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ARTICLECorticosterone primes the neuroinflammatory  
response to DFP in mice: potential animal model of  
Gulf War IllnessJames P. O'Callaghan,\* Kimberly A. Kelly,\* Alicia R. Locker,\*  
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Peoria, Illinois, USA**Abstract**

Gulf War Illness (GWI) is a multi-symptom disorder with features characteristic of persistent sickness behavior. Among conditions encountered in the Gulf War (GW) theater were physiological stressors (e.g., heat/cold/physical activity/sleep deprivation), prophylactic treatment with the reversible AChE inhibitor, pyridostigmine bromide (PB), the insect repellent, *N,N*-diethyl-meta-toluamide (DEET), and potentially the nerve agent, sarin. Prior exposure to the anti-inflammatory glucocorticoid, corticosterone (CORT), at levels associated with high physiological stress, can paradoxically prime the CNS to produce a robust proinflammatory response to neurotoxicants and systemic inflammation; such neuroinflammatory effects can be associated with sickness behavior. Here, we examined whether CORT primed the CNS to mount neuroinflammatory responses to GW exposures as a potential model of GWI. Male C57BL/6 mice were treated with chronic (14 days) PB/DEET, subchronic (7–14 days) CORT, and acute exposure (day 15) to diisopropyl fluorophosphate (DFP), a sarin surro-

gate and irreversible AChE inhibitor. DFP alone caused marked brain-wide neuroinflammation assessed by qPCR of tumor necrosis factor- $\alpha$ , IL6, chemokine (C-C motif) ligand 2, IL-1 $\beta$ , leukemia inhibitory factor, and oncostatin M. Pre-treatment with high physiological levels of CORT greatly augmented (up to 300-fold) the neuroinflammatory responses to DFP. Anti-inflammatory pre-treatment with minocycline suppressed many proinflammatory responses to CORT+DFP. Our findings are suggestive of a possible critical, yet unrecognized interaction between the stressor/environment of the GW theater and agent exposure(s) unique to this war. Such exposures may in fact prime the CNS to amplify future neuroinflammatory responses to pathogens, injury, or toxicity. Such occurrences could potentially result in the prolonged episodes of sickness behavior observed in GWI.

**Keywords:** CORT, DFP, GWI, microglia, minocycline, neuroinflammation.

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Gulf War Illness (GWI) is a multi-symptom disorder with features resembling 'sickness' behavior (Dantzer and Kelley 2007; Dantzer *et al.* 2008), for example, cognitive impairment, fatigue, depression, sleep disruption, muscle and joint pain, and gastrointestinal and dermatological problems

*Abbreviations used:* AChE, acetylcholinesterase; CB, cerebellum; CCL2, chemokine (C-C motif) ligand 2; CNS, central nervous system; CORT, corticosterone; DEET, *N,N*-diethyl-meta-toluamide; DFP, diisopropyl fluorophosphate; EtOH, ethanol; FC, frontal cortex; FDA, Food and Drug Administration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GW, Gulf War; GWI, Gulf War Illness; HIP, hippocampus; HYPO, hypothalamus; Iba1, ionized calcium-binding adapter molecule 1; IkBa, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IL6, interleukin 6; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; MINO, minocycline; NA, numerical aperture; NF- $\kappa$ B, nuclear factor kappa light chain enhancer of activated B-cells; OB, olfactory bulb; OSM, oncostatin M; PB, pyridostigmine bromide; PBS, phosphate-buffered saline; RAC, research advisory council; SDS, sodium dodecyl sulfate; STR, striatum; TNF- $\alpha$ , tumor necrosis factor-alpha.

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(Fukuda *et al.* 1998; Steele 2000; Haley *et al.* 2001; Golomb 2008). Typically, sickness behavior accompanies an inflammatory response to infection or injury and resolves over time with gradual restoration of homeostasis (Pavlov *et al.* 2003). However, in GWI the symptoms persist, findings indicative of a heightened or chronic neuroimmune/neuroinflammatory reaction for which the etiology remains unknown.

Enhanced expression of proinflammatory cytokines and chemokines serves as the basis for normal and pathophysiological inflammation states, including sickness behavior (Dantzer and Kelley 2007). Microglia and astrocytes, major subtypes of CNS glia, are thought to be the primary sources and targets of cytokines and chemokines that play a role in brain neuroinflammatory responses (Block and Hong 2005; Block *et al.* 2007; Sriram and O'Callaghan 2007; O'Callaghan *et al.* 2014). These glial cells also are speculated to play a key role in pain (Narita *et al.* 2008; Schreiber *et al.* 2008), depression (Miller and O'Callaghan 2005; Dantzer *et al.* 2008), and sickness behavior (Dantzer *et al.* 2008; Biesmans *et al.* 2013; Hines *et al.* 2013). Together, these observations lead us to hypothesize that conditions and exposures that result in exacerbated or chronic expression of proinflammatory mediators in the CNS may play a role in the symptoms exhibited by veterans suffering from GWI.

Several conditions and exposures have been hypothesized to serve as instigating events that precipitated the recurring/chronic symptoms associated with GWI (Federal Research Advisory Committee on Gulf War Veterans Illnesses' (RAC) Report 2008, 2014). Chief among these events are acute nerve agent exposures resulting from detonations at Khamisiyah and other Iraqi weapons facilities (RAC Reports 2008, 2014), prophylactic treatment with the reversible acetylcholinesterase (AChE) inhibitor pyridostigmine bromide (PB), and continuous exposure to pesticides/insect repellants in theater (RAC Reports 2008, 2014). Inhibition of AChE because of sarin and PB exposures has been put forth as a basis for the symptoms of GWI (Golomb 2008) and, at least with respect to sarin, such exposures can produce neuroinflammation in a rat model (Spradling *et al.* 2011). Whether exposure to the reversible AChE inhibitor, PB, or insecticides and insect repellants, like *N,N*-diethyl-meta-toluamide (DEET) can be neuroinflammatory alone, or when combined with sarin exposure, remains to be examined. Physiological stress experienced in theater also has been proposed as a potential contributor to GWI (Sapolsky 1998). Consistent with this view, we have observed that prior subchronic exposure to the rodent stress hormone corticosterone (CORT) can enhance the neuroinflammation associated with neurotoxicity (Kelly *et al.* 2012). Here, we show that the sarin surrogate diisopropyl fluorophosphate (DFP), results in neuroinflammation in the mouse brain, an effect markedly enhanced by prior exposure to CORT. These findings suggest that combined exposures to CORT and DFP may serve as an animal model of the pathobiology of GWI. Importantly, anti-inflammatory therapy with minocycline (MINO) suppressed

many of the neuroinflammatory effects observed in this mouse model, potentially providing an effective intervention for treating ill Gulf War (GW) veterans.

## Methods

### Materials

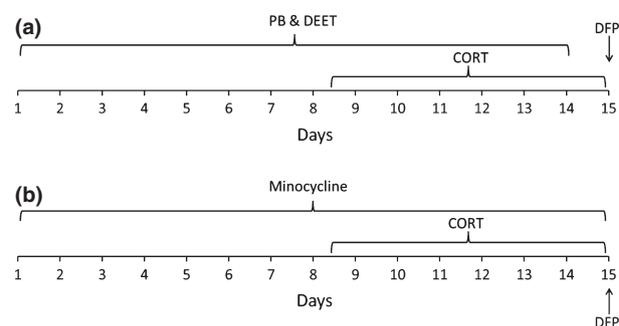
The following drugs and chemicals were kindly provided by or obtained from the sources indicated: DFP, (Sigma, St. Louis, MO, USA), PB (Sigma), DEET (Sigma), CORT (Steraloids, Inc., Newport, RI, USA), MINO (Sigma), bichononic acid protein assay reagent, and bovine serum albumin (Pierce Chemical Co., Rockford, IL, USA). The materials used in the glial fibrillary acidic protein (GFAP) assay previously have been described in detail (O'Callaghan 1991, 2002). All other reagents were of at least analytical grade and obtained from a variety of commercial sources.

### Animals

Adult male C57Bl/6J mice ( $n = 5$  mice per group) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All procedures were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, and the animal facility was certified by the American Association for Accreditation of Laboratory Animal Care. Upon receipt, the mice were housed individually in a temperature ( $21 \pm 1^\circ\text{C}$ ) and humidity-controlled ( $50 \pm 10\%$ ) colony room maintained under filtered positive-pressure ventilation on a 12 h light/12 h dark cycle beginning at 06:00 hours. The plastic tub cages were  $46 \times 25 \times 15$  cm; cage bedding consisted of heat-treated pine shavings spread at a depth of approximately 4 cm. Purina rat/mouse chow and water were available *ad libitum*.

### Dosing

The dosing paradigm is presented in Fig. 1. Mice were dosed once per day for 14 days with PB (2 mg/kg/day, *s.c.*) and DEET (30 mg/kg/day, *s.c.*). On days 8–15, mice received CORT in the drinking water (200 mg/L in 1.2% ethanol, EtOH). This CORT regimen is



**Fig. 1** Dosing paradigm. Time-line for administration of diisopropyl fluorophosphate (DFP) (4 mg/kg, *i.p.*) with and without prior administration of corticosterone (CORT) (200 mg/L in drinking water) and/or pyridostigmine bromide (PB) (2 mg/kg/day, *s.c.*) and *N,N*-diethyl-meta-toluamide (DEET) (30 mg/kg/day, *s.c.*) (a), or minocycline (MINO) (100 mg/kg/day, *s.c.*) (b).

known to be immunosuppressive as evidenced by decreased thymus weight (e.g., see O'Callaghan *et al.* 1991); thymus and spleen weights were confirmed to be decreased (> 20%) in all CORT (including PB/DEET+CORT) groups in this study. Control groups received saline injections and the 1.2% EtOH/water vehicle in the same paradigm and were controlled for the potential of handling stress. Finally, on day 15 mice were treated with a single injection of either DFP (4 mg/kg, i.p.) or saline (0.9%) (Fig. 1a). DFP causes behavioral seizures that abate over hours; DFP-induced seizure activity was not affected by PB/DEET pre-treatments and PB/DEET alone did not cause seizures. To compare the effects of handling, there was a non-handled control group which did not receive 14 days of saline injections. However, there were no significant effects of handling for our outcome measures; therefore, the non-handled group was not included in our final data analyses. For the MINO experiment, mice were dosed with MINO (100 mg/kg, s.c.) once per day for 15 days. On days 8–15 mice received CORT in the drinking water (200 mg/L in 1.2% EtOH). Control groups received saline injections and the 1.2% EtOH/water vehicle in the same paradigm. On day 15, mice were treated with a single injection of DFP (4 mg/kg, i.p.) or saline (0.9%) (Fig. 1b).

#### Brain dissection and tissue preparation

Mice were killed by decapitation and the brains rapidly removed. Frontal cortex (FC), hippocampus (HIP), striatum (STR), hypothalamus (HYPO), olfactory bulbs (OB), and cerebellum (CB) were dissected free-hand on a thermoelectric cold platform (Model TCP-2; Aldrich Chemical Co., Milwaukee, WI, USA). Brain regions from one hemisphere were frozen at  $-80^{\circ}\text{C}$  and used for subsequent isolation of total RNA; brain regions from the other hemisphere were used for total and specific protein analyses. These regions were weighed, homogenized with an ultrasonic probe (model XL-2005; Heat Systems, Farmingdale, NY, USA) in 10 volumes of hot ( $90\text{--}95^{\circ}\text{C}$ ) 1% sodium dodecyl sulfate, and stored at  $-70^{\circ}\text{C}$  for further analysis.

#### RNA isolation, cDNA synthesis, and qPCR

Total RNA was isolated at 2, 6, and 12 h after DFP using methods previously described (Kelly *et al.* 2012). Briefly, total RNA was isolated using Trizol<sup>®</sup> reagent (Invitrogen, Grand Island, NY, USA) and Phase-lock heavy gel (Eppendorf AG, Hamburg, Germany) and purification by RNeasy mini-spin column (Qiagen, Valencia, CA, USA). Reverse transcription of the total RNA (1  $\mu\text{g}$ ) to cDNA was achieved by SuperScript<sup>™</sup> II RNase H<sup>-</sup> and oligo (dT)<sub>12–18</sub> primers (Invitrogen) in a 20  $\mu\text{L}$  reaction. qPCR analysis of glyceraldehyde-3-phosphate dehydrogenase, tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin 6 (IL6), chemokine (C-C motif) ligand 2 (CCL2), interleukin 1beta (IL-1 $\beta$ ), leukemia inhibitory factor (LIF), and oncostatin M (OSM) was performed in an ABI PRISM 7500 sequence detection system (Applied Biosystems, Carlsbad, CA, USA) in combination with TaqMan<sup>®</sup> chemistry. Relative quantification of gene expression was performed using the comparative threshold ( $C_T$ ) method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Changes in mRNA expression levels were calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase. The ratios obtained after normalization are expressed as fold changes over corresponding controls.

#### GFAP immunoassay

GFAP protein was assayed at 72 h post DFP exposure as previously described (O'Callaghan 1991, 2002). In brief, a rabbit polyclonal antibody to GFAP was coated on the wells of Immulon 2 flat bottom microtiter plates (Thermo Labsystems, Franklin, MA, USA). The sodium dodecyl sulfate homogenates and standards were diluted in phosphate-buffered saline (PBS) (pH 7.4) containing 0.5% Triton X-100. After blocking non-specific binding with 5% non-fat dry milk, aliquots of homogenate and standards were added to the wells and incubated for 1 h at  $23^{\circ}\text{C}$ . Following washes, a mouse monoclonal antibody to GFAP (1 : 400; DAKO, Carpinteria, CA, USA) was added to 'sandwich' the protein between the two antibodies. An alkaline phosphatase-conjugated antibody directed against mouse IgG was then added and a colored reaction product was obtained by subsequent addition of the enzyme substrate *p*-nitrophenol. Quantification by spectrophotometry of the colored reaction product at 405 nm was performed on a microplate reader (Spectra Max Plus) and analyzed using Soft Max Pro Plus software (Molecular Devices, Sunnyvale, CA, USA). The amount of GFAP in the samples was calculated as  $\mu\text{g}$  GFAP per mg total protein. Total protein was determined by the bicinchoninic acid method (Smith *et al.* 1985) using bovine serum albumin as a standard.

#### Glial immunohistochemistry and neurodegeneration staining

Mice were anesthetized with Sleep Away<sup>®</sup> (0.1 mL; Fort Dodge Animal Health, Fort Dodge, IA, USA) and transcardially perfused with saline (0.9%) followed by 4% paraformaldehyde in 0.1 M PBS 24 h post DFP injection. Brains were removed and stored in 4% paraformaldehyde in 0.1 M PBS to be sent to FD Neurotechnologies (Columbia, MD, USA) for subsequent processing and staining for GFAP and ionized calcium-binding adapter molecule 1 (Iba1) (markers for activation of astrocytes and microglia, respectively), as well as FD NeuroSilver<sup>™</sup> and Fluoro-Jade B (markers for neuronal degeneration). The sections were visualized using an Olympus AX70 microscope (Olympus, Pittsburgh, PA, USA) with an UPlanFL 4 $\times$  0.13 numerical aperture (GFAP, Iba1, and Fluoro-Jade B) or 10 $\times$  0.30 numerical aperture (FD Neurosilver<sup>™</sup>) objective lens and images captured using Cell Sens Dimension software with the Olympus DP73 digital camera (Olympus, Pittsburgh, PA, USA) attached to the microscope. Post-processing included cropping images to isolate the HIP.

#### Statistics

All data analyses were performed using SPSS (version 22) software (IBM, Armonk, NY, USA). The tests of significance were performed on log transformed data using Student's *t*-tests, one-, or two-way ANOVAS. Fisher LSD test was used as a *post hoc* test for appropriate analyses. The type of statistical test is specified in each figure legend. Values were considered statistically significant at a 5% level of significance ( $p < 0.05$ ). Graphical representations are untransformed mean  $\pm$  SEM.

## Results

### DFP causes neuroinflammation in multiple brain regions

Administration of the sarin surrogate DFP caused a brain-wide neuroinflammatory response (FC, HIP, STR, HYPO,

OB, CB). The expression of multiple cytokine and chemokine mRNA was enhanced in multiple brain areas after a single dose of DFP (Fig. 2). Not all areas were affected equally, but all brain areas sampled showed various degrees of enhanced expression of multiple proinflammatory mediators. Increased expression of TNF- $\alpha$ , IL6, CCL2, IL-1 $\beta$ , LIF, and OSM mRNA was seen at 2 h post DFP, was increased further in most cases by 6 h, with a reduction toward control levels by 12 h post DFP (Fig. 3). These data are in general agreement with the effects noted for administration of sarin to the rat (Spradling *et al.* 2011) and together stand in contrast to the known anti-inflammatory responses expected for enhanced cholinergic agonism (Pavlov *et al.* 2003).

### CORT enhances DFP neuroinflammation in multiple brain regions

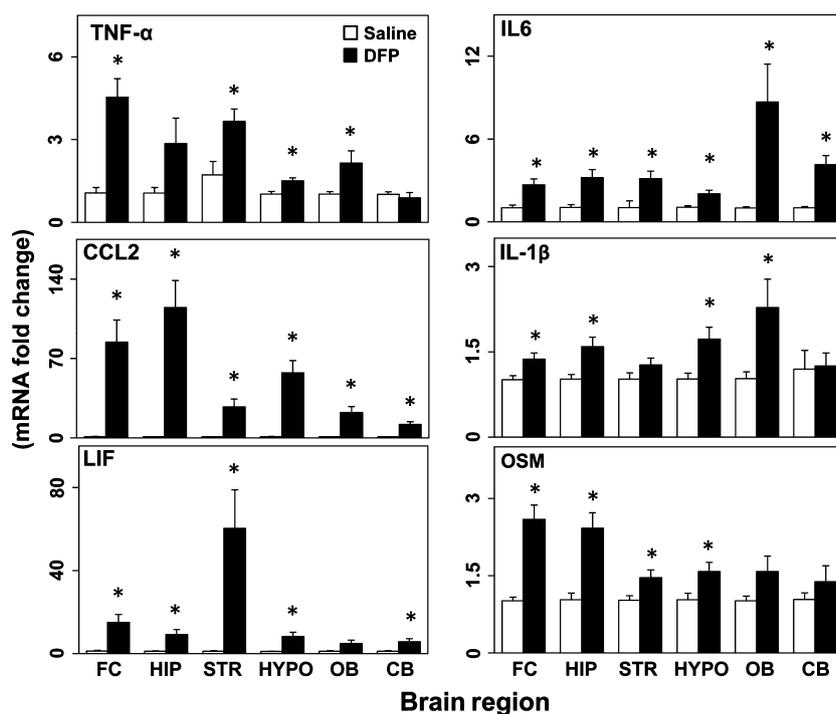
Many physiological stressors, for example, high and low ambient temperatures, threats of danger, physical exercise, were encountered in theater during the 1991 GW and may have contributed to the development of GWI (Sapolsky 1998). To emulate this possibility in our mouse model, we administered the stress hormone, CORT, in the drinking water for 7 days prior to administration of DFP. While an anti-inflammatory effect might be expected with a classic anti-inflammatory glucocorticoid, we found a marked exacerbation of the neuroinflammatory effect of DFP in all brain areas examined (Fig. 4). Again, as with DFP alone, the degree of the CORT-enhanced neuroinflammation varied by brain region and by cytokine/chemokine, but was observed in

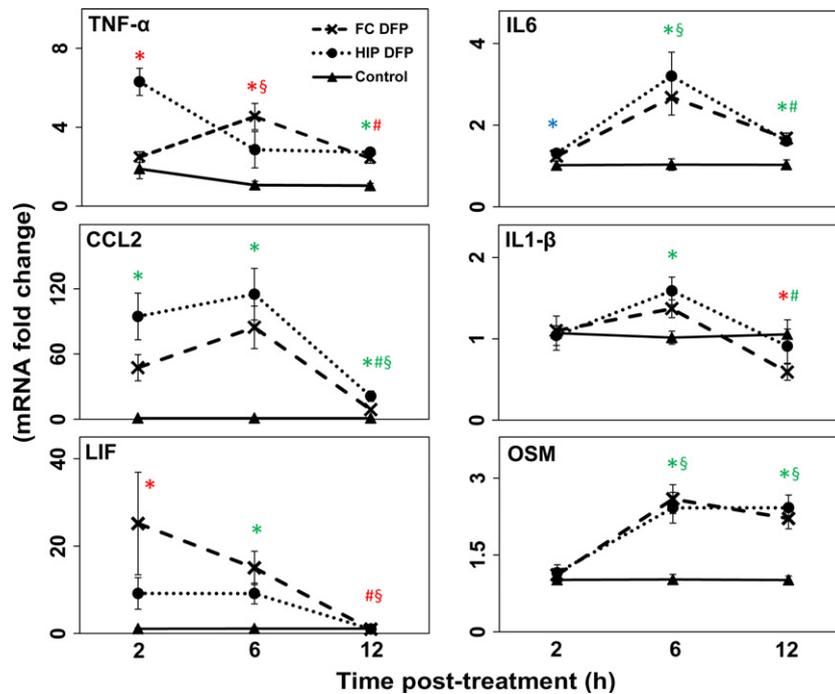
all areas sampled. Similar to the time course seen for DFP alone, CORT+DFP exposure resulted in a minimal neuroinflammatory response at 2 h post DFP dosing. At the 6 h time point, mRNA expression increased and then returned to control levels at 12 h in both the FC and HIP (Fig. 5). The potential for experimenter handling to be a stressor was assessed with a non-handled control group. Handling was found not to be a factor in any of the group values for DFP with or without CORT or with other pre-treatments (data not shown). It is noteworthy that exposure to DFP alone produced a 14% mortality rate, whereas exposure to CORT+DFP yielded a much lower mortality rate (4%).

### PB/DEET combination does not enhance DFP-induced neuroinflammation with or without prior CORT

Among the many potential exposures encountered by troops deployed to the 1991 GW were the prophylactic administration of PB to counteract nerve agent exposure and constant exposure to DEET to repel insects. Therefore, we administered both of these compounds for 2 weeks prior to acute exposure to DFP, and evaluated the subsequent proinflammatory response with and without prior administration of CORT. Across multiple brain regions and neuroinflammatory endpoints, PB/DEET alone did not produce a neuroinflammatory response. Furthermore, PB/DEET treatment did not enhance DFP-induced expression of proinflammatory cytokines and chemokines. Instead, for most proinflammatory mediators prior treatment with PB/DEET slightly suppressed the DFP-related neuroinflammation at both 2 (data not shown) and 6 h post DFP dosing time point (Fig. 6).

**Fig. 2** Diisopropyl fluorophosphate (DFP) causes neuroinflammation in multiple brain regions. Mice were administered DFP (4 mg/kg, i.p.) with saline (0.9%) as control and were killed at 6 h post dosing. Total RNA was prepared from frontal cortex (FC), hippocampus (HIP), striatum (STR), hypothalamus (HYPO), olfactory bulbs (OB), and cerebellum (CB) and for q-PCR analysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean  $\pm$  SEM,  $n = 5$ . Group differences between saline and DFP exposed mice were measured by Student's *t*-test, and statistical significant was measured at  $p < 0.05$  is denoted by \* as compared to saline controls.





**Fig. 3** Diisopropyl fluorophosphate (DFP) causes a time-dependent neuroinflammation in frontal cortex (FC) and hippocampus (HIP). Mice were administered DFP (4 mg/kg, i.p.) with saline (0.9%) as control and were killed at 2–12 h post dosing. Total RNA was prepared from FC or HIP for q-PCR analysis of tumor necrosis factor-alpha (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean  $\pm$  SEM,  $n = 5$ . Differences between mice in saline and DFP

groups and time course data were analyzed using a two-way ANOVA, with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \* as compared to saline control, # as compared to the 6 h time point within the appropriate brain region, and § as compared to the 2 h time point within the appropriate brain region. Symbols used to mark differences observed in the FC alone are in red, differences in HIP alone are in blue, and differences in both groups are marked in green.

Exposure to CORT during the pre-treatment with PB/DEET negated the slight protection PB/DEET had on DFP-related neuroinflammation (Fig. 7). The enhancement of DFP-induced neuroinflammation by prior exposure to CORT was largely unaffected by the 2-week exposure to PB/DEET (Fig. 7).

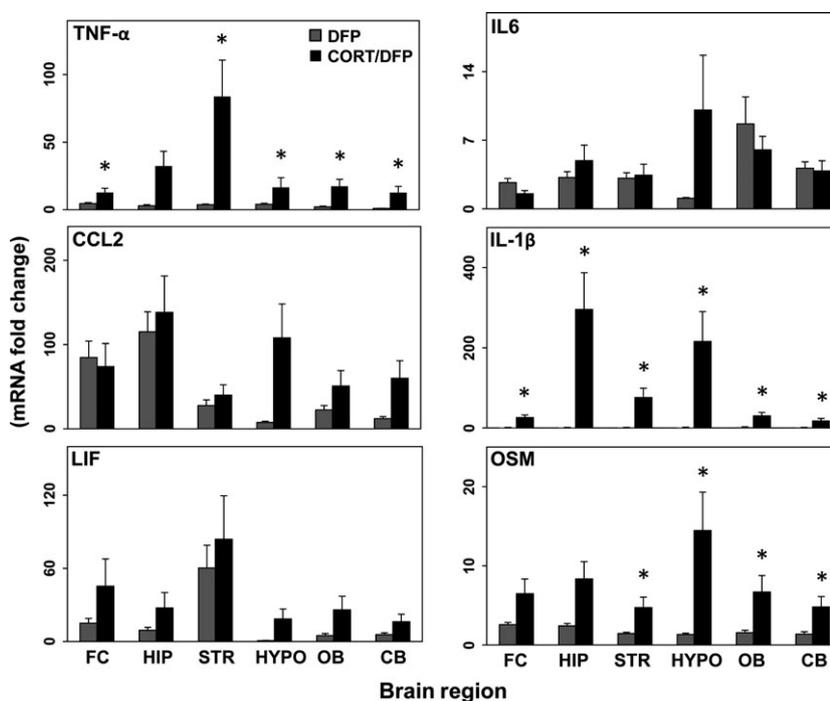
#### DFP with and without CORT+PB/DEET does not cause remarkable neurodegeneration or gliosis

Given the marked neuroinflammation observed with DFP, as well as the further exacerbation by CORT, we evaluated whether these effects were associated with neurodegeneration, astrogliosis, or microglial activation in the FC, HIP, STR, and HYPO. Representative images of the HIP are shown in Fig. 8. There was no evidence of neurodegeneration by sensitive FD Neurosilver™ staining in any brain region evaluated. One exception may be minor argyrophilic neurons in the dentate gyrus of the HIP (Fig. 8a); however, these changes are still not considered to be noteworthy. Fluoro-Jade B staining also was unremarkable in all regions in all treatment groups (Fig. 8b). Astrogliosis, as assessed by

immunostaining of GFAP, also was not observed in any brain regions, again with the possible exception of a minor increase in GFAP immunostaining in the CA1 region of the HIP (Fig. 8c). When we assayed GFAP content across multiple brain regions by ELISA, no treatment-related effects were observed (Fig. 9). Minor decreases in astrogliosis were observed in PB/DEET+DFP groups with and without CORT in the STR. Microglia activation based on Iba1 staining also did not reveal an activation of this cell type across the brain. Minor increases in microglial activation were seen in the CA1 region of the HIP in PB/DEET+DFP and CORT+DFP groups (Fig. 8d). Together these data are indicative of an absence of neurodegeneration and glial activation after DFP with and without prior administration of CORT and/or PB/DEET.

#### Minocycline pre-treatment suppresses neuroinflammation resulting from CORT+DFP

MINO is a tetracycline class antibiotic with anti-inflammatory properties. Previously, we showed that the neuroinflammatory response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine



**Fig. 4** Pre-treatment with corticosterone (CORT) enhances diisopropyl fluorophosphate (DFP)-induced neuroinflammation in multiple brain regions. Mice were administered CORT (200 mg/L) in the drinking water for 1 week prior to administration of DFP (4 mg/kg, i.p.) with saline (0.9%) as control and were killed at 6 h post dosing. Total RNA was prepared from frontal cortex (FC), hippocampus (HIP), striatum (STR), hypothalamus (HYPO), olfactory bulbs (OB), and cerebellum

(CB) for q-PCR analysis of tumor necrosis factor-alpha (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean  $\pm$  SEM,  $n = 5$ . Group differences between mice exposed to DFP and mice exposed to CORT+DFP treatments was measured using a Student's  $t$ -tests, and statistical significant was measured at  $p < 0.05$  is denoted by \* as compared to DFP alone.

(MPTP) and methamphetamine could be suppressed by prior treatment with MINO (Sriram *et al.* 2006). This raised the possibility that MINO could diminish the neuroinflammatory response to DFP with or without prior treatment with CORT. In both FC and HIP, MINO pre-treatment for 2 weeks suppressed some of the neuroinflammatory responses to CORT+DFP (TNF- $\alpha$ , IL6, CCL2, IL-1 $\beta$ , LIF, and OSM) with the drug exhibiting more effectiveness in the HIP than the FC (Fig. 10).

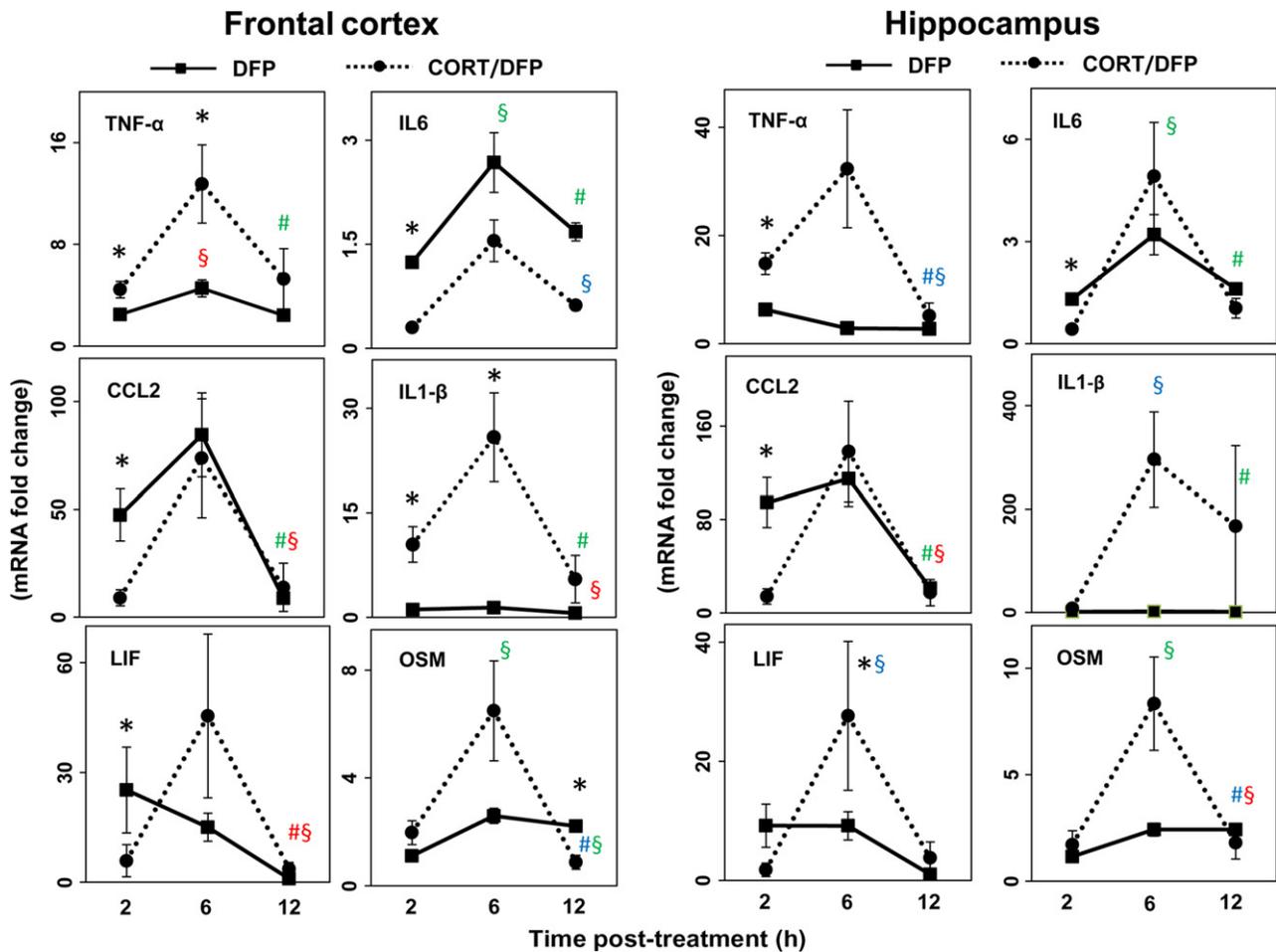
## Discussion

As many as 250,000 veterans of the 1990–1991 GW suffer from a chronic multi-symptom illness that resembles various aspects of sickness behavior, that is, symptoms that include fatigue, pain, memory problems, and GI disturbances. The etiology of GWI remains unknown but a variety of exposures in theater have been implicated including insect repellants, pesticides, the nerve agent sarin, and PB, the nerve agent antagonist widely self-administered during the War (RAC Reports 2008, 2014). The development of an animal model to mirror some of these exposures and their effects related to sickness behavior would provide a means to better define

GWI etiology and provide the basis for further research on effective treatments for this disorder.

Here, we demonstrate that administration of DFP, as a sarin surrogate, results in a brain-wide neuroinflammatory response affecting multiple cytokines and chemokines, effects known to underlie sickness behavior in animal models (Kelley *et al.* 2003; Dantzer *et al.* 2008; Henry *et al.* 2008; Huang *et al.* 2008). Pre-treatment with CORT as a high physiological stress mimic greatly exaggerated the neuroinflammatory response to DFP alone. Combined pre-treatment with PB and DEET, two other potential GW-relevant exposures, did not exacerbate neuroinflammatory responses following exposure to CORT and/or DFP, and in fact, had some suppressive effects on cytokine and chemokine responses.

While neuroinflammation can accompany or contribute to neural degeneration, it also can occur in the absence of damage (O'Callaghan *et al.* 2014) as a normal or exaggerated component of the acute phase response to an immune system insult (Godbout *et al.* 2005; Maes *et al.* 2007; Maes 2009; Wager-Smith and Markou 2011). These neuroinflammatory responses can be manifested chronically as episodic



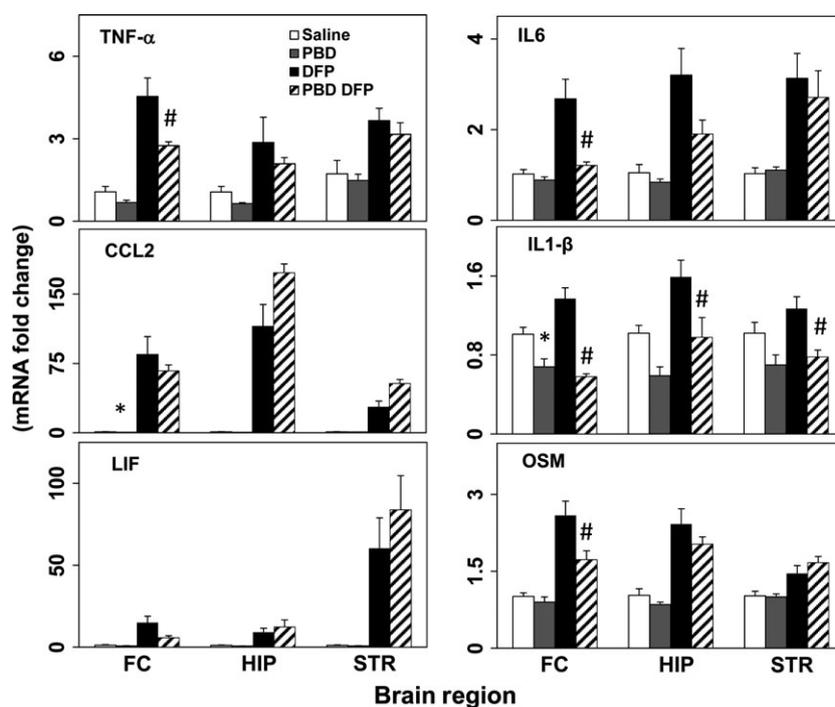
**Fig. 5** Pre-treatment with corticosterone (CORT) enhances diisopropyl fluorophosphate (DFP)-induced neuroinflammation in a time-dependent manner in frontal cortex (FC) and hippocampus (HIP). Mice were administered CORT (200 mg/L) in the drinking water for 1 week prior to administration of DFP (4 mg/kg, i.p.) with saline (0.9%) as control and were killed at 2, 6, and 12 h post dosing. Total RNA was prepared from FC or HIP for q-PCR analysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data

points represent mean  $\pm$  SEM,  $n = 5$ . Differences between mice in DFP and CORT + DFP groups and time course data were analyzed using a two-way ANOVA, with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \* as compared to DFP, # as compared to the 6 h time point and § as compared to the 2 h time point. Symbols used to mark differences observed in the DFP alone are in red, differences in CORT + DFP alone are in blue, and differences in both groups are marked in green.

sickness behavior, symptoms consistent with GWI. When we administered DFP to mimic the nerve agent exposures encountered in the GW theater, there were brain region 'hot spots' for a given cytokine/chemokine, but no clear pattern emerged that might provide insights for the basis of these effects. Indeed, it was surprising that DFP was proinflammatory as overwhelming enhancement of cholinergic signaling via irreversible inhibition of AChE should result in an anti-inflammatory effect (Pavlov *et al.* 2003). Nevertheless, these data are in general agreement with prior findings for sarin in a rat exposure model showing neuroinflammatory gene expression in multiple brain regions (Spradling *et al.* 2011). Increases in mRNA expression of inflammatory

markers often are attributed to activation of microglia in response to exposure to sickness behavior inducing compounds (e.g., LPS or poly I:C Buttini *et al.* 1996; Cunningham *et al.* 2007). Nevertheless, prior studies have reported that induction of inflammatory mediators by nerve agent (e.g., soman) may occur via neuronal rather than microglial or astrocyte activation (Angoa-Pérez *et al.* 2010). Regardless of the cell types involved in the neuroinflammatory responses we observed to DFP, it is tempting to assume that these effects are related to inhibition of AChE, a primary action of DFP. However, our findings may instead reflect organophosphorylation of other targets yet to be identified (e.g., see O'Callaghan 2003). AChE actions alone cannot account

**Fig. 6** Pyridostigmine bromide (PB)/*N,N*-diethyl-meta-toluamide (DEET) pre-treatment can reduce diisopropyl fluorophosphate (DFP)-induced neuroinflammation. Mice were administered PB (4 mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.) prior to administration of DFP (4 mg/kg, i.p). See Fig. 1 for dosing timeline. Saline (0.9%) was used as control and mice were killed at 6 h post dosing. Total RNA was prepared from frontal cortex (FC), hippocampus (HIP) and striatum (STR) for q-PCR analysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean  $\pm$  SEM,  $n = 5$ . The treatment effect of PB/DEET on mice exposed to DFP was analyzed using a two-way ANOVA, with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \*as compared to control, #as compared to DFP.

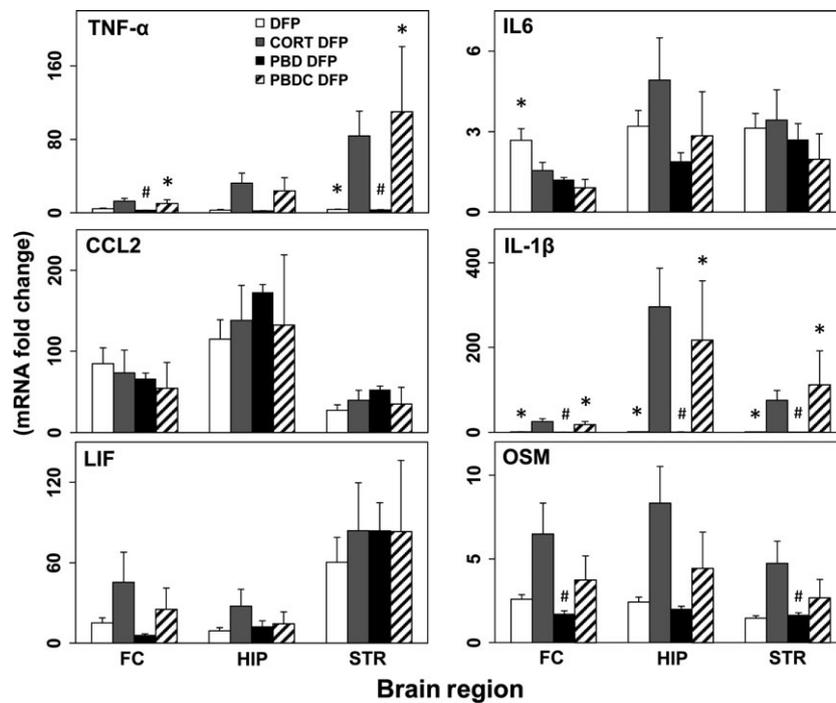


for the proinflammatory effects of DFP, because inhibition of AChE by PB was not proinflammatory (see also Revitsky *et al.* 2015).

A variety of physiological stressors were present during the GW and such stressors in combination with other GW exposures have been postulated to contribute to the development of GWI (Friedman *et al.* 1996; Sapolsky 1998). Moreover, physiological stress in the form of exercise can serve as a proinflammatory immune dysregulator (e.g., early and excessive activation of IL-1 $\alpha$ , IL-10, and IL-4) in ill veterans with associated adverse outcomes (e.g., increases in tender points, altered brain activation during memory recall tasks) (Cook *et al.* 2010; Broderick *et al.* 2013; Rayhan *et al.* 2013). When we used exogenous CORT as a stressor mimic prior to DFP, it was remarkably proinflammatory, causing a large increase in the DFP-induced expression of cytokines/chemokines brain wide. While it seems paradoxical that the classic anti-inflammatory stress hormone CORT can exacerbate neuroinflammation, such an effect has strong precedent. A number of papers demonstrate that prior treatment with CORT greatly exacerbates the neuroinflammatory effects of lipopolysaccharide (LPS) given systemically (Hains *et al.* 2011; Loram *et al.* 2011; Frank *et al.* 2014). In addition, we have shown that CORT given prior to a neurotoxic exposure to methamphetamine can enhance the neuroinflammatory and neurotoxic response to this drug (Kelly *et al.* 2012). Thus, at least when given prior to an inflammogen such as LPS or DFP or specific neurotoxins that cause neuroinflammation (e.g., methamphetamine) (Kelly *et al.* 2012; O'Callaghan *et al.* 2014), CORT can

exacerbate neuroinflammation. The basis for this CORT 'priming' response has not been delineated, and CORT does not necessarily generalize as a priming agent associated with other compounds that result in neuroinflammation. For example, while enhancing the neuroinflammation and neurotoxic effects of the dopaminergic neurotoxicant methamphetamine, CORT does not affect the neuroinflammation and neurotoxicity of another dopaminergic neurotoxicant, MPTP (O'Callaghan and Miller 2010). Regardless of the mechanism(s) underlying the propensity of prior CORT administration to exacerbate neuroinflammation, the CORT 'priming' of the DFP-related neuroinflammation is consistent with prior data obtained in animals for other agents and is also consistent with sickness symptoms observed chronically in GWI.

Among the many agents and conditions encountered in the GW, two were widely self-administered: PB pills to counteract any nerve agent exposure and DEET to ward off insects (RAC Reports 2008, 2014). We examined the effects of both of these compounds given repeatedly with and without CORT to mimic their ongoing usage in theater in the presence or absence of physiological stressors, and prior to DFP, to replicate a possible exposure to the nerve agent sarin. While epidemiological studies have implicated PB in GW etiology and symptoms (Schumm *et al.* 2002; Sullivan *et al.* 2003; Golomb 2008; Steele *et al.* 2012, 2015), we found little effect of this reversible AChE inhibitor and the insect repellent DEET on neuroinflammation. Indeed, PB/DEET appeared to somewhat diminish the neuroinflammatory responses to DFP, an action consistent with the utility of



**Fig. 7** Corticosterone (CORT) exposure negates the amelioration of diisopropyl fluorophosphate (DFP)-induced neuroinflammation by pyridostigmine bromide (PB)/*N,N*-diethyl-meta-toluamide (DEET). Mice were administered PB (4 mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.), with and without co-administration of CORT (200 mg/L) in drinking water prior to administration of DFP (4 mg/kg, i.p). See Fig. 1 for dosing timeline. Saline (0.9%) was used as control and mice were killed at 6 h post dosing. Total RNA was prepared from frontal cortex

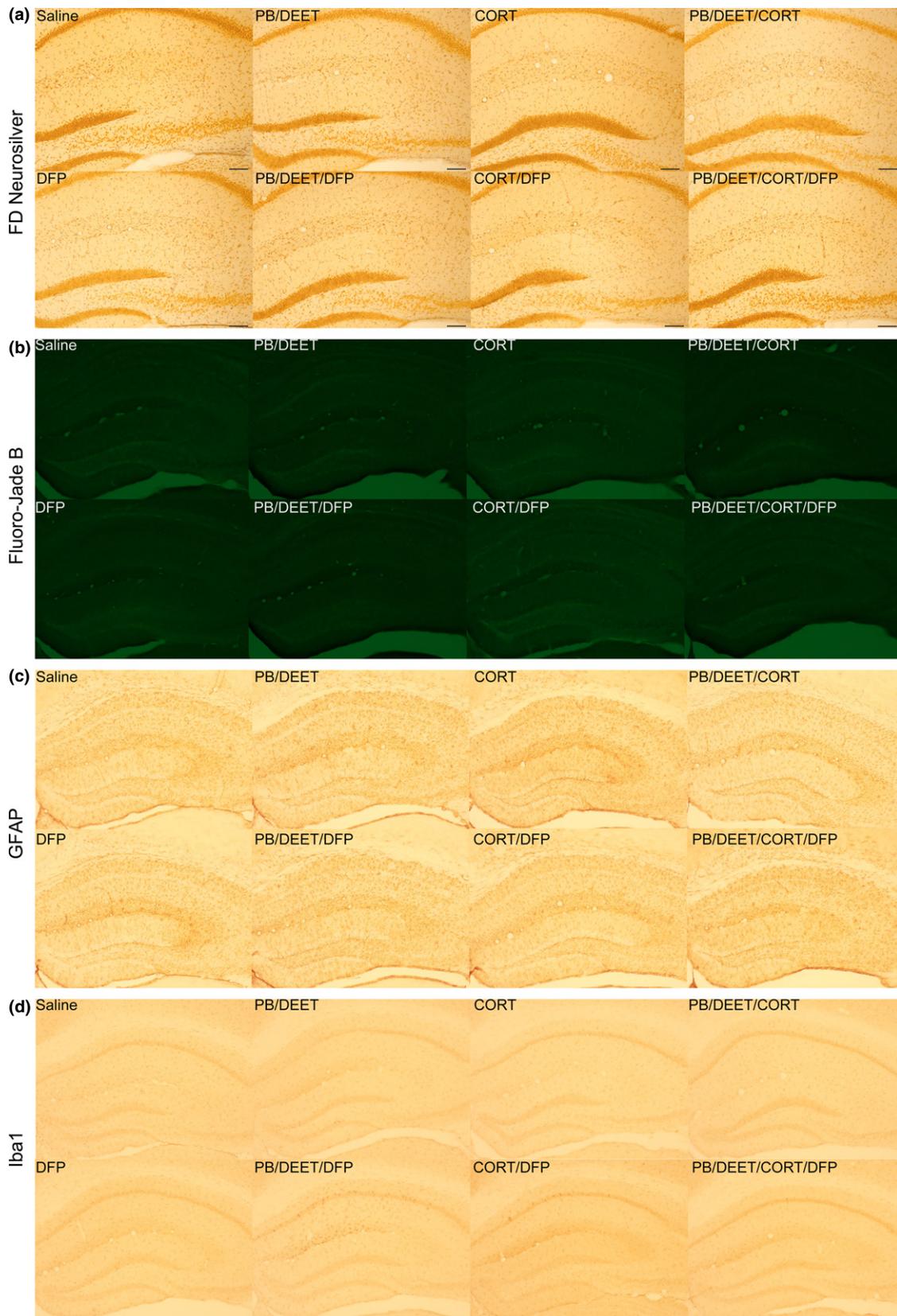
prior treatment with reversible AChE inhibitors to antagonize effects of irreversible AChE inhibitor nerve agents such as sarin and DFP. The combination of PB/DEET with CORT pre-treatments failed to enhance the CORT-primed neuroinflammatory effects of DFP and negated the PB/DEET-induced amelioration of DFP-induced neuroinflammation. These data argue that PB/DEET exposure are not major contributors to neuroinflammation caused by DFP, and serve to indicate that irreversible inhibition of AChE is involved in augmented neuroinflammation in the CNS. Together, these observations implicate non-AChE substrates of irreversible organophosphorylation as a basis of agent-related neuroinflammation.

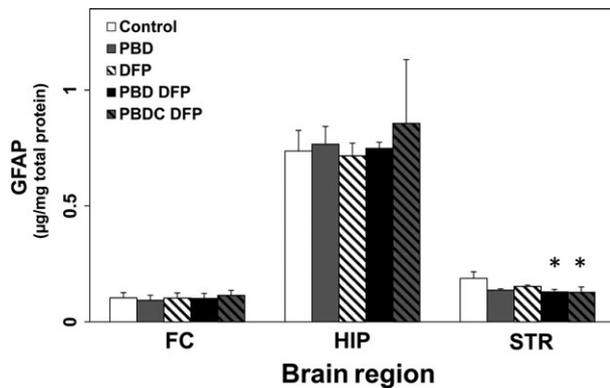
(FC), hippocampus (HIP), and striatum (STR) for q-PCR analysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL6, chemokine (C-C motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean  $\pm$  SEM,  $n = 5$ . Statistical significance was measured by one-way ANOVA with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \*as compared to DFP, #as compared to CORT+DFP.

Neuroinflammation can result from neurotoxicant-induced damage (O'Callaghan *et al.* 2008, 2014; Kelly *et al.* 2012), but also can occur in the absence of damage (O'Callaghan *et al.* 2014). To determine if the neuroinflammatory effects we observed were related to underlying neural damage, we used sensitive indicators (FD Neurosilver<sup>TM</sup> and Fluoro-Jade B staining) to assess neurodegeneration; astroglial (GFAP), and microglial (Iba1) activation markers were utilized to assess glial reactivity associated with possible neural damage. Our results showing the lack of remarkable changes in indices of neuronal damage as well as astrogliosis and microglial activation suggest that DFP, with and without prior treatment with CORT and/or PB/DEET, produced

**Fig. 8** Diisopropyl fluorophosphate (DFP) with and without prior pyridostigmine bromide (PB)/*N,N*-diethyl-meta-toluamide (DEET)+corticosterone (CORT) did not cause remarkable neurodegeneration or gliosis. Mice were administered PB (4 mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.), with and without co-administration of CORT (200 mg/L) in drinking water prior to administration of DFP (4 mg/kg, i.p). See Fig. 1 for dosing timeline. Saline (0.9%) was used as control and mice were killed at 24-h post dosing. Neurodegeneration as

indexed by silver disintegration staining (a) or Fluoro-Jade B staining (b) did not reveal evidence of neuronal damage in the hippocampus (HIP). Astroglial staining with glial fibrillary acidic protein (GFAP) (c) did not show evidence of astrogliosis; microglial staining with ionized calcium-binding adapter molecule 1 (Iba1) (d) did not reveal microglial activation in the HIP. Scale bars = 100  $\mu$ m (Silver) and 200  $\mu$ m (Fluoro-Jade B, GFAP, and Iba1).





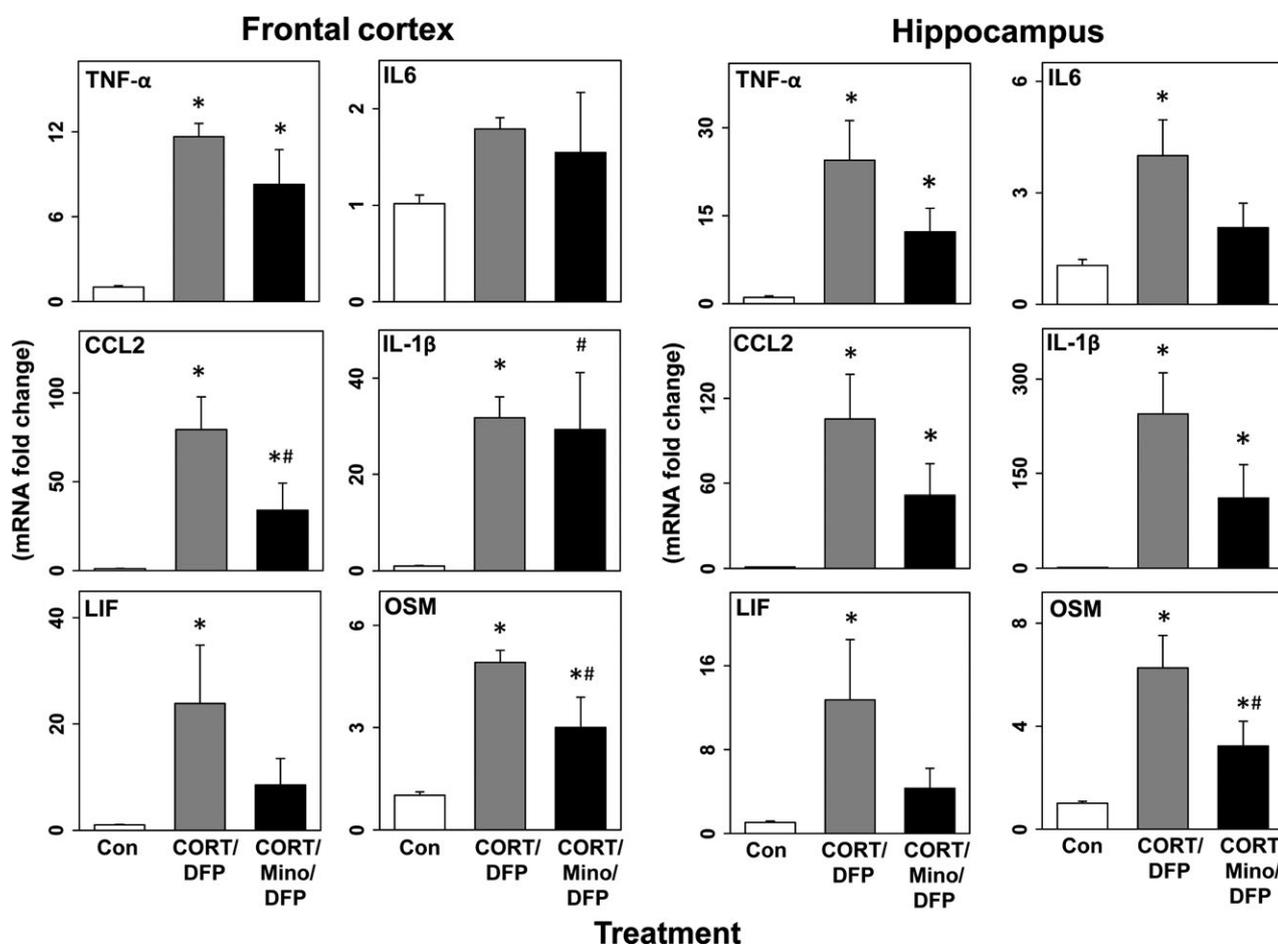
**Fig. 9** Diisopropyl fluorophosphate (DFP) with and without prior pyridostigmine bromide (PB)/*N,N*-diethyl-meta-toluamide (DEET)+ corticosterone (CORT) did not increase glial fibrillary acidic protein (GFAP) in multiple brain regions. Mice were administered PB (4 mg/kg/day, s.c.) and DEET (30 mg/kg/day, s.c.), with and without co-administration of CORT (200 mg/L) in drinking water prior to administration of DFP (4 mg/kg, i.p.). See Fig. 1 for dosing timeline. Saline (0.9%) was used as control and mice were killed at 72-h post dosing. Immunoassay of GFAP levels in frontal cortex (FC), hippocampus (HIP) or striatum (STR), as a quantitative index of astrogliosis did not reveal any significant effects as a function of any of the treatments. All data points represent mean  $\pm$  SEM,  $n = 5$ . Statistical significance was measured by one-way ANOVA with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \*as compared to control.

enhanced neuroinflammation in the absence of underlying damage. These findings stand in stark contrast to neuronal and glial responses we have seen following administration of a variety of known neurotoxicants (for reference images, see: Brock and O'Callaghan 1987; Balaban *et al.* 1988; Benkovic *et al.* 2004; Kelly *et al.* 2012; Little *et al.* 2002, 2012). Thus, the data from this study suggest that a brain-wide and perhaps persistent neuroinflammation can occur in the absence of underlying neural damage, as assessed by multiple sensitive markers of neuronal damage and glial activation. As these assessments were made soon after the dosing regimens were employed, it is nevertheless possible that neurodegeneration could emerge over days or weeks post-exposure that would not be evident in our analysis (e.g., see Abdullah *et al.* 2011; Ojo *et al.* 2014).

A main goal of developing an animal model of GWI was to provide a means to assess potential therapies. Because neuroinflammation was a central feature of the effects of DFP with and without prior CORT, we attempted to suppress these effects with an anti-inflammatory compound. Previously, we showed suppression of neurotoxicant-induced neuroinflammation by pre-treatment with MINO (Sriram *et al.* 2006); therefore, we used this non-traditional anti-inflammatory to attempt suppression of the neuroinflammation observed in our GWI animal model. In general, we observed a partial inhibition of the expression of several proinflammatory mediators, especially in HIP, by pre-

treating with MINO along with CORT prior to DFP. These data suggest that downstream effectors of the cytokines and chemokines targeted by MINO, for example, NF- $\kappa$ B and related transcription factors, are amenable to pharmacological intervention in our GWI animal model, a view consistent with past findings showing suppression of the phosphorylation of I $\kappa$ B $\alpha$  and NF- $\kappa$ B by MINO following exposure to inflammatory agents (Nikodemova *et al.* 2006; Bernardino *et al.* 2009; Tai *et al.* 2013). We administered MINO as a pre-treatment in this study to determine if it would suppress the development of neuroinflammation as it had in prior neurotoxicant exposure studies (Sriram *et al.* 2006). In future studies, we will administer MINO as a post-treatment to DFP and CORT+DFP to determine if neuroinflammation can be suppressed after it has fully developed, as may be the case for most ill GW veterans. As an already FDA-approved pharmacotherapeutic, MINO may prove of use in suppressing neuroinflammation not only in our animal model, but also in ill veterans.

Among the advantages of animal models of GWI are (i) they permit direct examination of tissue (especially brain), (ii) they allow for evaluation of persistent molecular, cellular, and functional effects associated with individual and combined exposures/conditions encountered in the GW, and (iii) they can be used to test specific hypotheses and evaluate potential therapeutic interventions. Because multiple classes of chemical exposures are implicated in GWI, and because non-chemical stressors also may have played a role in development of GWI symptoms, we evaluated several of these variables in our mouse model. Other investigators have also utilized rodents to develop models of GWI and have shown persistent behavioral and neurochemical changes following GWI-relevant exposures (Amourette *et al.* 2009; Barbier *et al.* 2009; Lamproglou *et al.* 2009; Abdullah *et al.* 2012; Parihar *et al.* 2013); these include evidence of delayed damage emerging months after exposures (Abdullah *et al.* 2012; Fiona Crawford, personal communication). The evidence obtained in our mouse model presented here primarily implicates nerve agent exposure, combined with high physiological levels of stress hormone, to produce a brain-wide neuroinflammatory response consistent with the molecular basis of sickness behavior, that is, symptoms exhibited by ill GW veterans. Data will be presented in a subsequent manuscript indicating that the mouse GWI phenotype presented here can persist in time to the equivalent of 20 years in man. The efficacy of MINO in suppressing expression of proinflammatory cytokines in our model provides a promising lead for future experiments in animals designed to determine if anti-inflammatory therapies can be used successfully to treat neuroinflammation long after it develops, as may be the case in GWI. A positive outcome of such studies should be of translational value for implementing therapeutic interventions in ill GW veterans. In summary, our findings are suggestive of a possible critical interaction



**Fig. 10** Pre-treatment with minocycline (MINO) partially suppresses neuroinflammation induced by corticosterone (CORT)+diisopropyl fluorophosphate (DFP) in frontal cortex (FC) and hippocampus (HIP). Mice were pre-treated with CORT (200 mg/L in drinking water) with and without co-administration of MINO (100 mg/kg/day, s.c.) prior to administration of DFP (4 mg/kg, i.p). See Fig. 1 for dosing timeline. Saline (0.9%) was used as control and mice were killed at 6-h post DFP dosing. Total RNA was prepared from FC and HIP for q-PCR analysis of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL6, chemokine (C-C

motif) ligand 2 (CCL2), IL-1 $\beta$ , leukemia inhibitory factor (LIF), and oncostatin M (OSM). All data points represent mean + SEM,  $n = 5$ . Statistical significance was measured by one-way ANOVA with Fisher's LSD Method of *post hoc* analysis,  $p < 0.05$  is denoted by \*as compared to control and #as compared to CORT+DFP. Previously, we have shown that MINO alone does not affect cytokine or chemokine expression using the regimen employed in this study (Sriram *et al.* 2006; O'Callaghan *et al.* 2008).

between physiological stressors of the GW theater and agent exposure(s) unique to this war; exposures in which the CNS is primed to amplify future exposures to pathogens, injury or toxicity and result in symptoms of sickness behavior characteristic of GWI.

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All experiments were conducted in compliance with the ARRIVE guidelines.

### Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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