of psychological disorders in Australian veterans of the 1991 Gulf War. Br. J. Psychiatry 185, 116–126. https://doi.org/10.1192/bjp.185.2.116.

Johnson, G.J., Slater, B.C.S., Leis, L.A., Rector, T.S., Bach, R.R., 2016. Blood biomarkers of chronic inflammation in Gulf War illness. PLoS One 11, e0157855. https://doi.org/10.1371/journal.pone.0157855.

Khaiboullina, S.F., DeMeirleir, K.L., Rawat, S., Berk, G.S., Gaynor-Berk, R.S., Mijatovic, T., Blatt, N., Rizvanov, A.A., Young, S.G., Lombardi, V.C., 2015. Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis. Cytokine 72, 1–8. https://doi.org/10.1016/j.cyto.2014.11.019.

Kim, J.J., Jung, M.W., 2006. Neural circuits and mechanisms involved in Pavlovian fear conditioning: a critical review. Neurosci. Biobehav. Rev. 30, 188–202. https://doi.org/10.1016/j.neubiorev.2005.06.005.

Macintosh, F.C., 1941. The distribution of acetylcholine in the peripheral and the central nervous system. J. Physiol. 99, 436–442. https://doi.org/10.1113/jphysiol.1941.sp003913.

Magarinos, A.M., McEwen, B.S., 2000. Experimental diabetes in rats causes hippocampal dendritic and synaptic reorganization and increased glucocorticoid reactivity to stress. Proc. Natl. Acad. Sci. U. S. A. 97, 11056–11061. https://doi.org/10.1073/pnas.97.20.11056.

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., Davis, U.G., Parsons, B., Pfaff, D.W., 1979. The brain as a target for steroid hormone action. Annu. Rev. Neurosci. 2, 65–112.

McLaughlin, K.J., Gomez, J.L., Baran, S.E., Conrad, C.D., 2007. The effects of chronic stress on hippocampal morphology and function: an evaluation of chronic restraint paradigms. Brain Res. 1161, 56–64. https://doi.org/10.1016/J.BRAINRES.2007.05.042.

Nettleman, M., 2015. Gulf war illness: challenges persist. Trans. Am. Clin. Climatol. Assoc. 126, 237.

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. https://doi.org/10.1038/npp.2013.158.

Parkitny, L., Middleton, S., Baker, K., Younger, J., 2015. Evidence for abnormal cytokine expression in Gulf War illness: a preliminary analysis of daily immune monitoring data. BMC Immunol. 16, 57. https://doi.org/10.1186/s12865-015-0122-z.

Perconte, S.T., Wilson, A.T., Pontius, E.B., Dietrick, A.L., Spiro, K.J., 1993. Psychological and war stress symptoms among deployed and non-deployed reservists following the Persian Gulf War. Mil. Med. 158, 516–521.

Phillips, R.G., LeDoux, J.E., 1992. Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behav. Neurosci. 106, 274–285.

Reagan, L.P., Grillo, C.A., Piroli, G.G., 2008. The As and Ds of stress: metabolic, morphological and behavioral consequences. Eur. J. Pharmacol. 585, 64–75. https://doi.org/10.1016/J.EJPHAR.2008.02.050.

Sharko, A.C., Fadel, J.R., Kaigler, K.F., Wilson, M.A., 2017. Activation of orexin/hypocretin neurons is associated with individual differences in cued fear extinction. Physiol. Behav. 178, 93–102. https://doi.org/10.1016/J.PHYSBEH.2016.10.008.

Smylie, A.L., Broderick, G., Fernandes, H., Razdan, S., Barnes, Z., Collado, F., Sol, C., Fletcher, M.A., Klimas, N., 2013. A comparison of sex-specific immune signatures in Gulf War illness and chronic fatigue syndrome. BMC Immunol. 14, 29. https://doi.org/10.1186/1471-2172-14-29.

Starkman, M.N., Gebarski, S.S., Berent, S., Schteingart, D.E., 1992. Hippocampal formation volume, memory dysfunction, and cortisoi levels in patients with cushing's syndrome. Biol. Psychiatry 32, 756–765.

Steele, L., Lockridge, O., Gerkovich, M.M., Cook, M.R., Sastre, A., 2015. Butyrylcholinesterase genotype and enzyme activity in relation to Gulf War illness: preliminary evidence of gene-exposure interaction from a case-control study of 1991 Gulf War veterans. Environ. Health 14, 4. https://doi.org/10.1186/1476-069X-14-4.

Üçeyler, N., Valenza, R., Stock, M., Schedel, R., Sprotte, G., Sommer, C., 2006. Reduced levels of antiinflammatory cytokines in patients with chronic widespread pain. Arthritis Rheum. 54, 2656–2664. https://doi.org/10.1002/art.22026.

Wilson, M.A., Grillo, C.A., Fadel, J.R., Reagan, L.P., 2015. Stress as a one-armed bandit: differential effects of stress paradigms on the morphology, neurochemistry and behavior in the rodent amygdala. Neurobiol. Stress 1, 195–208. https://doi.org/10.1016/j.ynstr.2015.06.001.

Wolfe, J., Erickson, D.J., Sharkansky, E.J., King, D.W., King, L.A., 1999. Course and predictors of posttraumatic stress disorder among Gulf War veterans: a prospective analysis. J. Consult. Clin. Psychol. 67, 520–528.