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

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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

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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

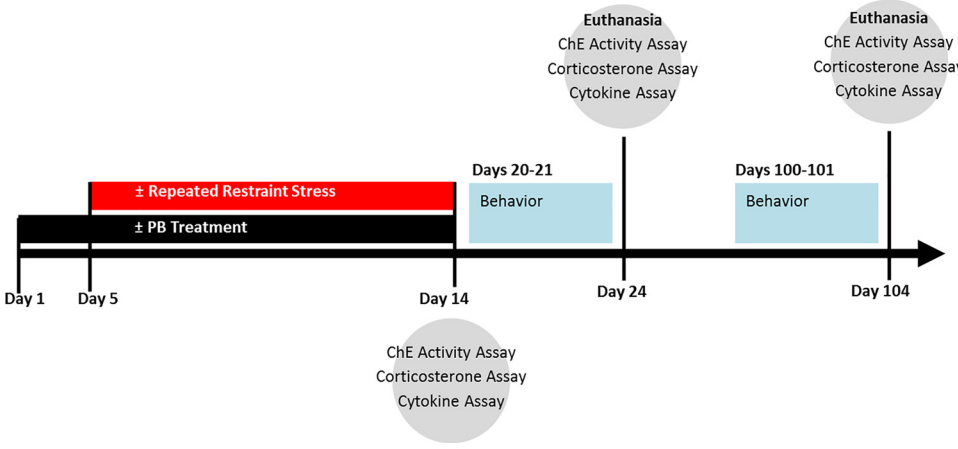

Fig. 1. Experimental Timeline. All rats underwent the GWI paradigm with 2 levels of drug treatment (vehicle, PB) and 2 levels of stress (non-stressed control, repeated restraint stress). Thirty minutes following gavage on day 14, tail bleeds were performed to assess plasma levels of ChE activity, CORT, 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.