# Interactions between pyridostigmine bromide and stress on glutamatergic neurochemistry: Insights from a rat model of Gulf War Illness

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## ABSTRACT

Pyridostigmine bromide (PB) was administered to soldiers during the first Gulf War as a prophylactic treatment to protect against toxicity in the event of exposure to nerve agents. Although originally thought to pose minimal risk to soldiers, epidemiological studies have since correlated PB administration with the development of a variety of symptoms, including cognitive dysfunction, termed Gulf War Illness (GWI). We previously demonstrated in a rodent model of GWI that central cholinergic responses were altered to various stimuli. In the current study we used *in vivo* microdialysis to examine how combinations of PB and repeated restraint stress (RRS) altered extracellular glutamate levels in response to an innate immune challenge (lipopolysaccharide; LPS) and an immobilization stress challenge in the prefrontal cortex (PFC) and hippocampus. There were four groups in this study: vehicle non-stressed control (Veh-NSC), vehicle-stressed (Veh-RRS), PB-NSC, and PB-RRS. While LPS decreased glutamate levels in PB-treated rats relative to vehicle-treated rats in the PFC, PB and stress interacted to attenuate LPS-induced decreases in hippocampal glutamate levels. Although immobilization stress increased glutamate in the PFC, glutamate levels in PB-NSC rats failed to recover in the post-stress period relative to vehicle-treated rats. In the hippocampus, PB-stressed rats failed to exhibit habituation of the glutamate response to immobilization stress relative to vehicle-stressed rats. Collectively, these results indicate that PB and stress interacted to produce brain-region specific effects on glutamate neurochemistry, providing insight into the potential mechanisms underlying interactions between the immune system and persistent cognitive dysfunction in veterans with GWI.

## 1. Introduction

Following return from the Gulf War (GW), veterans have exhibited a constellation of symptoms - designated Gulf War Illness (GWI) - that cannot be associated with a single disease. Such symptoms include cognitive-psychological disturbances such as memory loss, confusion, inability to concentrate, irritability, and somnolence, as well as chronic fatigue and musculo-skeletal pain. Ten-year follow-up surveys found that deployed GW veterans reported a significantly higher rate of multi-symptom illnesses and mental disorders, plus increased onset of additional adverse health events, than non-deployed veterans (Li et al., 2011a; Kang et al., 2009). With upwards of 25% of soldiers from the first Gulf War presenting with this constellation of progressive and treatment-resistant symptoms, determining the etiology and

pathophysiology of this illness has become a priority for the Departments of Defense and Veterans Affairs (Ikin et al., 2004; Li et al., 2011b; Steele, 2000). While several factors have been implicated in the etiology of GWI, research currently supports two primary contributors to the presentation of these symptoms: stress, and use of the anti-nerve agent, pyridostigmine bromide (PB), which was administered to soldiers in zones at high-risk for chemical warfare exposure (Steele, 2000). PB reversibly inhibits acetylcholinesterase and butyrylcholinesterase, and although PB was not thought to cross the blood-brain-barrier, its use has consistently been correlated with the presentation of working and long-term memory deficits in both clinical studies (Hubbard et al., 2014; Tillman et al., 2017) and preclinical models (Hattiangady et al., 2014; Parihar et al., 2013; Zakirova et al., 2015; Lamproglou et al., 2009). This suggests that PB either directly or indirectly alters neural
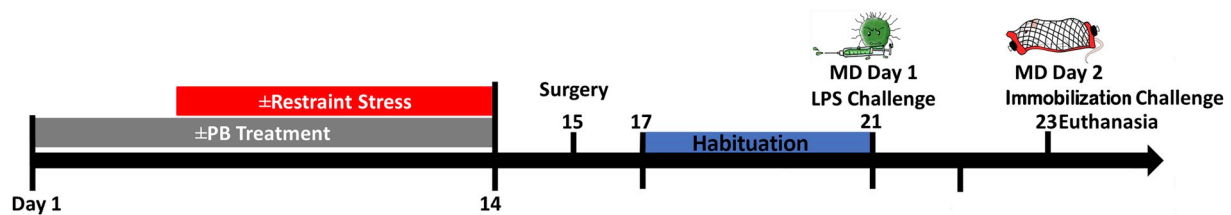
**Fig. 1. Experimental Timeline.** The GWI paradigm consisted of 14 days of gavage with either PB or vehicle. On day 5, rats were subdivided into restraint stress or non-stressed control conditions. Restraint stress was conducted for 6 h/day beginning at 10:00 a.m. for 10 consecutive days; non-stressed controls were housed separately to eliminate visual, auditory and olfactory cues of stress. Upon completion of the stress-PB paradigm, cannula surgery was performed. Rats were given 2 days of recovery followed by 4–5 days of habituation before the first microdialysis (MD) session (LPS challenge) on day 21. Forty-eight hours later a second microdialysis session was performed in which rats were subjected to an immobilization stress challenge. Rats were immediately euthanized following termination of the second microdialysis session.

networks that mediate these functions. Preclinical models of GWI have further demonstrated that stress can exacerbate these cognitive deficits (Hattiangady et al., 2014; Macht et al., 2018, 2019; Parihar et al., 2013), although the mechanisms through which PB and stress interact to impact cognitive functioning are currently unknown.

Previously, we reported that repeated restraint stress (RRS) and PB interact to impair central cholinergic responses to an acute stress challenge and an acute immune challenge (lipopolysaccharide; LPS) in a preclinical model of GWI (Macht et al., 2019). However, while this study demonstrated that cholinergic neurochemistry is dysregulated in PB-treated rats subjected to repeated stress, the combination of PB and repeated stress also likely impacts additional central systems. In this regard, two non-cholinergic systems which may contribute to the pathophysiology of GWI are the immune system and glutamatergic system. Indeed, unique immune signatures in response to stressful or inflammatory stimuli are emerging as hallmark features of GWI, and alterations in glutamatergic systems have long been associated with chronic stress, organophosphate poisoning, and dysregulated immune function. The link between glutamate and immune function is unsurprising given that glutamate exhibits dynamic interactions with immunocompetent glial cells: microglia and astrocytes. Microglia and astrocytes can both release and reuptake glutamate under various conditions and these cells also express a variety of glutamate receptors and transporters. As such, it is unsurprising that glutamate is a critical mediator between balancing the appropriate glial immune response and neurotoxicity. Since aberrations in immune function are emerging as hallmark features of GWI, it is possible that glutamatergic systems are a critical point of intersection between stress and PB on immunological and neurological systems.

To address whether PB and RRS interact to prime the glutamatergic response to inflammatory stimuli, the current study used a rodent model of GWI in combination with *in vivo* microdialysis to examine the neurochemical response to an immune challenge and an acute stress challenge. The glutamatergic response was assessed in two brain regions: the prefrontal cortex (PFC) and the hippocampus. These brain regions were targeted due to: 1) the critical roles of glutamate in their facilitation of cognitive function; and 2) their different susceptibilities to stress-induced inflammation. We tested the hypothesis that combinations of PB and stress increased the glutamatergic response to an innate immune challenge, thus potentially contributing to cognitive deficits evidenced in veterans with GWI. In addition, we tested whether an acute psychological stressor (immobilization stress) produced similar changes in the glutamatergic response in rats with a prior history of PB and repeated stress.

## 2. Methods and materials

### 2.1. GWI model

While the exact etiology of GWI remains to be unequivocally determined, 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; see (Macht et al., 2018, 2019). Specifically, adult male Sprague Dawley rats (250–300 g) were individually housed 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. All procedures were performed in accordance with all guidelines and regulations of 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. Pyridostigmine bromide (Sigma-Aldrich; St. Louis,. MO) 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. 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 (Macht et al., 2019). Interlocking intracerebral guide cannulae and stylets from Bioanalytical Systems Incorporated (BASi: MD-2251; West Lafayette, IN) 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 two days to recover from surgery undisturbed, followed by four days of habituation to the microdialysis bowls prior to microdialysis. As such, microdialysis did not commence until approximately one week following the date of surgery. There were no differences in surgical recovery between any groups.

### 2.3. In vivo microdialysis

*In vivo* microdialysis was performed as described in our previous study (Macht et al., 2019). 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 bw LPS challenge, and the second session was an immobilization stress

challenge. This LPS dose was selected based on pilot data as described in our previous study (Macht et al., 2019). 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$_2$, 0.183 mM MgCl$_2$, 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. All rats responded to LPS as evidenced by elevated levels of pro-inflammatory cytokines (for details see Macht et al., 2019). Forty-eight hours later, rats were subjected to a second microdialysis session that included a 1 h 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 glutamate 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 stress challenge.

### 2.4. Probe placement verification

Following microdialysis, rats were anesthetized with isoflurane and transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. Brains were removed and placed in a 30% sucrose/0.1 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 (Macht et al., 2019). Only rats with accurate probe placements were used for analysis.

### 2.5. High performance liquid chromatography

In random order from sample collection, 7.5 μL of previously frozen microdialysate sample was loaded onto an EiCom GU-GEL polymer resin based analytical column where glutamate was isolated from other biogenic compounds in interaction with a mobile phase consisting of 50 mM ammonium chloride-ammonia, 250 mg/L hexadecyl-trimethylammonium bromide, and 10 μL/L of 5 mg/mL Na$_2$ EDTA, pH 7.2. Afterwards, a glutamate oxidase enzyme column metabolized glutamate into hydrogen peroxide and 2-ketoglutarate. The hydrogen peroxide was oxidized at the platinum electrochemical detector with an applied current of +500 mV. The potential was read using the Epsilon computer controlled detector system from BASi. Concentration of glutamate in samples was interpolated against a three-point standard curve of 3, 1, and 0.1 μM glutamate. It is important to note that HPLC analysis for glutamate was performed on the same microdialysis samples that were used for the assessment of acetylcholine levels in our prior study

(Macht et al., 2019). These samples were analyzed simultaneously on two separate machines. Under these conditions, microdialysis samples were not subjected to a freeze-thaw cycle prior to HPLC analysis.

### 2.6. Plasma CORT and ChE activity analysis

Plasma from all rats was collected 30 min after the start of restraint on the last day of restraint stress (Day 14). Cholinesterase (ChE) activity was measured using the Abcam acetylcholinesterase assay kit (#ab138871) according to the manufacturer's instructions, as described in our previous studies (Macht et al., 2018, 2019). Plasma corticosterone (CORT) was measured using commercially available ELISAs as described in our previous studies (Macht et al., 2018, 2019). Briefly, plasma CORT was assessed using an ELISA kit from Enzo-Life Sciences (#ADI-900-097) according to the manufacturer's instructions. Samples were diluted 1:40 with steroid displacement reagent and kept on ice. CORT levels were interpolated from standards using a 4-parameter logistic curve. ELISAs were read using a BioTek Synergy microplate reader (BioTek Instruments Inc., Winooski, VT).

### 2.7. Statistical analysis

Results were calculated as a 2 × 2 × 16 mixed ANOVA for the LPS session and a 2 × 2 × 12 mixed ANOVA for the immobilization stress challenge session. For between-subjects factors, this experiment had 2 levels of drug treatment (vehicle, PB), 2 levels of stress (NSC, stressed). Within-subjects repeated measures consisted of 16 levels, representing the 16 consecutive collections during microdialysis for the LPS challenge. The restraint stress challenge had a total of 12 collections: 4 baseline, 4 during restraint, 4 post-restraint. Following significant interactions, simple main effects post hoc tests were performed with Bonferroni post-hoc corrections for family-wise error. For sample sizes in each group, see Supplemental Table 1.

## 3. Results

### 3.1. Plasma endocrine analysis

In agreement with our previous findings (Macht et al., 2018, 2019), on Day 14 of the GWI paradigm plasma ChE activity was significantly decreased in PB-treated rats compared to vehicle-treated rats irrespective of stress conditions [F(3,31) = 1.413; p = 0.0001; Fig. 2, Panel A]. From these same plasma samples isolated on Day 14 it was determined that rats subjected to restraint stress exhibited significant increases in plasma CORT levels compared to non-stressed control rats irrespective of PB treatment [F(3,30) = 0.3865; p < 0.0001; Fig. 2, Panel B]. These findings verify the efficacy of PB treatment to reduce plasma ChE activity and restraint stress to increase plasma CORT levels.

### 3.2. LPS challenge

On the first day of microdialysis, prior to the LPS challenge, neither prior history of PB nor restraint stress significantly impacted basal
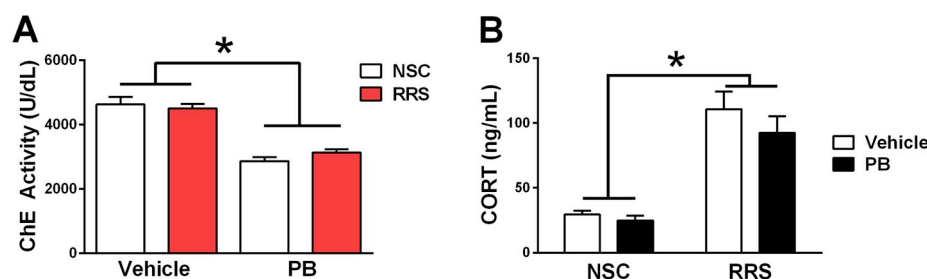
**Fig. 2. Plasma measures of the efficacy of PB treatment and RRS administration.** Panel A: In agreement with prior studies, PB administration (1.3 mg/kg by gavage) significantly reduced plasma cholinesterase activity compared to vehicle-treated control rats. Panel B: Rats subjected to repeated stress exhibited significant increases in plasma corticosterone (CORT) levels compared to non-stresed control rats. For these measures, plasma was isolated approximately 30 min following the initiation of stress or handling (NSC group) on Day 14, which is approximately 60 min following oral gavage of PB or vehicle. [*: p ≤ 0.05].

**Table 1**
Basal levels of glutamate (Glu) prior to LPS administration or immobilization stress challenge.

| Prefrontal Cortex (µM) | | | |
| --- | --- | --- | --- |
| Group | Basal Glu prior to LPS Rx | Basal Glu prior to stress Rx | P value |
| Veh-NSC | 2.60 ± 0.50 | 1.185 ± 0.40 | 0.052 |
| Veh-RRS | 1.084 ± 0.38 | 0.58 ± 0.08 @ | 0.227 |
| PB-NSC | 2.12 ± 0.54 | 0.58 ± 0.07 @ | 0.026 |
| PB-RRS | 2.86 ± 1.13 | 1.11 ± 0.29 | 0.214 |
| **Hippocampus (µM)** | | | |
| **Group** | **Basal Glu prior to LPS Rx** | **Basal Glu prior to stress Rx** | **P value** |
| Veh-NSC | 2.91 ± 0.75 | 1.81 ± 0.89 | 0.365 |
| Veh-RRS | 3.84 ± 1.22 | 3.37 ± 0.46 | 0.750 |
| PB-NSC | 2.75 ± 0.94 | 0.55 ± 0.21 | 0.100 |
| PB-RRS | 4.71 ± 1.69 | 2.38 ± 1.07 | 0.288 |

@: significant difference between PB-NSC and vehicle-stressed rats relative to vehicle-NSC and PB-stressed rats.
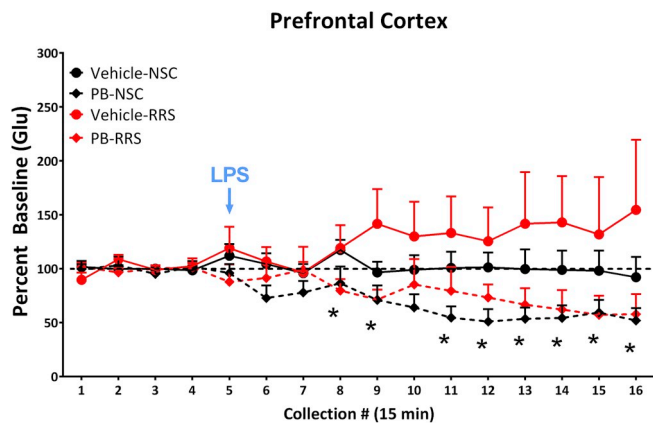
### Prefrontal Cortex



**Fig. 3. Glutamate Response to an LPS Challenge in the PFC.** There was a main effect of drug treatment on glutamate levels following LPS in the PFC. Specifically, although extracellular glutamate levels were unchanged in response to LPS in vehicle-NSC conditions, extracellular glutamate levels were decreased in response to LPS selectively in PB-treated rats starting at the 8th collection with the exception of collection 10. [*: Significant effect of PB relative to vehicle-treated rats, $p < 0.05$. Collection 10 p value = 0.056].

glutamate levels in the PFC [$F_{(1,22)} = 0.62$, $p = 0.44$; Table 1]. However, there was a significant interaction between time and PB treatment on the glutamatergic response to an LPS challenge [$F_{(15, 360)} = 3.82$, $p < 0.001$ (Fig. 3)]. Specifically, after intraperitoneal injection of LPS, extracellular glutamate levels were significantly decreased in PB-treated rats compared to vehicle-treated rats in collections 8–9 and 11–16 (p = 0.022, 0.008, 0.056, 0.019, 0.006, 0.016, 0.010, 0.037, 0.031, collections 8–16 respectively). Decreased glutamate levels in the PFC following LPS could indicate sensitivity of this brain region to LPS-induced deficits in synaptic plasticity. Moroever, these data suggest that PB primes glutamatergic systems for later immune challenges in the PFC, resulting in a significant reduction in extracellular glutamate levels in this brain region.

Similar to observations in the PFC, neither prior history of PB nor restraint stress significantly impacted basal glutamate levels in the hippocampus prior to LPS challenge[$F_{(1, 24)} = 0.18$, $p = 0.67$; Table 1]. However, there was a significant interaction between a prior history of PB treatment and time on the glutamatergic response to LPS [$F_{(15, 360)} = 1.77$, $p = 0.037$ (Fig. 4)]. There was also a trend for an interaction between prior stress history and drug treatment on the glutamatergic neurochemical response to LPS [$F_{(1, 24)} = 4.00$;
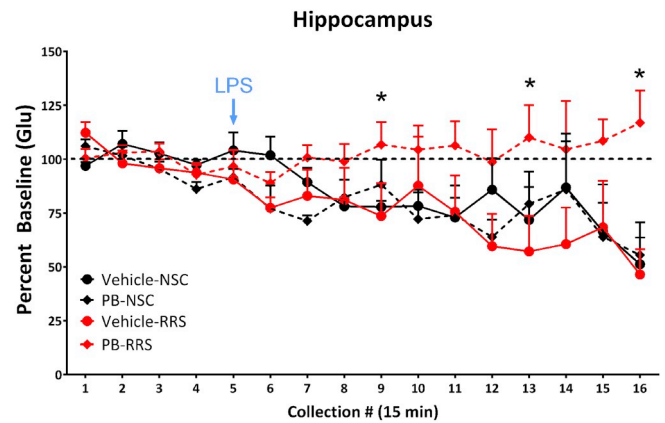
### Hippocampus



**Fig. 4. Glutamate Response to an LPS Challenge in the Hippocampus.** Unlike in the PFC, vehicle-NSC rats exhibit a depression in glutamate levels in response to LPS, suggesting that this brain region may be more sensitive to the effects of LPS. PB-stressed rats fail to show this LPS-induced depression in glutamate, suggesting that PB and stress interact to influence how the hippocampus responds to a systemic stressor. [*: PB-stressed rats significantly different from vehicle-treated rats, $p < 0.05$].

p = 0.057]. Specifically, while LPS reduces extracellular glutamate levels in vehicle-treated rats with a prior history of restraint stress, glutamate failed to decrease in response to LPS in PB-stressed rats relative to their vehicle-stressed counterparts at collections 9, 13, and 16 (p = 0.04, 0.037, and 0.004, respectively). This suggests that PB interacts with stress to influence the glutamatergic response to a novel immune stressor in the hippocampus.

### 3.3. Acute immobilization stress challenge

Prior stress history and prior drug history interacted over time to influence extracellular glutamate levels in response to an acute
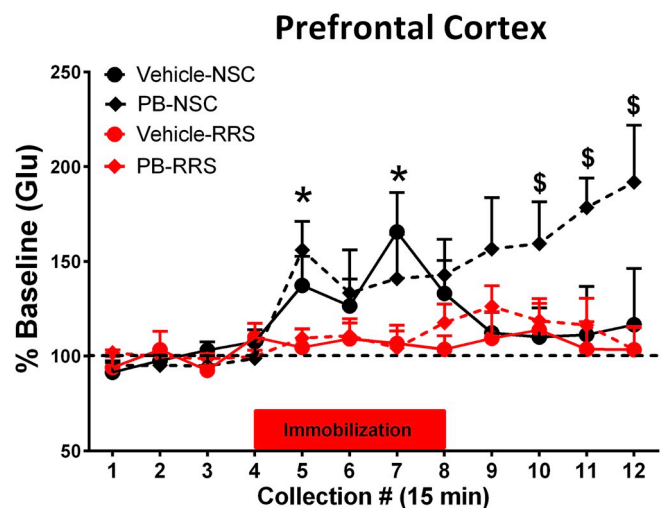
### Prefrontal Cortex



**Fig. 5. Glutamate Response to an Immobilization Stress Challenge in the PFC.** Restraint stress produces an acute increase in glutamate levels in the PFC during the immobilization stress challenge, followed by a rapid return to baseline after the stressor is removed. There is no increase in glutamate levels in rats with a prior stress history in this brain region. In contrast, rats with a prior history of PB but not restraint stress exhibit climbing levels of glutamate even after removal of the stressor, possibly suggesting an inability of glutamatergic systems to recover from this experience. [*: significant effect of stress, $p < 0.05$; $: PB-NSC rats significantly different from vehicle-NSC rats, $p < 0.05$].

immobilization challenge in the PFC [F(11, 209) = 1.98, p = 0.031 (Fig. 5)]. Specifically, unlike Veh-NSC rats, rats with a prior stress history did not exhibit an increase in extracellular glutamate levels in response to the immobilization stress challenge (collections 5 and 7; p = 0.008 and 0.022, respectively). In contrast, glutamate levels remained elevated during the post-stress period in PB-NSC rats relative to Veh-NSC rats (collections 10, 11 and 12; p = 0.048, p = 0.017 and 0.029, respectively), suggesting that PB interferes with the ability of the glutamatergic system to recover from an acute stress challenge in the PFC. Interestingly, there was a significant carry-over interaction between PB and stress on basal glutamate levels in the PFC [F(1, 21) = 12.32, p = 0.045 (Table 1)]. Specifically, glutamate was reduced in PB-NSC and Veh-RRS rats relative to vehicle-NSC and PB-RRS counterparts. Additionally, basal glutamate levels were significantly decreased in PB-NSC rats prior to restraint stress challenge relative to baseline levels measured prior to LPS administration. Similarly, basal glutamate levels in Veh-NSC rats prior to restraint stress challenge were reduced relative to basal levels prior to LPS administration, although these decreases did not achieve statistical significance; see Table 1. These shifts in basal glutamate levels in the PFC on the second day of microdialysis could result from several factors including residual effects of LPS.

In the hippocampus, there was a significant time × drug treatment [F(11, 198) = 2.28, p = 0.01] and time × stress interaction [F(11, 198) = 3.87, p < 0.001]. Specifically, in rats with a prior history of restraint stress, there is a significant effect of drug treatment at collection 5, 6, 7, 8, and 9, (p = 0.019, 0.005, 0.041, 0.035, and 0.037, respectively; Fig. 6). At each of these time points, a prior stress history decreased the glutamatergic response to restraint, but a combined history of PB treatment blocked this stress effect. These results suggest that PB impairs the habituation of the hippocampal glutamatergic neurochemical response to a novel psychological stress. On the second day of microdialysis, prior to the restraint challenge, the hippocampus did not exhibit any differences in basal glutamate levels between any groups [F(1, 18) = 0.03, p = 0.87; Table 1].
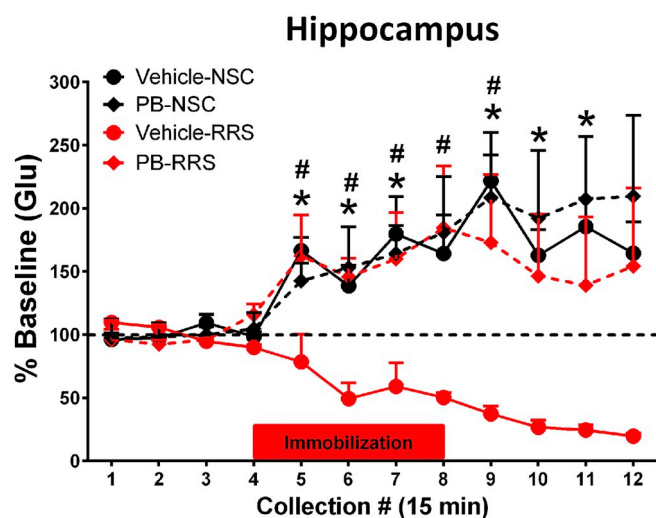


**Fig. 6. Glutamate Response to an Immobilization Stress Challenge in the Hippocampus.** Immobilization stress increases hippocampal glutamate levels in vehicle non-stress control ratsbut depresses the glutamatergic response to a restraint stress challenge in vehicle-treated rats. However, PB blocks this stress-adaptation of the glutamatergic response in rats with a prior stress history. This suggests that PB impairs the ability of glutamatergic circuits to adapt to prior experience, providing a potential underlying mechanism for the memory impairments evidenced in veterans with GWI. [*: Significant effect of prior stress history in vehicle-treated rats, p < 0.05; #: Significant effect of PB in rats with a prior stress history, p < 0.05].

## 4. Discussion

With no existing effective treatments for cognitive deficits, GWI research must first determine the potential underlying neurological changes induced by exposure to Gulf War chemicals. While much of the existing literature has focused on cholinergic changes, the current study is the first to use a rodent model of GWI to examine *in vivo* dynamics of glutamatergic systems to novel stressful stimuli. These results illustrate how PB and stress may have interacted in veterans with GWI to shift the function of excitatory neural circuits. Importantly, these results demonstrate that 1) the response of glutamatergic systems to systemic or psychological stress is disrupted by combinations of PB and RRS, and 2) the effect of PB on glutamatergic systems is dependent upon the brain region, the type of challenge, and the stress history.

In rats with no history of restraint stress, PB preferentially increases the glutamatergic response relative to Veh-NSC rats following an immobilization stress challenge in the PFC but not the hippocampus. In contrast, when LPS was administered as a systemic stress challenge, there was a decrease in the glutamatergic response to LPS in PB-treated rats regardless of their stress history. This suggests that PB disrupts how the glutamatergic system in the PFC processes novel stressful stimuli. Because cortical processing of environmental stimuli is an important precursor for how the hippocampus encodes the engram of that event, shifts in cortical processing of stressful stimuli could underlie the interactive effects between PB and stress on the hippocampal response following repeated exposures to stress.

When the acute stressor is distinct from prior stressful experiences (e.g. LPS challenge is heterotypic to restraint stress), then the hippocampal glutamatergic response to that challenge is similar in vehicle-NSC rats to both vehicle-stressed and PB-NSC rats. However, in rats with both a history of PB and RRS, LPS fails to induce a change in glutamate levels. This suggests that PB and RRS interact to selectively attenuate how the hippocampus responds to a systemic stressor. This selective effect of PB and RRS on glutamatergic systems is also evident following a restraint stress challenge. In this case, rats with a prior history of PB and restraint stress exhibit a parallel glutamatergic response to the restraint stress challenge as restraint stress naïve rats. That is, glutamate levels in the hippocampus are similar in PB-stressed rats as vehicle or PB-NSC rats following a restraint challenge. These results have important implications for how veterans with GWI may continue to process novel and repeated stressful events, thereby potentially contributing to the progressive presentation of symptoms in veterans.

The failure of glutamatergic systems to adapt in the hippocampus to repeated exposures of a stressful stimulus could be attributable to a combination of factors. One possible mechanism is that PB-driven shifts in the cortical processing of stressful stimuli could impair the ability of the hippocampus to form an appropriate memory circuit to encode the stressful stimulus. Another possibility is that PB-driven changes in the cytokine response to stress may impair the ability of the glutamatergic systems to facilitate long-term potentiation in the hippocampus. The subsequent sections will address each of these possibilities in conjunction to their relation to neurocognitive symptoms exhibited by veterans with GWI and preclinical models of GWI.

An important caveat of the current study is that LPS was administered to all rats prior to the immobilization stress challenge, and as such, a potential interaction between these two effects cannot be eliminated. However, the dose of LPS used in the current study is extremely low. In addition, the effects of LPS and restraint stress appear to be divergent, and restraint stress responses of control and repeatedly restrained rats are similar to results seen in prior studies in the absence of LPS pre-treatment (Grillo et al., 2015; Reznikov et al., 2007; Moghaddam, 1993). As such, while it is impossible to eliminate the possibility that prior LPS exposure influenced glutamate responses, this effect is unlikely to explain the group differences to the immobilization stress challenge in the current study.

### 4.1. Overarching behavioral and neurocognitive consequences of PB and stress

Clinical studies on GWI have documented that PB exposure is correlated to chronic cognitive impairments including deficits in attention, information processing speed, and working and long-term memory deficits (Hom et al., 1997; Sullivan et al., 2018). These clinical findings have been supported by a variety of preclinical models which indicated that various combinations of PB, pesticides, and stress can interact to exacerbate deficits in behavioral performance in working and long-term memory tasks, including the Morris water maze (Parihar et al., 2013) and novel object recognition (Hattiangady et al., 2014). In addition, using the same GWI model employed in the current study, we have previously determined that PB and stress interact to produce recall deficits to both context and tones in a classical fear conditioning paradigm (Macht et al., 2018, 2019). As PFC and hippocampal glutamatergic circuitry are critical mediators of performance in these tasks, examining how these brain regions respond to aversive stimuli provides insight as to how PB and stress-induced aberrations in excitatory neurochemical responses may influence learning and memory processes.

### 4.2. PB produces an exaggerated PFC-glutamatergic response to immobilization challenge in rats with no prior restraint stress history

In previous studies, we determined that PB and restraint stress interact to impair contextual recall in fear conditioning, and PB alone impairs recall of fear associations with a cue (tone) (Macht et al., 2018, 2019). These learning and memory impairments could be directly related to shifts in glutamate responses to novel and repeated stressful stimuli. For example, blocking glutamate activation of NMDA receptors with selective administration of an antagonist into the PFC also blocks contextual freezing and decreases the heart-rate response to the context upon re-exposure (Resstel et al., 2008). This suggests that not only does PFC glutamate regulate the cognitive memory of the fear-associated context re-exposure, but it also impacts the physiological stress-response to that memory.

Given these findings, one interpretation of the current study is that a failure of extracellular glutamatergic levels to subside following a novel immobilization stress challenge in PB-treated rats indicates a failure of appropriate processing of stress-associated contexts and cues. This suggests that PB alters the cortical processing of novel stressful stimuli such that when veterans returning from the Gulf War are subjected to new stressors, glutamatergic systems may not respond in an adaptive manner. For example, climbing glutamate levels following the termination of restraint stress in PB-treated rats could create a predisposition to neurotoxicity following psychological stress.

Surprisingly, PB and a history of RRS did not interact to produce an exaggerated effect on the glutamatergic response to immobilization stress in the PFC. Rather, PB alone altered the glutamatergic response to a stress challenge, and only in the absence of a prior stress history. One important consideration is that the current paradigm examined the effects of PB one-week following the conclusion of the GWI paradigm, potentially allowing for the recovery of neural systems in the RRS group. However, it is possible that a prior exposure to PB in conjunction with restraint stress will alter the recovery of these systems. In this context, a PB-stress interaction in the PFC may be more apparent only at a more delayed time point following the GWI paradigm.

### 4.3. PB impairs the adaptation of hippocampal glutamatergic responses to immobilization challenge in rats with a prior stress history

The hippocampus, particularly the hippocampal-septal pathway, is also a critical mediator of contextual fear conditioning, indicating a close relationship between glutamate, acetylcholine, and recall of memories associated with aversive stimuli. Following exposure to an imobilization stressor after RRS exposure, the cholinergic hippocampal stress-response is attenuated (Macht et al., 2019) and the glutamatergic response is suppressed. However, in PB-stressed rats, both hippocampal neurotransmitter systems exhibit matched stress responses to their restraint stress-naive counterparts. One way to interpret this finding is that PB is protective against the maladaptive effects of stress on hippocampal circuitry. However, ten days of restraint stress was selected for the current GWI paradigm as it is insufficient to produce remodeling of glutamatergic pyramidal neurons in the hippocampus (McLaughlin et al., 2007), which typically requires a minimum of 21 days (Watanabe et al., 1992). Based on these morphological observations, it is unlikely that the neurochemical responses to repeated stress exposure in these conditions are maladaptive. In addition, considering the plethora of learning and memory impairments in veterans with GWI which are consistently linked to PB exposure, as well as the cognitive impairments in contextual fear conditioning in our model, PB's effect on hippocampal glutamate is unlikely to be neuroprotective. An alternative interpretation is that PB prevents typical habituation of hippocampal glutamatergic systems to repeated stress. This could have important implications for veterans who were exposed to PB during the Gulf War and how their neurological systems respond to stressors even after returning from deployment.

What could be the consequence of this failure of glutamatergic systems to exhibit habituation to repeated stress? Returning from active combat deployment and reintegrating into society poses many repeated stressful challenges for veterans. A failure of neural systems to habituate to the repeated presentation of stressors could indicate a failure in learning appropriate stress-associated contexts and cues, indicating a repeated and exaggerated neurochemical response. Repeated and exaggerated glutamatergic responses could also have toxic consequences to local circuits. In support of this, other preclinical studies of GWI have demonstrated increased oxidative stress in response to combinations of stress, pesticide exposure and PB (Shetty et al., 2017). Pharmacologically targeting PB and stress-induced aberrations in glutamatergic systems could help protect against oxidative damage and be an important potential therapeutic target for GWI, which currently remains treatment-resistant.

### 4.4. Cortical processing of innate immune challenges

Shifts in the innate immune response are emerging as a hallmark feature of GWI. While most studies in veterans with GWI have focused on peripheral immune signatures, the brain is also sensitive to innate immune challenges. Therefore, we tested the hypothesis that PB and RRS would alter the neurochemical response to a relatively modest innate immune challenge (30 μg/kg LPS). In the PFC, a prior history of PB accentuates the glutamatergic response to immobilization stress but attenuates the glutamatergic response to LPS in rats with no prior stress history. An important distinction between the glutamatergic response for each of these stressors is that the PFC is typically resistant to the effects of LPS but sensitive to the effects of restraint stress (de Pablos et al., 2006; Moghaddam, 1993). As such, a decrease in the glutamatergic response following LPS administration in PB-treated rats could indicate that PB increases the sensitivity of the PFC to inflammatory effects. In addition, glutamatergic levels in the PFC fail to recover following the termination of immobilization stress in rats with no prior restraint history. In contrast, depression of glutamatergic levels in response to LPS may indicate sensitivity to sickness behaviors, including impairments in complex cognitive tasks (Moghaddam et al., 1997; Verma and Moghaddam, 1996; Walker et al., 2013). Further studies would need to be conducted to discern whether LPS exacerbates cognitive deficits following PB and whether PFC systems are sensitized to excitotoxicity following a restraint challenge.
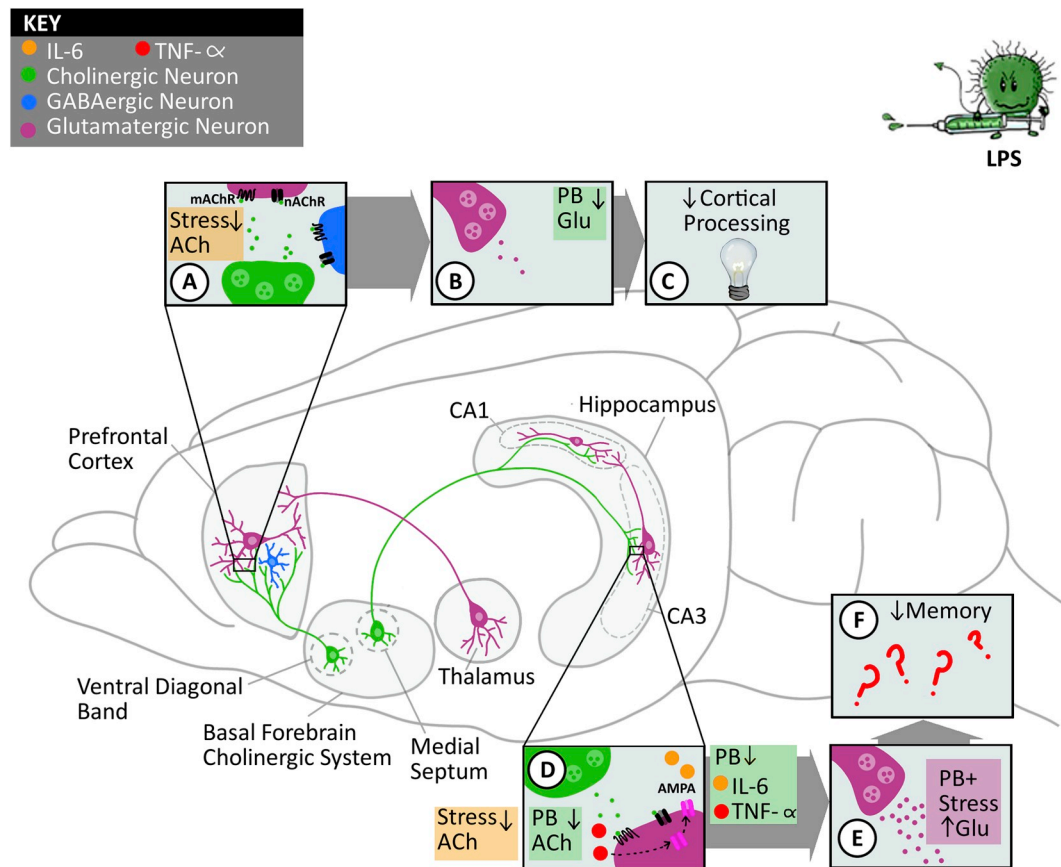
**Fig. 7. Neurochemistry of LPS in a Model of GWI. A.** Cholinergic terminals project diffusely across the PFC to modulate the excitatory-inhibitory balance via muscarinic (mACh) and nicotinic (nACh) receptors. A history of stress decreases the cholinergic response to LPS in both vehicle and PB-treated rats. **B.** PB decreases glutamate in the PFC in response to LPS, irrespective of stress-history. This decrease in glutamate could be attributable to reductions in local signaling or afferents from distal cortical or subcortical brain regions, such as the thalamus. **C.** Decreases in cholinergic and glutamatergic processing of new stressful stimuli could provide an underlying mechanism for impairments in PFC-driven cognitive function in veterans with GWI. **D.** Both PB and stress decrease the cholinergic response to LPS in the hippocampus. PB also decreases plasma levels of IL-6 and TNF-α. In the brain, IL-6 regulates the temporal specificity of LTP; TNF-α regulates stability of synaptic plasticity by mediating AMPA trafficking. **E.** Combinations of PB and stress produce interactive effects on the glutamatergic response to LPS in the hippocampus. **F.** Combined deficits in cholinergic, glutamatergic, and cytokine systems could impair the stability of LTP and thus decrease memory function.

### 4.5. Summarizing across studies: modeling PB and stress and the neurochemical mechanisms for learning and memory

In addition to interacting at multiple points in peripheral systems, the current study elaborates on an expanding literature suggesting that stress and PB also produce many convergent and synergistic changes related to how the brain responds neurochemically to either an LPS (Fig. 7) or immobilization stress challenge (Fig. 8). As these models demonstrate, some of the neurochemical changes in PB-stressed rats are driven independently by PB or stress whereas others are a result of interactive effects between PB and stress. For example, while PB and stress both independently and in combination produce decreases in the cholinergic and glutamatergic response to a challenge (either LPS or immobilization stress) in the PFC, glutamatergic systems in the hippocampus of PB-RRS rats continue to respond as if they have no prior stress history. This failure in hippocampal glutamatergic systems to adapt to repeated stressors following PB could have multiple causes and consequences. For example, decreases in cortical processing of stressful stimuli induced by PB and stress could impair the salience of the stressful event which would have downstream effects on memory-formation.

In addition, future studies should examine levels of pro-inflammatory cytokines in the hippocampus. Our previous work suggests that IL-6 and TNF-α responses are attenuated in plasma following an LPS challenge (Macht et al., 2019). If similar responses are evident in

the hippocampus, then this would have important consequences for the appropriate assimilation of the cellular mechanisms for memory: LTP. IL-6 and TNF-α are constitutively expressed under normal physiological conditions. IL-6 is associated with proper temporal tuning of LTP, exhibiting a negative regulatory feedback. TNF-α facilitates AMPA receptor trafficking, influencing the stability of newly formed synapses. Either too much or too little IL-6 or TNF-α produces deficits in synaptic plasticity and the stability of new memories. As such, deficits in cortical processing of stressful stimuli in addition to deficits in cytokine-mediation of synaptic plasticity could underlie some of the surprising neurochemical responses in the hippocampus to PB and stress. These shifts in the neurochemical response to a stressful challenge provide a potential mechanism underlying cognitive deficits in soldiers with GWI.

Our studies provide insight as to how shifts in acetylcholine, glutamate, and cytokines may be influencing the cellular processes underlying learning and memory in a model of GWI. In addition, as deficits in contextual fear conditioning in PB-stressed rats appear transient, these acute shifts in cognitive processing of stressful stimuli may have important consequences for maintenance and progression of GWI as veterans returning from deployment face a host of stressors upon reintegrating into society. An inability of the central nervous system to adapt to repeated stressors may increase the sensitivity to neural damage from oxidative stress, thus exacerbating the effects of PB and stress. If shifts in cognitive processing in response to recurrent stressors is a factor in perpetuating the progression of GWI, treatment strategies
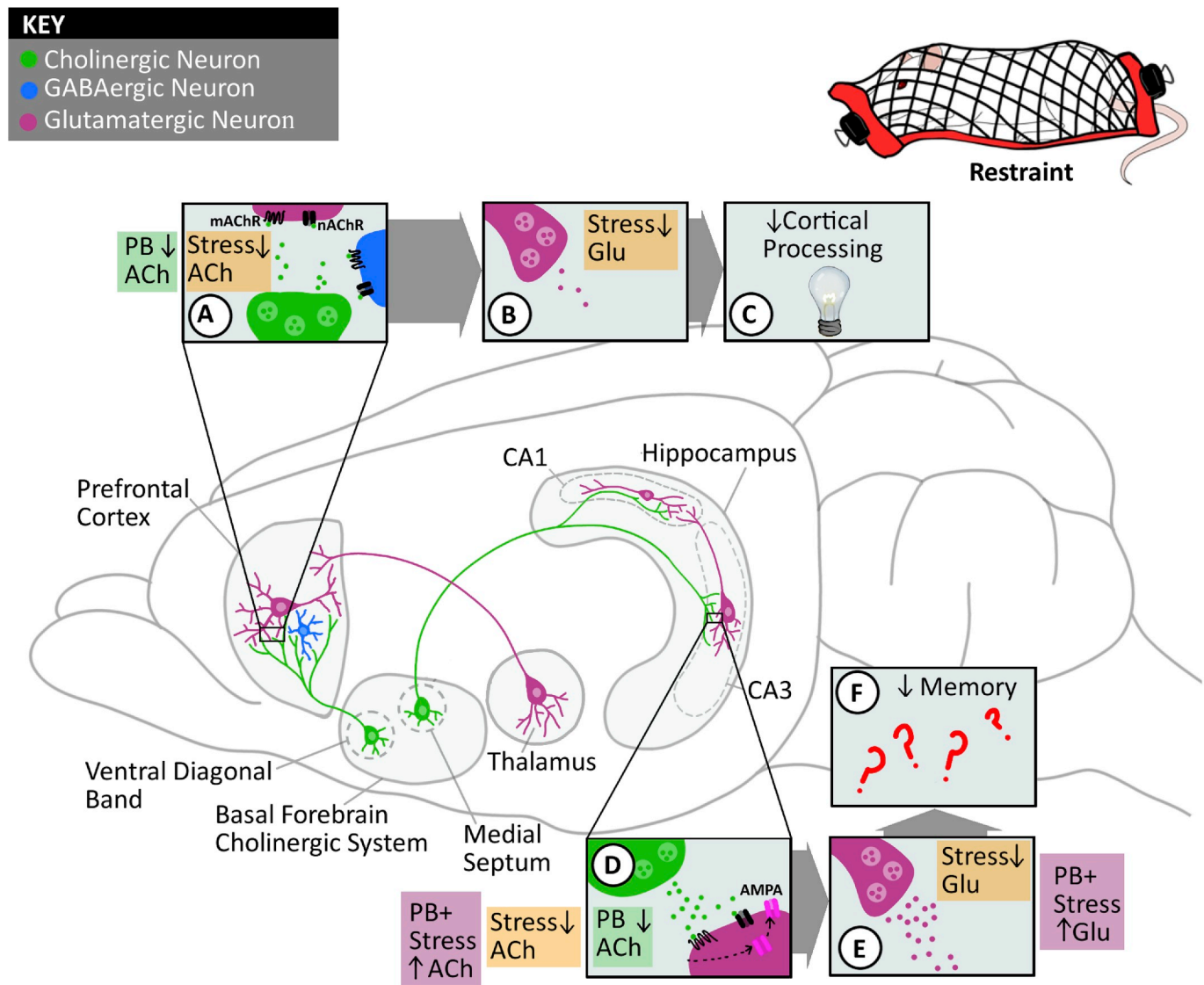
**Fig. 8. Neurochemistry of Stress Responses in a Model of GWI. A.** A prior history of PB or restraint stress both independently and in combination decrease the cholinergic response to a stressful stimulus in the PFC. **B.** A history of stress decreases the glutamatergic response to stressful stimuli in the PFC. This decrease in glutamate could be attributable to reductions in local signaling or afferents from distal brain regions, such as the thalamus. **C.** Decreases in cholinergic and glutamatergic processing of new stressful stimuli could provide an underlying mechanism for impairments in PFC-driven cognitive function in veterans with GWI. **D.** While both PB and stress decrease the cholinergic response to stressful stimuli in the hippocampus independently, combinations of PB with stress increase the cholinergic responses to an immobilization stress challenge. **E.** PB and stress also produce interactive effects on the glutamatergic response to a stressful challenge. **F.** Failure in the cholinergic and glutamatergic systems' adaptation to stress in the hippocampus could be reflective of impaired hippocampal-dependent memory in veterans with GWI.

would need to emphasize two primary goals: 1) to stop the progression of GWI, and 2) to reverse physiological and cognitive deficits in GWI.

## 5. Conclusions

In sum, these results emphasize that PB and stress interact to impact a variety of immunological and neurological systems, including excitatory neurotransmission. Alterations in the ability of excitatory neurotransmitter systems to respond appropriately to stressful events may underlie memory consolidation problems in veterans with GWI, suggesting the glutamatergic system may also be an important target for future studies which focus on potential treatment options. As the current study focused on the short term consequences of PB and stress on glutamatergic neurochemistry, future studies should also examine the persistence of these neurological effects following PB and stress. This is particularly important as veterans with GWI have exhibited

persistent and progressive deficits, continuing for decades post-deployment. However, what the current study does highlight is that PB and stress interact to shift brain excitatory neurochemistry, and as such, changes in neural responses to stressors after the Gulf War may also play an important role in this progressive pathology in veterans.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynstr.2019.100210.

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