

AWARD NUMBER: W81XWH-13-2-0072

TITLE: Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

PRINCIPAL INVESTIGATOR: Kimberly Sullivan, Ph.D

CONTRACTING ORGANIZATION: Boston University Medical Campus
Boston, MA 02118-2526

REPORT DATE: OCT 2018

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			<i>Form Approved</i> OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. REPORT DATE: OCTOBER 2018		2. REPORT TYPE ANNUAL		3. Report Type and Period Covered 30 Sep 2017 - 29 Sep 2018
4. Title and Subtitle Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)			5. Award Number W81XWH-13-2-0072	
6. Author(s) Kimberly Sullivan, Ph.D. E-Mail: tty@bu.edu				
7. Performing Organization Name Boston University Medical Campus Boston, MA 02118			8. Performing Organization Report Number (Leave Blank)	
9. Sponsoring/Monitoring Agency Name and Address U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. Sponsoring/Monitoring Agency Report Number (Leave Blank)	
11. Supplementary Notes				
12a. Distribution/Availability Statement Approved for public release; distribution unlimited			12b. Distribution Code	
13. Abstract (Maximum 200 Words) The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain- immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies. These studies are incorporating sufficient overlap of scientific content area to inform each other in a bench-to-bedside-to-bench approach. Results to date from the preclinical (animal) studies suggest a strong neuroinflammatory component to the illness model and provide important leads for treatment development approaches in the animal model before translation to the clinic. Clinical study recruitment is ongoing and has shown correlations between proinflammatory cytokine markers and behavioral and neuroimaging outcomes. Larger samples sizes will continue to make these inter-relationships clearer.				
15. Subject Terms: Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, pesticides, DFP, sarin 16. Security Classification			19a. NAME OF RESPONSIBLE PERSON: USAMRMC	
			19b. TELEPHONE NUMBER	
of Report a. U	b. ABSTRACT U	c. THIS PAGE U	17. LIMITATION OF ABSTRACT UU	

Table of Contents

	<u>Page</u>
1. Introduction.....	4
2. Keywords.....	4
3. Overall Project Summary.....	4
4. Key Research Accomplishments.....	37
5. Conclusion.....	38
6. Publications, Abstracts, and Presentations.....	38
7. Inventions, Patents and Licenses.....	42
8. Reportable Outcomes.....	42
9. Other Achievements.....	45
10. References.....	46
11. Appendices.....	49

1. INTRODUCTION:

The primary function of the Gulf War Illness (GWIC) consortium is to identify the pathobiological mechanisms of Gulf War Illness. The ultimate goal is to discover and characterize biomarkers of Gulf War illness and then identify targeted treatment strategies. The GWIC allows for the development of multidisciplinary collaborations targeting suspected brain-immune signaling alterations in GWI. The GWIC consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent priming of glial responses that cause a chronic activation loop of stronger and longer proinflammatory signaling effects between the immune system and the brain. The GWIC includes both clinical (human) and preclinical (animal and cell) studies and researchers in the 10 funded sub-studies.

2. KEYWORDS:

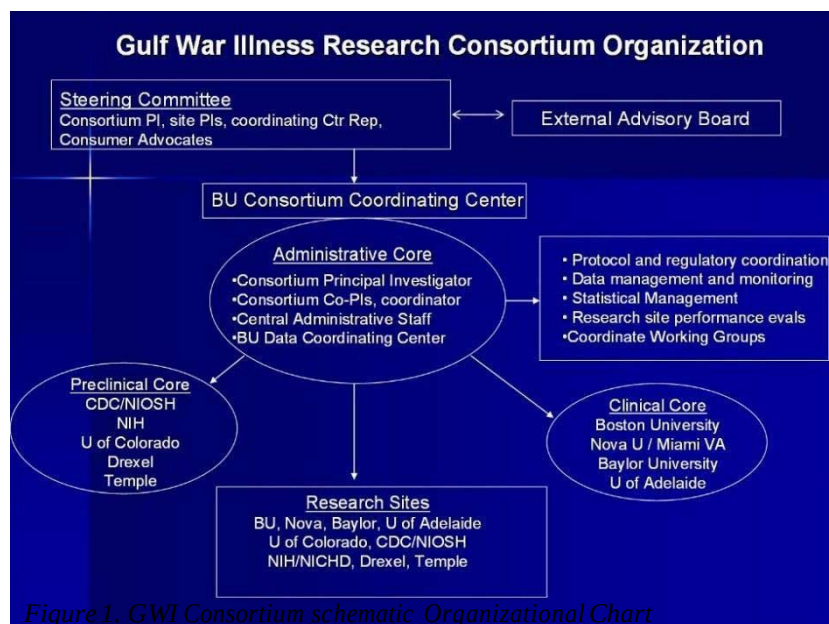
Gulf War Illness, consortium, CNS, innate immunity, cytokines, MRI neuroimaging, cognitive deficits, pesticides, DFP, sarin, CORT, genetics, objective biomarkers, treatment development

3. OVERALL PROJECT SUMMARY:

INTRODUCTION

Background. Twenty seven years after the 1991 Gulf War, 25-30% of the nearly 700,000 U.S. troops who served in the war still suffer from the debilitating symptomatic illness known as Gulf War Illness (GWI) (White et al., 2016; RAC, 2008, 2014, IOM, 2010). A growing body of evidence indicates that GWI is associated with diverse central nervous system (CNS) and immune alterations, but the specific pathobiological processes driving GWI symptoms have not been clearly elucidated (Zhang et al., 1999; Sullivan et al., 2003; Heaton et al., 2007; Toomey et al., 2009; Whistler et al., 2009; Broderick et al., 2011; Chao et al., 2011; Sullivan et al., 2013; White et al., 2016; Abou-Donia et al., 2017; Janulewicz et al., 2017). Animal studies indicate that a chronic CNS inflammatory state can develop in response to an insult—chemical injury, infection, or physical trauma (including mild traumatic brain injury)—that mobilizes CNS defense systems via activation of glia, the brain’s primary immune response cells, and release of chemical messengers that precipitate a complex of “sickness behavior symptoms” identified by measures of impaired memory and learning, increased pain sensitivity, and persistent fatigue, a symptom complex similar to that of GWI (Rathbone et al., 2015; Banks & Lein, 2012; Watkins et al., 2007; 2009; Zhang et al., 2010). Recent studies have also demonstrated CNS inflammatory effects of GW-related exposures and additional immune and cellular processes that plausibly explain the mechanisms contributing to the full spectrum of GWI symptoms (Janulewicz et al., 2018; Abou-Donia et al., 2017; Koo et al., 2017; Rao et al., 2017; Qiang et al., 2017; Locker et al., 2017; Emmerich et al., 2017; Abdullah et al., 2016; O’Callaghan et al., 2016; O’Callaghan et al., 2015; Milligan et al., 2009; Rivest et al., 2009; Spradling et al., 2011).

Consortium Management and Expertise. This multidisciplinary collaboration brings together established GWI researchers, and leading experts in brain-immune processes associated with neurotoxicology and neuroinflammation, damage to white matter and axonal transport, immunology, and immunogenetics. This team has designed a body of interrelated studies linked together by a cohesive model of ‘brain-immune interactions’ as the basis for GWI. The consortium is led by Dr. Kimberly Sullivan, at Boston University (BU), whose extensive background in GWI research includes contributions in identifying effects of Gulf War exposures on brain structure and function (Sullivan et al., 2003; Sullivan et al., 2013; Yee et al., 2015; Yee et al., 2017; Janulewicz et al., 2017; Janulewicz et al., 2018; Sullivan et al., 2018). BU serves as the Coordinating Center for the Gulf War Illness Consortium (GWIC) and provides the Administrative and Data Management Cores (figure 1). The consortium also includes a Preclinical Core, consisting of experts at five sites who are working collaboratively to characterize the persistent neurological and immune effects of GW exposures at the physiological, tissue, and cellular levels. This is done



in parallel with human studies conducted by the Clinical Core at three recruitment sites (and two additional laboratory sites) to characterize the specific profile of brain, immune, and genetic measures that distinguish veterans with GWI from healthy controls. The GWIC Steering Committee and External Advisory Committee monitors research progress and findings, and advises on research modifications and follow-up.

Objective. The primary objective of the Boston GWI consortium is to provide a cohesive understanding of the pathobiological mechanisms for the symptoms of GWI in order to provide a rational and efficient basis for identifying beneficial treatments and diagnostic markers.

Research Plan. The consortium is undertaking a coordinated series of clinical and preclinical studies aimed at providing a comprehensive understanding of the pathobiology of GWI. This includes clinical case-control studies conducted in parallel at 3 subject recruitment sites—Boston, Miami, and Houston—that include a total of 300 Gulf War veterans. Clinical assessments include a) advanced neuroimaging protocols (MRI, DTI, fMRI, PET) that assess brain volumetrics, white matter integrity, and CNS inflammatory indicators, b) neuropsychological assessment of cognitive function, c) blood levels of cytokines and other immune signaling molecules, d) genetic expression of immune markers, e) pilot assessment of cerebrospinal fluid levels (CSF) of cytokines and neurotransmitters (in subgroup of Boston cohort), f) immunogenetic markers of innate immune responsivity, f) longitudinal assessment of brain-immune measures. Parallel preclinical studies are evaluating persistent effects of GW neurotoxicants *in vitro* and in rodent models of GWI. Preclinical studies are evaluating cellular effects of GW neurotoxicants on a) axonal transport, b) glial cytokine production, c) neurotransmitter signaling, d) myelination, and e) oligodendrocyte proliferation. Animal studies are determining the effects of GW exposures on: a) priming and maintaining glial activation, differentiating effects on astrocytes vs. microglia, b) glial activation in relation to development of learning impairment and chronic pain sensitivity, c) brain and blood levels of proinflammatory cytokines, and d) genetic expression of immune and inflammatory markers in brain and blood. Findings from clinical and preclinical studies are being compared and used to identify specific brain-immune pathways that can be targeted for intervention by a variety of glial modulating and other currently available treatments. Treatment compounds are being tested in animal models to determine their effectiveness for resolving or ameliorating the pathobiological processes associated with GWI. Figure 1 represents the hypothesized mechanisms for GWI that are being tested by this planned series of preclinical and clinical experiments.

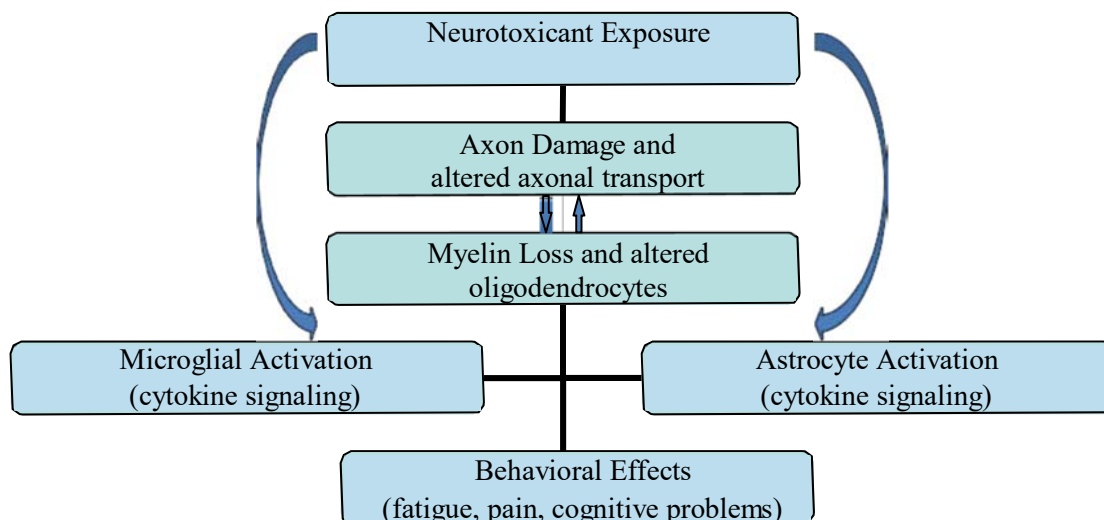
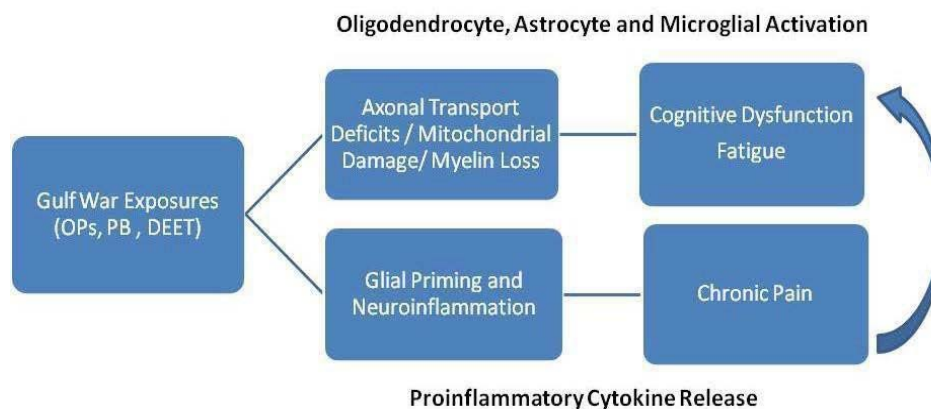


Figure 2. Schematic Representation of Hypothesized GWI Mechanisms

The GWI consortium central hypothesis identifies chronic neuroinflammation as an end result of initial glial activation and subsequent *priming* of glial responses that cause a chronic activation loop of stronger and longer proinflammatory effects between the immune system and the brain. Figure 2 below represents the integrated theory of GWI being tested in the consortium studies.

INTEGRATED THEORY OF GWI



The overall aims of this integrated multidisciplinary consortium scientific focus are to (1) To identify validated markers of GW illness by using state of the art neuroimaging, behavioral, genetic and blood markers of neuroinflammatory activation in both clinical and preclinical models that will elucidate targeted and validated treatment strategies (2) To create a Neuroinflammation Risk Profile for GWI (3) To identify viable mechanistic treatments based on identified pathophysiological pathways of GWI that have been validated in preclinical treatment models.

BODY

The approved statement of work for the entire study period is below:

STATEMENT OF WORK

Table 1. Brain-Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium

Task 1. Obtain necessary authorization prior to initiation of human subjects' and animal studies research (months 1-8)
1a. Attend pre-award meeting with CDMRP GWIRP program staff
1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements
1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals
1d. Obtain data use agreement from Hines VA for stored blood sample study
1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center for Disease Control/NIOSH, National Institutes of Health, Drexel University, Temple University and University of Colorado
1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members
Task 2. Preparation for consortium clinical studies (months 1-9)
2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.
2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.
2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies.
2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.
Task 3. Preparation for consortium preclinical studies (months 9 - 24)
3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-Colorado for planned studies of axonal transport, myelin integrity and learning and pain assessments.
3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.
Task 4. Perform preclinical cell and animal studies (months 9-42)
4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel - 30 Sprague Dawley rats, Temple - 27 Sprague Dawley rats).
4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (NIH – 624 NIH/S mice and 208 rats).
4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the chronic nature of GWI (CDC – 100 C57BL/6 mice).

4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate “drugable” targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129-Cx3CR1 mice).
4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado – 120 rats).
4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from GWI neuroinflammatory rodent models (CDC – 60 rats, Nova).
4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado)(20 animals per treatment).
Task 5. Screening, recruitment and assessment of Gulf War veterans from three sites (months 9-42)
5a. Obtain informed consent from potentially eligible GW veterans
5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva samples.
5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis.
5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.
5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.
5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis
5g. Conduct preliminary analyses of clinical data
Task 6. Recruitment and assessment for Boston CSF and PET studies (months 24-42)
6a. Perform lumbar punctures to obtain cerebrospinal fluid markers of neuroinflammation in 50 GW veterans.
6b. Perform positron emission tomography (PET) scanning with novel EAAT2 ligand in partnership with RIO pharmaceuticals in 15 GW veterans.
6c. Perform FDG-PET scan imaging with 30 GW veterans after a computerized CPT cognitive challenge task.
Task 7. Interim Analyses, Grant Submission, and Annual Reporting (Months 18-42)
7a. Data entry of all questionnaires, evaluations and quality control measures will be ongoing
7b. Interim Statistical analyses of data obtained from cognitive evaluations, blood markers, neuroimaging and questionnaire data will be performed periodically.
7c. Grant submissions to relevant funding agencies for further collaborative studies based on initial results and preliminary data targeted toward treatment strategies will be ongoing.
7d. Annual reports of progress will be written.
Task 8. Final analysis and Report Writing (months 42-48)
8a. Statistical analyses comparing brain MRI volumetrics, cognitive functioning, health symptom report and cytokine/chemokine markers in veterans with and without GWI

8b. Statistical analyses of correlations between clinical and preclinical neuroinflammatory markers of GWI models
8c. Perform longitudinal assessments of imaging, cognitive, health symptom and cytokine functioning in veterans with and without GWI
8d. Perform validation analysis studies of identified biomarkers of GWI using an unrelated sample of stored blood and cognitive health symptom data from a prior CSP study.
8e. Write final study report
8f. Present findings at scientific meetings
8g. Prepare manuscripts for submission
8h. Write grant proposals based on consortium findings and identified treatment avenues for GWI.

The statement of work for year 5 is inclusive of Tasks 1-8 above. The statement of work for year 5 primarily describes the completion of the initial 10 sub-studies. In addition, in year 5, the plan was to have cell and animal studies all underway or completed, reporting final results and publishing manuscripts. The plan was also to continue with subject recruitment for the clinical studies and to recruit 145 study participants for the study protocol including cognitive evaluations, interviews, neuroimaging and specimen collection. Progress toward completing each task is listed below and due to some delays in finishing the ten studies, a no-cost extension was requested and approved for a sixth year to complete the GWIC studies.

TASK 1. OBTAIN NECESSARY AUTHORIZATION PRIOR TO INITIATION OF HUMAN SUBJECTS' AND ANIMAL STUDIES RESEARCH (MONTHS 1-8)

Task 1a. Attend pre-award meeting with CDMRP GWIRP program staff

Due to delays in funding the consortium as a result of the government shutdown, the pre-award meeting was held in February 2013 and was considered a post-award meeting. The meeting included an overview of study hypotheses and plans as well as a review of the consortium administrative and core center structure. The Consortium PI, Dr. Sullivan and other steering committee members were present at the meeting in addition to CDMRP commanders, grants officer's representative (GOR) and administrative staff. Required External Advisory Board (EAB) meetings have also begun to meet with the first meeting being held in September 2014. Subsequent EAB meetings were held in April 2015, October 2015, May 2016, November 2016; May 2017 and November 2017. The EAB provided helpful suggestions and comments for study progress and discussions for future meetings that have occurred semi-annually during the consortium funding period.

Task 1b. Obtain final Institutional Review Board (IRB) approval for clinical research sites at Boston University School of Public Health (BUSPH), Baylor University and Miami VA/Nova University for protocols and advertisements

IRB and HRPO approvals have been submitted and approved for all three clinical sites at Miami VA/NOVA University, Boston University (BU) and Baylor College of Medicine and renewed as required. University of Adelaide received exempt status from their local IRB.

Task 1c. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals

HRPO submissions have been submitted and approved for all three sites at Miami VA/NOVA University, Boston University and Baylor Medical College.

Task 1d. Obtain data use agreement from Hines VA for stored blood sample study

All relevant blank study forms from CSP 458 have been obtained and reviewed in order to generate a definition of CMI based on the Kansas definition. GWIC investigators have finalized the definition to compare how many subjects meet criteria for the CDC definition of CMI, the Kansas definition or both. This

has informed the selection of the blood samples to analyze. Clarification from Dr. Steele regarding a few details of the variables in the algorithm to apply the Kansas GWI definition to CSP#458 data have been obtained and subjects can now be compared for CMI and Kansas criteria and cytokine outcomes once the Data Use Agreements between Hines VA and BU and Miami VA are approved.

The DUA document has been finalized between BU and Hines VA and signed by the BU attorney. We are still waiting for the DUA to be finalized between Dr. Klimas at Miami VA and the Hines VA. The Hines VA will not submit the BU DUA to Central Office without also having the Miami DUA to submit with it at the same time. Once the DUA is approved, we can begin sending blood samples to Dr. Klimas at the Miami VA. Dr. Klimas will perform bloodwork and send results to Hines. Hines will conduct statistical analyses.

Task 1e. Obtain final protocol approval by the respective Institutional Animal Care and Use Committees (IACUC) approval for the preclinical animal research sites at Center for Disease Control/NIOSH, National Institutes of Health, Drexel University, Temple University and University of Colorado

All local IACUC approvals have been obtained from CDC, NIH, Temple and University of Colorado. BU offsite IACUC approvals have been obtained for all animal study sites. ACURO final approvals have also been obtained for all pre-clinical sites and renewals are submitted for approval as they are required for 3-year re-writes.

Task 1f. Complete hiring of necessary staff and ensure all mandatory IRB and IACUC research related trainings are completed by all staff members

Hiring of local post-docs and research assistants has been ongoing for each site. BUSPH hired and trained a new consortium project coordinator and Dr. Steele hired and trained a research assistant for the clinical studies at Baylor. All current staff have completed IRB and IACUC trainings necessary for their work with animal and human studies. The Miami site has hired and trained an additional research assistant.

TASK 2. PREPARATION FOR CONSORTIUM CLINICAL STUDIES

The consortium coordinating center and Administrative Core at Boston University has led many monthly web and in-person meetings during the past year to oversee and interact with the clinical study sites. A significant amount of time and effort was devoted to maintain all required study and test administration materials, training new staff members and to developing centralized web-based data collection materials for the consortium studies. Table 2 lists these planning meetings. Smaller working group meetings were also held during the past year to plan for particular consortium topic areas. The Working Groups are described in Table 3. Since subject recruitment has begun, considerable time has been spent with training and quality control assurance meetings of clinical staff to ensure consistent inter-rater reliability and to reduce any administration drift from standard testing and scoring procedures. Baylor College of Medicine site in Houston has begun recruitment and clinical appointments. Boston, Miami and Texas sites added new clinical staff to their teams. With these new changes, training on test administration and quality control measures have continued for all three sites.

Table 2. GWIC Monthly Planning and EAB Meetings for 2018

Date	Type of Meeting	Discussion Items
01-03-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
02-07-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
03-07-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
04-04-2018	Monthly Web-meeting	Pre-clinical and clinical study updates

05-02-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
06-06-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
07-04-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
08-01-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
09-05-2018	Monthly Web-meeting	Pre-clinical and clinical study updates
10-03-2018	Monthly Web-meeting	Pre-clinical and clinical study updates

Table 3. Consortium Working Groups

Working Group	Tasks	Members
Data Management Service Group	Assist with QC issues, data cleaning, data management and sharing, website management.	Joe Palmisano, DCC Consortium PI, co-PIs
Statistics Service Group	Perform analyses and provides statistical planning and advice for study investigators and research site PIs.	Timothy Heeren, Joe Palmisano, Consortium PI/co-PIs
Translational Working Group	Forum for Intellectual property and material (IP) issues, translation of results into papers, abstracts, new grant submissions and how clinical and preclinical results can inform each other.	Michael Pratt – BU Tech Transfer office Consortium PI, co-PIs Research site PIs, RIO
Behavioral Studies Working Group	Plan imaging protocols and provide quality control for multiple imaging sites. Plan behavioral testing protocols and coordinate preclinical and clinical studies for comparability.	Drs. Sullivan, Killiany, Krengel, Toomey, Steele, Klimas, Collier, Hutchinson, Maier, Watkins
Histopathology Working Group	Plan tissue studies of proinflammatory, glial, axonal transport and mitochondrial markers in similarly dosed animal and cell models.	Drs. Baas, Black, O’Callaghan, Fields, Maier, Watkins
Immune Genetics Working Group	Plan and implement studies assessing brain-immune interactions involving glia and proinflammatory cytokines/chemokines through genetic SNPs and mRNA and miRNA protein studies.	Drs. Collier, Hutchinson, Klimas, Steele, Sullivan, Watkins, Maier
Gulf War Veterans Advisory working Group	Update fellow GW veterans about GWIC research efforts and results, assist with recruitment efforts by making fellow vets aware of GWIC studies.	Denise Nichols, Frances Perez Wilhite, Lynn Santosuosso, Tim Demers, Christine Tron, Jim Arrocho

Task 2a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire consortium including all preclinical and clinical sites.

Consortium website (<http://sites.bu.edu/gwic/>) and other social media pages are finalized and approval was obtained from each institution to use their logos on the site. They are updated regularly and are a primary source of subject recruitment. Electronic data collection forms using REDCap software and CATI recruitment software

are finalized and in use for subject screening and data collection. The study is utilizing Frontier Science's LDMS specimen tracking system for shipping samples to collaborating sites and for biorepository tracking. Training for the specimen tracking system has also been completed by all necessary staff. A refresher REDCap training was completed for all clinical staff and is in use as subjects are recruited and complete online questionnaires and screened for eligibility through the CATI system.

Task 2b. Develop manuals for the neuropsychological testing protocol, imaging protocols, specimen collection protocols and recruitment.

All cognitive administration and scoring manuals, specimen collection protocols have been finalized. All clinical staff has been trained to ensure proper quality control measures are in place for the clinical studies. This has been followed up by videotaping practice testing to ensure tester drift is not occurring and weekly phone calls are being conducted with testing staff as subject recruitment gets underway to answer any questions or discuss problems with test administration/scoring issues. This has proven helpful to ensure consistent test administration and scoring at all study sites.

Task 2c. Train researchers and staff on protocols and quality control measures for the clinical and preclinical studies

Training for researchers and clinical staff was completed at in-person meeting in Boston in August 2014 and continued to be monitored as described above. Working groups have finalized training procedures and protocols for cognitive, neuroimaging and laboratory procedures that are currently being used. As new staff members are added, they are trained appropriately.

Task 2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.

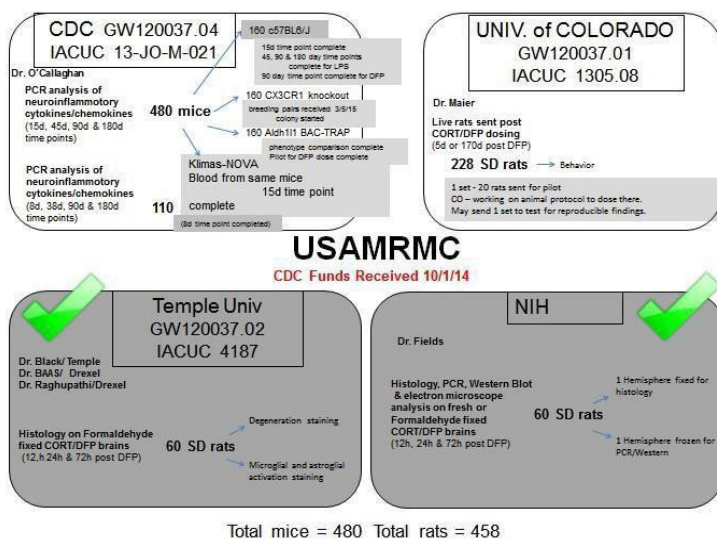
Dr. Toomey has obtained local Boston University exempt IRB status for this project. Dr. Klimas has obtained local IRB approval from Miami VA IRB for the study. The DUA document that was finalized between BU and the VA and signed by the BU attorney is being put on a newer DUA template from Hines VA staff and we are currently waiting on the new version for review and signature. The Miami VA IRB has approved the DUA and sent it to the Hines VA and we are waiting for their revisions on the draft.

Following this review and approval, they will be submitted to VA Central Office. Dr. Toomey submitted an application to HRPO for the BU and Miami VA sites and they concurred with the exempt determination of the protocol. Once the HRPO approval is final, the Hines VA will submit all materials to VA Central Office for approval and then the blood samples can be sent to Dr. Klimas for analysis.

TASK 3. PREPARATION FOR CONSORTIUM PRECLINICAL STUDIES (MONTHS 9 - 24)

Monthly web meeting and working group meetings were ongoing during the past quarter to prepare for the planned preclinical treatment and new pilot studies and to coordinate overlap of the studies and to ensure that the same neurotoxicant dosing and exposure model of GWI are used in the pilot studies. The CDC site was tasked with comparing the mouse and rat models of GWI to ensure comparability for planned studies and to distribute dosed animals and animal tissue to the preclinical sites which they have successfully done.

Figure 3. Distribution schematic of animals from CDC



This has resulted in several published preclinical and translational papers (Kelly et al., 2018; O'Callaghan et al., 2017; Koo et al., 2017; Rao et al., 2017; Qiang et al., 2017; Locker et al., 2017; Emmerich et al., 2017; Abdullah et al., 2016; O'Callaghan et al., 2016; Fields et al., 2017). Dr. O'Callaghan has traveled to UCSF to assist with the PET animal pilot dosing study with Dr. Gerdes at RIO pharmaceuticals to begin the pilot study.

Task 3a. Prepare rat dosing models at CDC and distribute to other sites at NIH, Drexel, Temple and U-COLORADO for planned studies of axonal transport, myelin integrity and learning and pain assessments.

Dr. O'Callaghan at the CDC site prepared and validated rat dosing models based on his initial mouse GWI dosing models from prior DOD funded studies using chronic daily corticosterone (CORT) and 1 dosage of the sarin-surrogate DFP (O'Callaghan et al., 2015).

CDC sacrificed 20 adult male rats [control group (n= 5), CORT only exposed group (n= 5), acute DFP exposed group (n= 5), and chronic CORT and DFP group (n= 5)]. Animals in CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). Rats were perfused and brains were preserved in 10% formalin brains. Perfused brains were then sent out to Dr. Killiany and Boston University collaborators for brains to be structurally imaged. These results were published in Koo et al., 2017.

CDC has exposed rats to the same conditions described above and has shipped flash frozen and formalin preserved brains of rats sacrificed at 7, 14 and 21 days post-DFP exposure to NIH collaborators. Animals in the CORT groups were exposed to 200mg/L in their drinking water for 4 days followed by a single s.c. injection of saline (CORT group) or 1.5mg/kg DFP (CORT and DFP). Rats in the control and DFP groups were given standard tap water for 4 days followed by a single s.c. injection of saline (Control) or 1.5mg/kg DFP (DFP). The 7, 14, and 21 day time points have allowed for investigation of neuronal changes or degradation at further time points post condition exposure and showed important changes over time. Initial NIH investigator results were published in Fields et al., 2017.

Task 3b. Develop co-cultures of rodent oligodendrocytes in cell culture chambers for electrical stimulation of axons and development of myelination in vitro at NIH.

Dr. Fields and Dutta have made cell cultures from embryonic mice and rats for studies of myelination and the co-cultures are developed in the cell culture chambers for the ongoing oligodendrocyte and myelin studies.

Cholinergic neurons were myelinated in co-culture with oligodendrocytes, providing a framework to study the effects of GW agents on axonal myelination by oligodendrocytes. Techniques were developed to produce purified motor neuron cultures. OPCs have now been combined with these neuronal cultures in multicompartments chambers for electrical stimulation of axons and treatment with pharmacological agents.

GW agents primarily disrupt cholinergic neurotransmission (Fields et al., 2017). Like neurons, oligodendrocytes express several muscarinic receptors, types 1-5, and our preliminary research shows that oligodendroglia can respond to cholinergic stimulation. We have acquired and housed muscarinic receptor 1-5 KO mice, from which oligodendrocytes have been isolated and cultured with GW agents, so as to definitively delineate the role of these agents on oligodendrocyte biology and development.

NIH investigators also received brain tissue from CDC to perform histopathology studies of myelin from exposed animals. The brains from rats exposed to neurotoxicants by Dr. O'Callaghan's lab were analyzed for changes in

myelin and other proteins by western blot. This included 60 samples (brains), 12 treatment conditions, 3 time points, (12, 24 and 72 hr) for the proteins olig 2, MBP, and GAP-43. The results show that myelin proteins are affected, but the result is surprising. Rather than decreasing, as would be expected with myelin damage, we find an increase in myelin basic protein (MBP) levels increase significantly 72 hrs after treatment. This result, however, shows that myelinating glia are being affected by the treatments modeling exposures that patients with GWI are likely to have experienced. This is an important finding with respect to the research on white matter damage found in GWI. We suspected that the increase in MBP at this time point may reflect an adaptive response to the toxicants in an attempt to recover from white matter injury. Histological analysis of the same brains have allow us to investigate this. We performed microtome sectioning of these brains and immunocytochemical staining to assess this further.

Dr. Field's lab also investigated levels of olig2, a transcription factor for oligodendrocytes, to determine if oligodendrocytes are dying or increasing in number after treatment. No change in this protein was observed. Dr. Field's lab also tested for changes in GAP-43, because this protein is a marker for axon sprouting. One possibility for an increase in MBP after treatment might have been that axons were damaged and had begun to sprout in recovery. These sprouts would then need to be myelinated, which could account for the increase in MBP protein we found after treatment. This hypothesis was not supported. GAP-43 levels are not different among treatment groups.

Interestingly, the only treatment condition where MBP levels changed was in the CORT+DFP group. Neither DFP nor CORT alone caused a change. This result is consistent with other results that Dr. O'Callaghan has reported at CDC and in a recent rat brain imaging publication (Koo et al., 2017; O'Callaghan et al., 2015).

The next step was to extend the treatment conditions beyond 72 hours and analyze MBP levels, because myelination is a long-term process and the shorter time points may not have captured the full response of what was occurring. Based on that finding Dr. Fields planned longer-term treatments to study effects on myelin. In the results of these longer-term analyses, we saw that increase in MBP levels persisted for the CORT+DFP condition in the longer time-points in Dr. O'Callaghan's rats, as compared to controls. Moreover, unlike in earlier time-points (72 hours and less), MBP levels seem to increase modestly with CORT alone and DFP alone treatments too at 7 days and 21 days post-exposure. We are trying to find a biological basis for why this would be so. Additional study has shown that this increase in MBP is not due to myelination of new axonal sprouts as assessed by expression of GAP43, a marker of axonal sprouting and there is no evidence of astrogliosis in the animal model of GWI, as assessed by expression of GFAP. There is an increase in cell proliferation 21 days post treatment however it is not clear why this is happening.

Returning to the earlier aims identifying the cholinergic receptors on oligodendrocytes by using calcium imaging. This work shows a rich array of cholinergic receptors are active in these cells and thus they would be affected by exposure to nerve agents. A review article about this phenomena was recently published (Fields et al., 2017). The main conclusion was that DFP (50 uM) significantly disrupts 1 uM ACh-treatment mediated OPC intracellular calcium kinetics, response frequency, and amplitude of response.

An important second component of Dr. O'Callaghan's in vivo model of GWI is prior exposure to corticosterone. Corticosterone has complex cellular and systematic effects, which make it difficult at this point to fully understand its mechanism of action in the GWI model. Dr. Fields therefore began testing oligodendrocytes in cell culture and found that these cells do respond to corticosterone treatment by undergoing a sharp rise in intracellular calcium. He found complex interactions between corticosterone and cholinergic receptor activation, which can be additive, synergistic, or antagonistic to the rise in intracellular calcium caused by cholinergic receptor activation. This and the prior MBP and immunocytochemistry findings have been submitted for publication to *Glia*.

Task 4. Perform preclinical cell and animal studies (months 9-42)

Specific progress to date is listed below for each of the sub-studies.

Studies to assess axonal transport and microtubule integrity in vitro and in vivo have been progressing as expected. Our research team continues to explore the effects of GW neurotoxins and cytokines on axonal transport and neuronal function and we have now shifted from the animal studies to the human induced pluripotent stem cell (hiPSC) and organoid (mini-brain) studies.

Items completed for the axonal transport and microtubule integrity group include:

- 1) Dr. Liang (Oscar) Qiang used neurons from human stem cell lines and from those differentiated from GW veteran-derived induced pluripotent stem cells as part of a recently completed New Investigator proposal to Dr. Peter Baas in collaboration with Dr. Sullivan (GW140086).
- 2) Gulf War neurotoxicants such as organophosphate pesticides and sarin gas have been shown to alter microtubule dynamics, axonal transport, and mitochondrial health, and these deficits are exacerbated by pretreatment with cortisol to mimic the stress of the battlefield. The funded iPSC work was designed to assess axonal transport and microtubule dysfunction in GW relevant exposures including DFP and cortisol (human equivalent of CORT) so that translation from animal to human studies can be compared for biomarkers and treatment development. The hypothesis is that exposure of neurons and/or neuroinflammatory cells to GW toxins caused long-lasting axonal transport/microtubule defects in neurons, and that these defects lead to a loss of microtubule mass, a change in the proportions of stable and labile microtubule mass, and/or flaws in the lattice of the microtubule that lead to abnormalities in how molecular motor proteins and other microtubule-related proteins interact with the microtubule and move down the axon. Dr. Qiang differentiated human induced pluripotent stem cells (hiPSCs) from GWIC veterans into mature neurons, and then exposed to one of three experimental conditions: vehicle control, Cortisol + the sarin gas analog diisopropylfluorophosphate (DFP), or Cortisol+DFP + Monastrol, an inhibitor of the molecular motor protein kinesin-5. Inhibition of kinesin-5 has been shown to increase microtubule mobility and improve the vitality of axons.

Dr. Qiang's lab is currently investigating the effects of the GWI treatment regimen of DFP+cortisol on hiPSC-derived glutamatergic neurons. They have shown that this treatment reduces MT stability, MT dynamics, axonal transport of mitochondria. Although these changes are important characteristics of the cellular effects of DFP+cortisol treatment, it is important to have a functional readout of how these changes alter higher level processes, and the gold standard functional assay in neuroscience is to examine electrical activity. They will use multi-electrode arrays from MultiChannel Systems, in which hiPSC-derived neural progenitor cells are differentiated directly on the electrodes on the array, to record local field potentials of spontaneous and stimulated electrical activity. They will examine relevant parameters such as firing rate, burst frequency, and network synchrony to assess changes due to DFP+cortisol treatment. A preliminary experiment on our hiPSC-derived glutamatergic neurons indicates that exposure to DFP+cortisol increases neuronal activity, and that hiPSC-derived neurons from veterans with GWI show increased activity compared to control veterans, suggesting a possible role of hyperexcitability in GWI. This is meaningful because aberrant glutamatergic activity is implicated in numerous cognitive disorders, and GW-relevant organophosphates have been shown to enhance glutamatergic neurotransmission. More importantly, changes in electrical activity can link back to some of the cognitive symptoms of GWI, such as altered information processing speeds and memory deficit.

Results: Electrical activity of hiPSC-derived neurons. Extracellular field potentials recorded from neurons differentiated on a multielectrode array (MultiChannel Systems) after exposure to the GWI neurotoxicant regimen of Cortisol+DFP were assessed. Preliminary data indicate that Cortisol+DFP increases electrical activity, and that hiPSC-derived neurons from veterans with GWI showed increased activity compared to control veterans.

Dr. Qiang is also currently working on the DOD funded project “Tau Pathology as a contributor to Gulf War Illness and a basis for potential therapy” (GW160151) which builds from the prior stem cell grant that were made from GWIC participant blood samples. Human induced pluripotent stem cells (hiPSCs) from veterans with and without Gulf War Illness (GWI) are being differentiated into mature neurons in order to study how Gulf War neurotoxins produce tau pathology and alter microtubule dynamics in human neurons.

Dr. Qiang’s team differentiated the hiPSCs into neurons using 2-dimensional and 3-dimensional differentiation protocols. Neurons will be exposed to the GWI regimen of Diisopropyl fluorophosphate (DFP) plus Cortisol to examine the effects on tau and microtubules.

Dr. Qiang had previously shown that GW-relevant organophosphates induce abnormalities in neuronal microtubules (MTs) and impair axonal transport. The neurotoxicant regimen of Diisopropyl fluorophosphate (DFP), a sarin analog, plus the rodent stress hormone corticosterone resulted in MTs with reduced stability, dynamics, axonal transport, and neurotransmitter release. These alterations can have a variety of deleterious effects in neurons that might contribute to some of the long-lasting cognitive symptoms of GWI, such as chronic fatigue or reduced information processing speeds.

The GWIC-related hiPSCs were differentiated into glutamatergic neurons because glutamate is the primary excitatory neurotransmitter in the brain and is intricately involved in synaptic plasticity, learning, and memory, which links back to some of the cognitive symptoms of GWI. Future studies can harness the power of hiPSCs to differentiate them into different neuronal subtypes to examine whether there are subtype-specific effects of GW-relevant neurotoxicant exposure.

Differentiating hiPSCs into glutamatergic neurons is also the most straightforward differentiation procedure. Differentiation protocol was based on the Stem Cell Technologies neuronal differentiation protocol. Briefly, hiPSC colonies were dissociated and cultured in an ultra-low attachment plate to enhance formation of embryoid bodies (EBs) with uniform size and shape. EBs were cultured in neural induction media to form neural progenitor cells (NPCs), which were then differentiated into mature neurons. Figure 2 shows neuronal validation for mature neuronal and glutamatergic markers via immunocytochemistry (ICC).

Next, the hiPSC-derived glutamatergic neurons were exposed to our previously characterized model of neurotoxicant exposure in GWI by combining DFP with the human stress hormone cortisol. We started with a DFP concentration of 200nM, which we used in our previous studies and which is believed to be just below the level that inhibits acetylcholinesterase (GWI thought to be caused by low-level organophosphate exposures that do not inhibit acetylcholinesterase). Initially, the neurons were exposed to cortisol for three days, followed by DFP+cortisol for two days, and then fixed or live-cell imaged the cells three days later.

We would like to experiment with additional time points and treatment durations to further characterize pathology in these neurons.

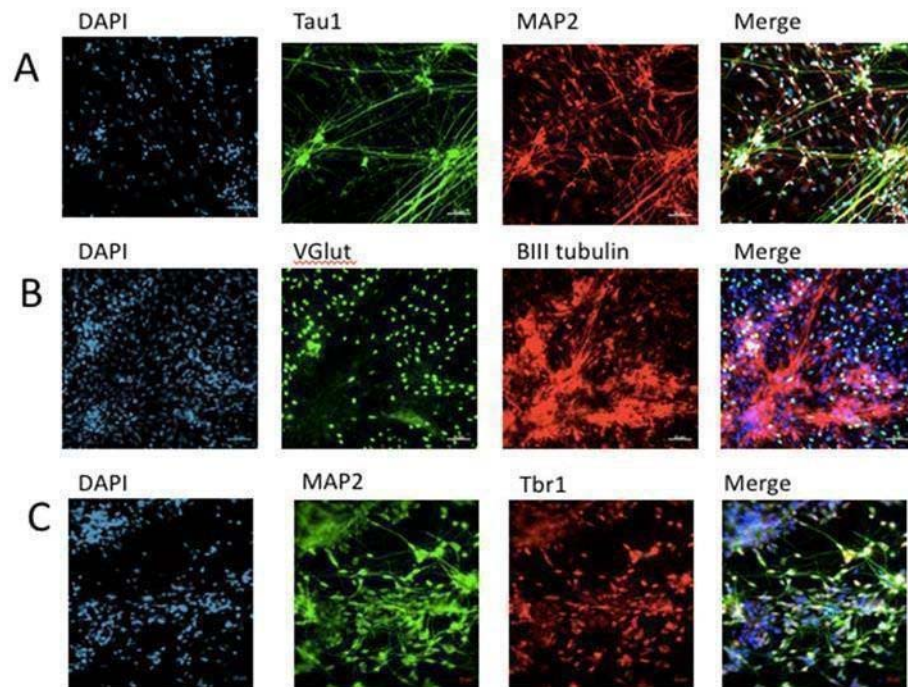


Figure 2. Neuronal validation of hiPSC-derived neurons.

(A) Tau1 stains for total tau and is used to mark mature neurons. MAP2 is a microtubule-associated protein.

(B) VGlut marks glutamatergic neurons and excitatory synapses. BIII tubulin is a neuron-specific tubulin marker.

(C) Tbr1 is a transcription factor for mature forebrain glutamatergic neurons.

The first experiment confirmed that the GWI regimen of DFP+cortisol causes similar deficits in the axonal transport of mitochondria in hiPSC-derived neurons as in the rodent neurons that we previously documented (Figure 3). The cell-permeable dye tetramethylrhodamine, ethyl ester (TMRE) was added to the cell culture media for 30 minutes prior to live-cell imaging. TMRE is actively taken up by mitochondria with an intact membrane potential, and so the dye can be used to track mitochondrial transport. The GWI treatment regimen decreased the percentage of mitochondria that move, and among moving mitochondria, the neurotoxicants reduced the distance and speed of traveling mitochondria (Figure 3). These impairments in axonal transport, and of mitochondria in particular, can have a variety of deleterious effects on neurons, including disrupting energy production in the cell by mitochondria, as well as disrupting the transport and release of secretory vesicles carrying neurotransmitters to the synapse. Alterations in these processes might contribute to some of the long-lasting cognitive symptoms of GWI, such as chronic fatigue, reduced information processing speeds and memory deficits, synaptic dysfunction, and other cognitive complaints.

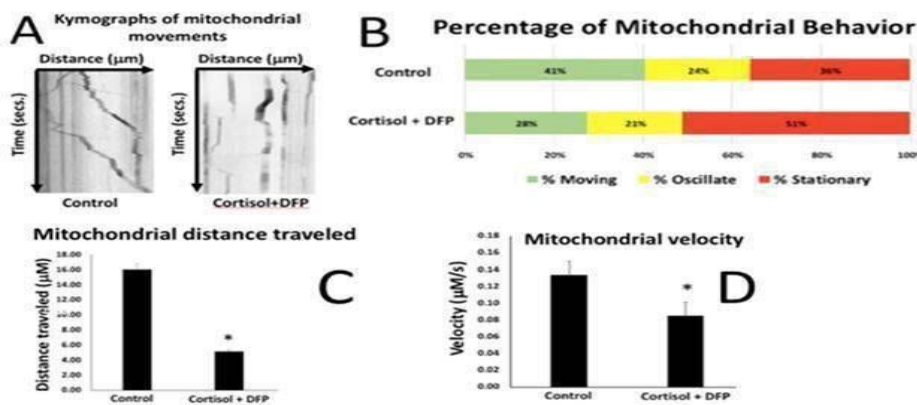


Figure 3. Neurotoxicant/stress-mediated impairment of mitochondrial transport. **A.** Representative kymographs showing mitochondrial movements along axons over time. **B.** Percentage of mitochondrial behavior. The graph shows a significant decrease in the number of moving mitochondria and a significant increase in the number of stationary mitochondria after Cortisol+DFP treatment. **C.** Distance traveled by mitochondria shows decreased distance in exposed group.

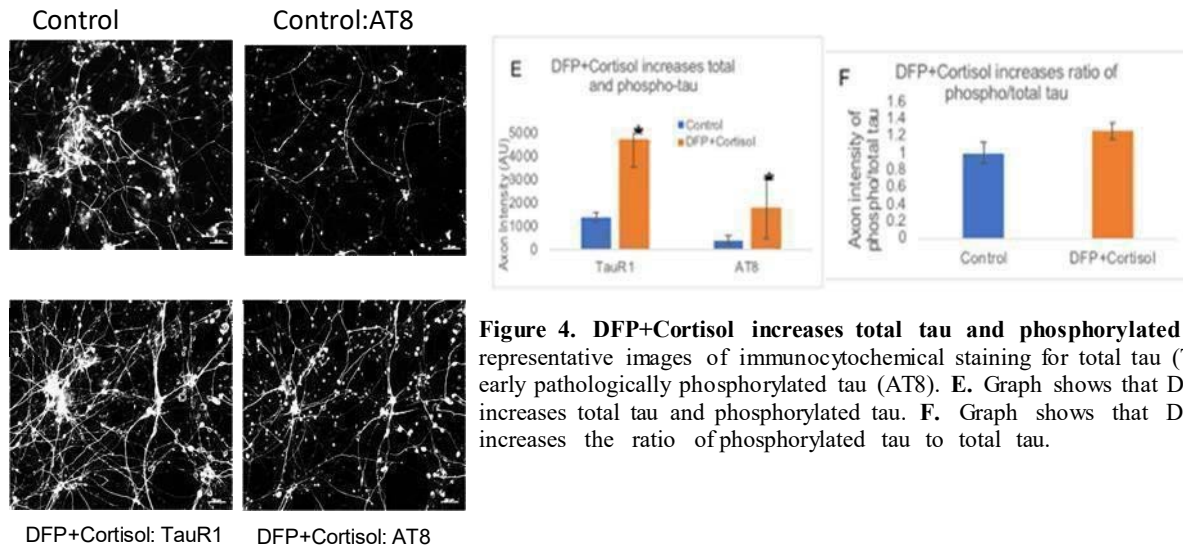


Figure 4. DFP+Cortisol increases total tau and phosphorylated tau. **A-D.** representative images of immunocytochemical staining for total tau (TauR1) and early pathologically phosphorylated tau (AT8). **E.** Graph shows that DFP+cortisol increases total tau and phosphorylated tau. **F.** Graph shows that DFP+cortisol increases the ratio of phosphorylated tau to total tau.

The hypothesis that hiPSC-derived neurons exposed to GW neurotoxicants develop tau pathology was addressed. The hiPSC model is well-suited to study tau pathology because this is a human-specific phenotype; pathological human tau behaves very differently from rodent tau. Neurofibrillary tangles composed of hyperphosphorylated tau cannot be recapitulated in rodent models without overexpressing human mutant transgenes. Tau is also more highly expressed in human neurons compared to rodent neurons, and so human tau is more vulnerable to insult. It is well-known that tau is susceptible to a variety of cellular insults, which can lead to disruptions in the interactions among numerous MT-associated proteins, as well as obstruct the cell with aggregates of hyperphosphorylated tau. One of the questions in GWI is whether the neurotoxicants cause tau pathology, and whether this pathology might contribute to some of the cognitive deficits that are common to other tauopathies such as Alzheimer's disease or Frontotemporal dementia. Figure 4 shows that the GWI regimen of DFP+cortisol increases total tau, as measured by the TauR1 antibody, and hyperphosphorylated tau, as measured by the AT8 antibody that detects paired helical filament (PHF) tau, which is an early pathologically phosphorylated tau and is a major component of neurofibrillary tangles in Alzheimer's disease. We would like to confirm these findings by Western blot and by staining for additional antibodies to total tau and pathologically phosphorylated tau.

In order to better evaluate the effects of DFP+cortisol in a more physiologically relevant model, a 3-dimensional cerebral organoids from the GW-veteran hiPSCs was generated. These "mini-brains" are increasingly being used to model neurological disorders in 3D, instead of traditional 2D cell culture methods, because the organoids can form a more complex neural structure, complete with ventricles, layers of neurons mirroring the cortical plate, and mature synapses. The organoids were cultured for several months to attain mature neurons. Figure 6 shows immunocytochemical neuronal validation for mature neurons as well as neural progenitor cells.

The organoids were exposed to our GWI regimen and replicated the tau pathology of increased total tau and hyperphosphorylated tau (data not shown). In addition, DFP+cortisol resulted in increased neuroinflammation, as shown by staining for glial fibrillary acidic protein (GFAP) (Figure 7). The pronounced astrogliosis indicates increased activation of astrocytes, possibly reacting to neuronal stress signals. The presence of neuroinflammation in response to Gulf War neurotoxins is consistent with studies that have posited neuroinflammation as one of the main drivers of GWI. We would like to further investigate neuroinflammation in our model, including what kinds of interactions exist between tau pathology, MT abnormalities, and neuroinflammation.

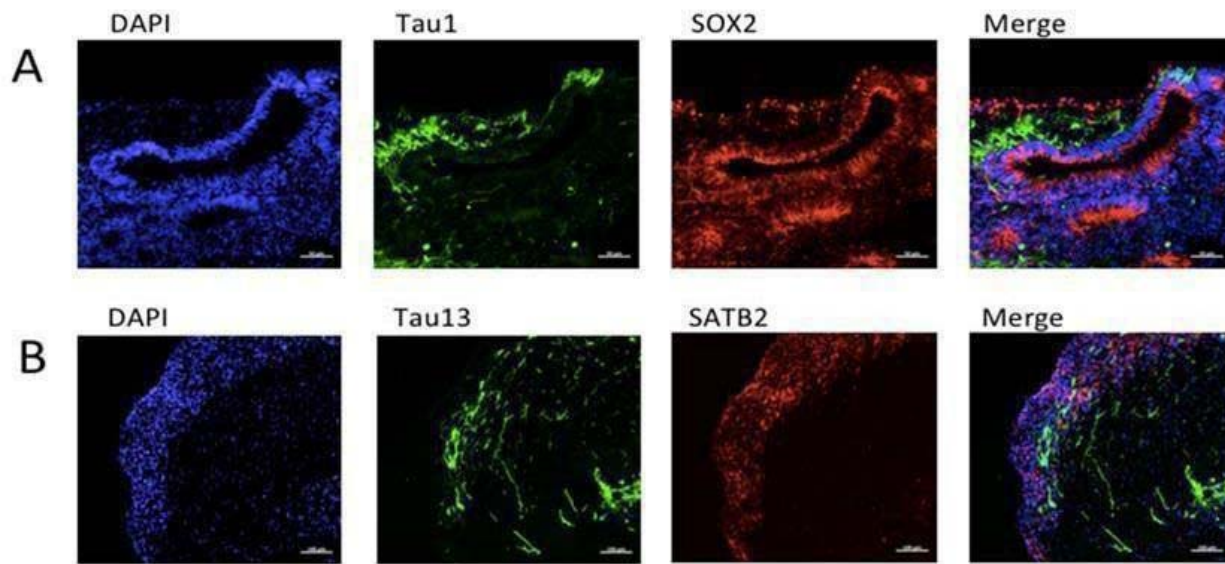


Figure 6. Neuronal validation of cerebral organoids. (A-B) Day 43 organoids. DAPI is a nuclear stain. Tau1 mark de- phosphorylated tau. SOX2 is a neural progenitor marker. Tau13 is a total tau marker. SATB2 stains surface layer neurons

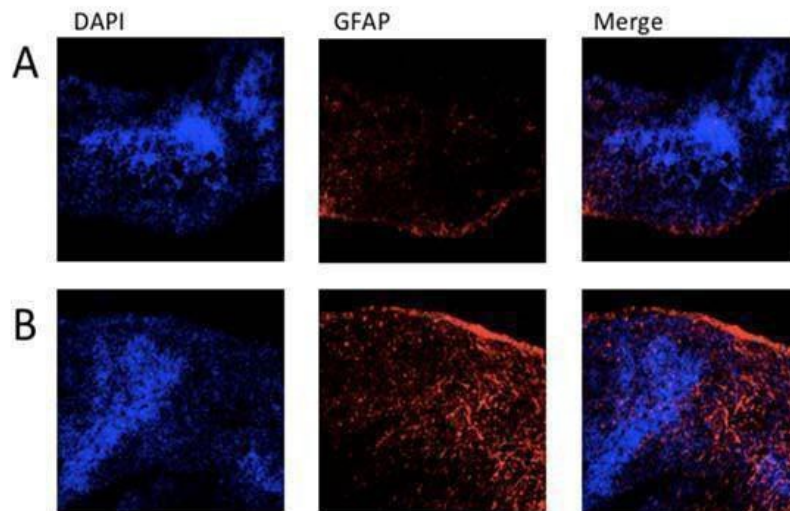


Figure7. Reactive astroglial neuroinflammation in cerebral organoids. Organoids were treated at 2 months with DFP+cortisol, then fixed 1 day after the end of neurotoxicant treatment. (A) Organoids treated with vehicle. (B) Organoids treated with DFP+cortisol. DAPI is a nuclear stain. GFAP is an astroglial marker.

Dr. Qiang's team is currently investigating whether the GWI neurotoxins induce changes in neurogenesis (Figure 8). The cerebral organoids are critical for this study because we can observe changes over time to the developing cortical layers as well as to the ventricle-like structures.

Lastly, Dr. Qiang is investigating changes in neuronal activity due to the GWI treatment regimen of DFP+cortisol in both 2D and 3D cultures. GWI hiPSCs were differentiated into neurons and organoids on multi-electrode arrays from which spontaneous local field potentials from different areas of the culture will be recorded. They will also stimulate the cells to observe the connections among the active neuronal networks in real time. They will then test whether the activity can be modulated by excitatory and inhibitory compounds, as well as what alterations in neuronal activity occur due to the GWI treatment regimen.

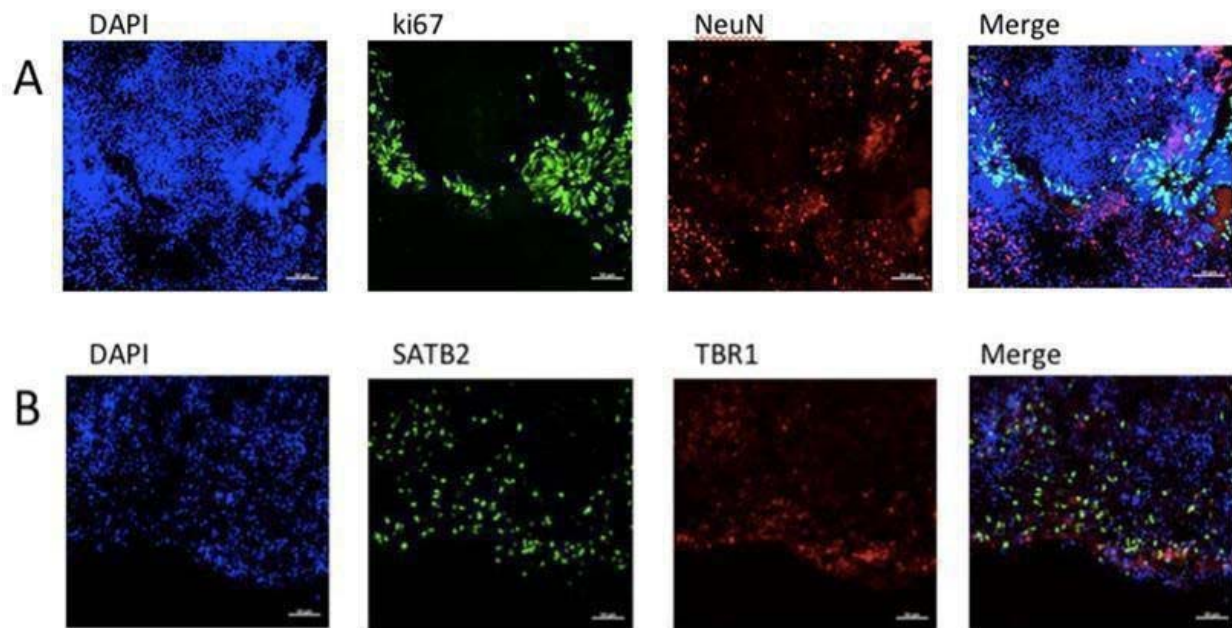


Figure 8. Neurogenesis in cerebral organoids. Organoids were treated at 2 months with DFP+cortisol, then fixed 1 day after the end of neurotoxicant treatment. DAPI is a nuclear stain. ki67 marks dividing cells. NeuN marks mature neurons. SATB2 and TBR1 stain for neurons in the developing cortical plate.

4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxins or cytokines (NIH). –

The main conclusions from the NIH work that is now completed can be summed up as:

GW toxicants (DFP and CORT) primarily affect:

1. Calcium signaling in oligodendrocytes, in-vitro
2. Oligodendrocyte biology, especially their survival and proliferation, in-vitro
3. We have characterized these findings in the O'Callaghan rat model of GWI and prepared and submitted a manuscript for publication. Although the O'Callaghan model shows no correlation between cholinergic signaling and chronic neuroinflammation in GWI animal models, the cholinergic signaling does seem to affect myelination as described in the abstract below and recent review publication (Fields et al., 2017). These are two distinct and perhaps not mutually exclusive phenomena. Neuroinflammation and myelination changes in GWI can and likely do work through different mechanisms. However, OP induced neuronal and myelin damage caused by increased calcium signaling through cholinergic and/or glutamatergic neurotransmitters can lead to 'danger signals' to TLR4 receptors on microglia to illicit chronic neuroinflammatory cytokine signaling. This seems to validate a main GWIC hypothesis regarding at least one mechanism for chronic neuroinflammatory signaling in GWI; that neural debris in the extracellular spaces can and likely does illicit microglial activation and chronic neuroinflammatory signaling.
4. A poster was presented to the Society for Neuroscience summarizing our findings with cholinergic signaling in myelination is listed below:

Cholinergic signaling has been recently implicated in myelination and as a promising target for demyelinating disorders. Despite established roles as a major neurotransmitter and source of choline metabolite, the contribution of acetylcholine (ACh) to oligodendrocyte development and myelin plasticity remains to be elucidated. Here, we show that oligodendrocytes express the receptors and enzymes necessary to engage in cholinergic signaling and respond to ACh with robust and heterogeneous intracellular calcium kinetics. To investigate the purpose of cholinergic signaling in oligodendrocytes, we studied the anticholinergic pathology associated with Gulf War Illness (GWI), the heterogeneous condition that afflicts a third of US veterans deployed in 1990-1991 Gulf War. Based on our previous studies, we hypothesized that the myelin abnormalities reported in GWI veterans, were due to atypical cholinergic signaling in oligodendrocytes. This hypothesis was tested using an animal model of GWI, mimicking the exposure to anticholinesterase agents (modeled by Sarin gas analog diisopropyl fluorophosphate, DFP) and extreme stress (exogenous corticosterone, CORT). Western blot data revealed increased myelin basic protein levels, a key protein in myelin production, in the combined CORT+DFP condition in whole brain homogenate at 24 hours and persisting through 21 days post exposure (One-way ANOVA, N=3, p=0.01). At the molecular level, live cell calcium imaging data showed that pretreatment with DFP significantly increased the number of wild type oligodendrocytes that responded to acetylcholine in vitro (two-tailed two-sample t-test, N= 5, p=0.005) in a wild type rat monoculture. The DFP-mediated increase in oligodendrocyte responsiveness is not due to acetylcholine produced by oligodendrocytes

or astrocytes ($N=3$, T-test, $p=0.55$) suggesting GWI is a pathology of neuron-glia rather than a glial cell- autonomous glia-glia cholinergic signaling. Taken together, this work demonstrates GW agents disrupt oligodendrocyte development in vivo and at the molecular level. These findings both reveal the importance of cholinergic signaling for proper myelin development and indicate that the anticholinergic and corticosterone mediated signaling by GW agents, and more generally by commercial-use pesticides, may be largely responsible for myelin changes in veterans with Gulf War Illness and broader myelin- related pathologies.

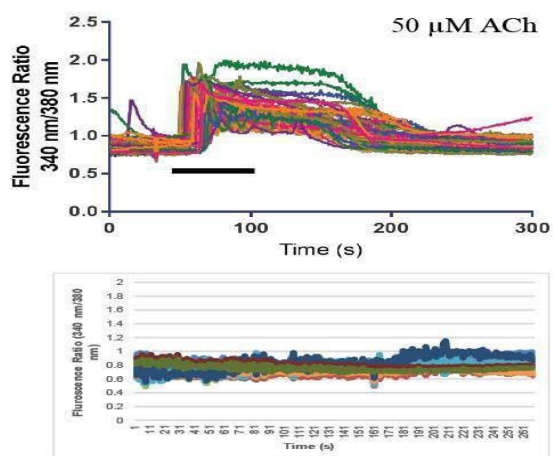
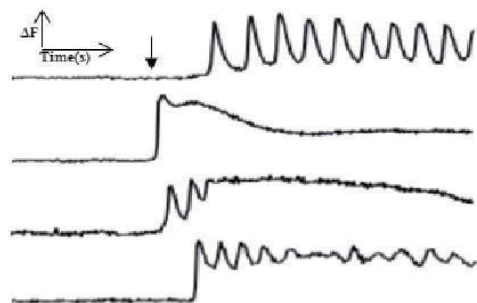
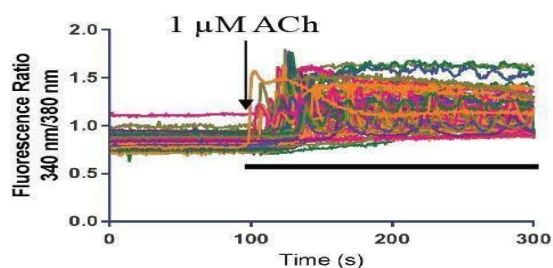


Fig 1. ACh induces OPC intracellular calcium response. a) Schematic for ACh treatment during live-cell calcium imaging. b) 50 μ M ACh ($N=5$, $n=56$). Inset: inhibition with mAChR M1 inhibitor pirenzepine (PZP, 50 μ M) to confirm specificity of ACh-induced calcium response ($N=3$, $n=9$). Left arrow at 40 seconds is PZP pretreatment onset, right arrow at 160 seconds indicates PZP + 50 μ M ACh treatment onset. c) 1 μ M ACh ($N=5$, $n=91$) d) representative waveform traces of data presented in part (c).



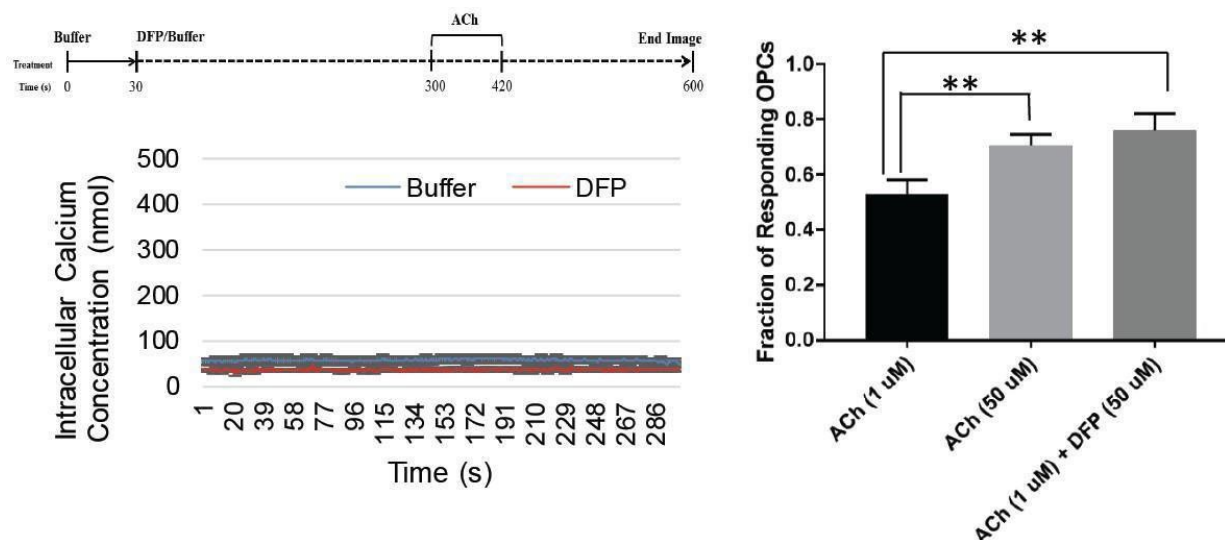


Fig 2. DFP increases fraction of OPCs responding to ACh. a) DFP (50 μ M) alone has no effect on intracellular calcium mobilization in OPCs. b) DFP pretreatment followed by ACh significantly increases fraction of cells that respond (t-test, p-value = 0.005). 50 μ M ACh significantly increases number of cells that respond compared to 1 μ M ACh (t-test, p value = 0.01).

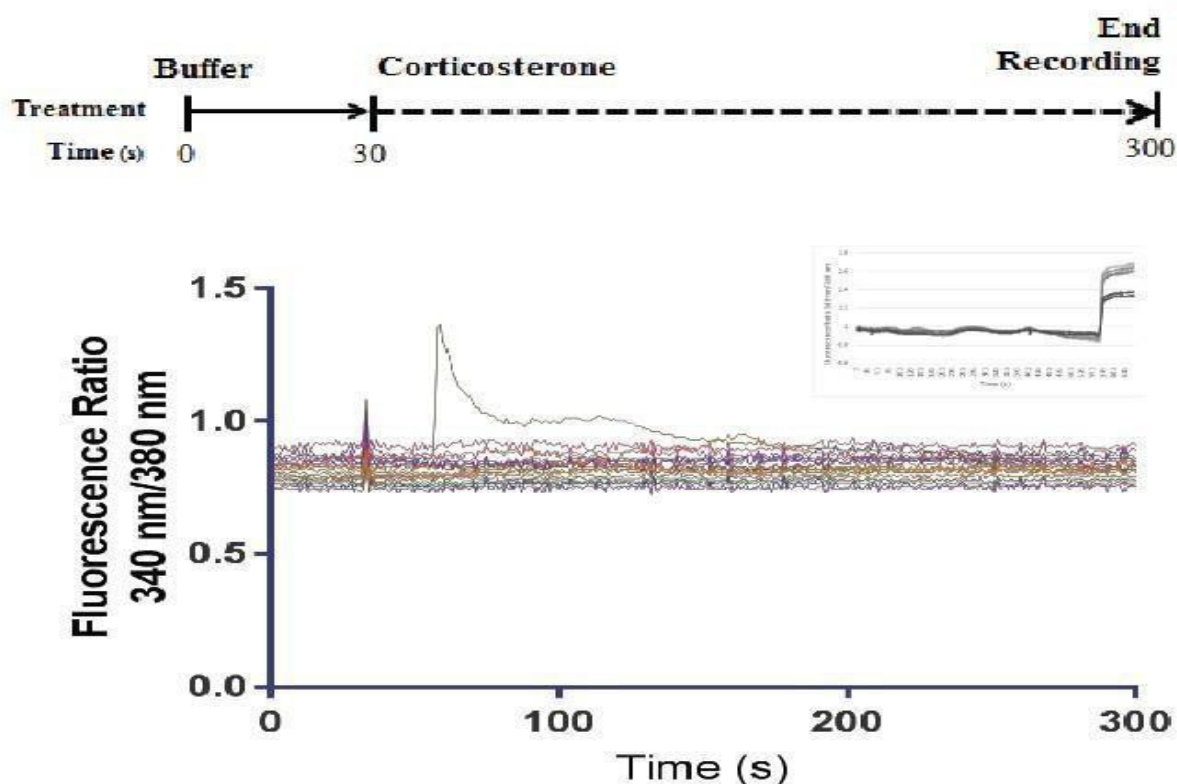


Fig 3. Corticosterone has no effect on intracellular calcium mobilization. a) Treatment paradigm used. b) intracellular calcium measurement. Each line indicates recording from one cell (N=3, n= 36). Inset box indicates positive control using calcium ionophore A23187.

In vitro OPC monoculture

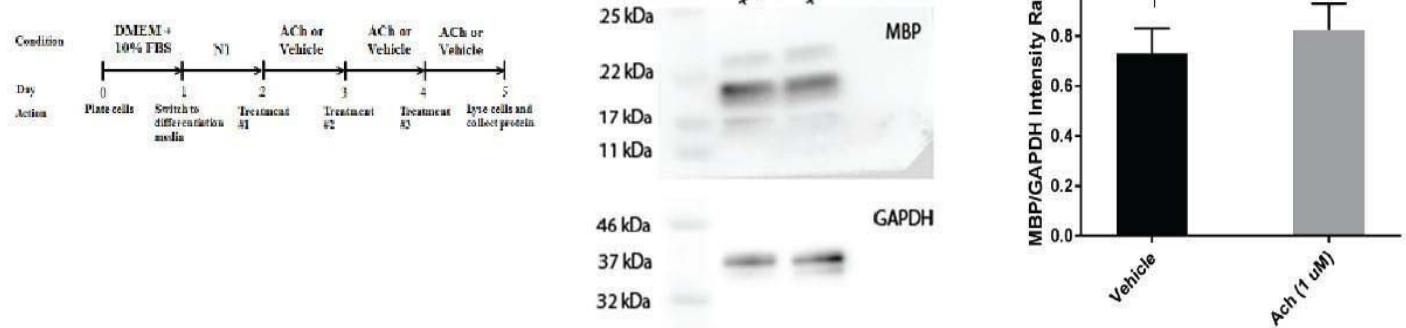
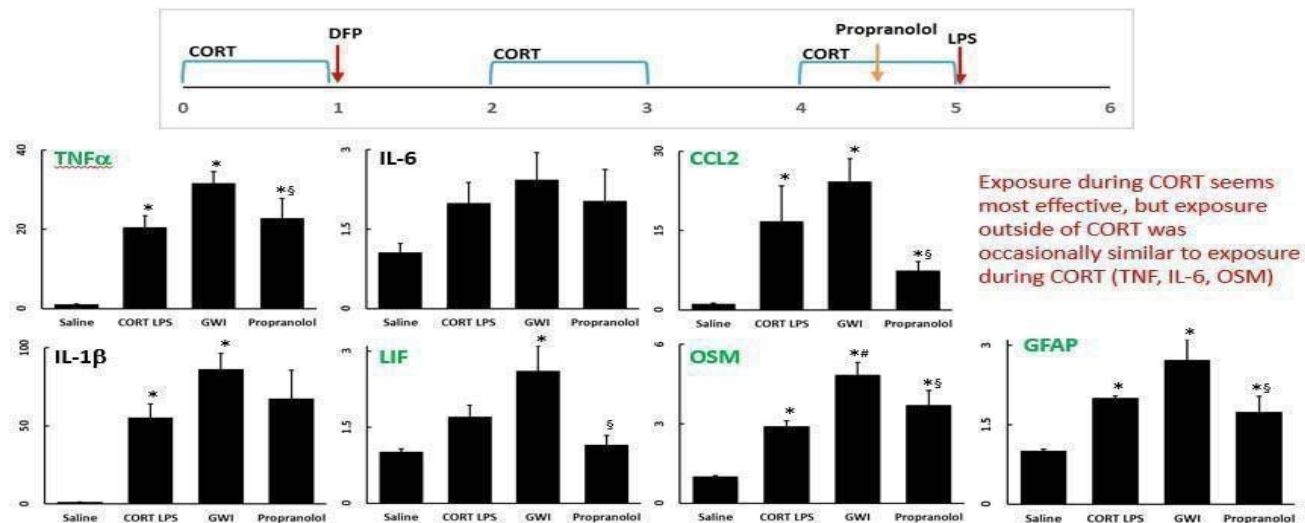


Fig. 4: ACh alone not sufficient to induce MBP changes in OPCs a) Treatment paradigm used for in vitro OPC monoculture experiments. b) Western blot for MBP and GAPDH on differentiating oligodendrocytes with Vehicle (differentiating media) and ACh (1 μM in N1 differentiation media). c) Quantified western blots as MBP/GAPDH (N = 3, Vehicle = 0.731 ± 0.099 SEM, ACh = 0.824 ± 0.105, two-sample, two-tailed t-test p-value = 0.554). Western blots quantified with Image J. Individual MBP isoform bands were summed to give a single MBP value for each sample.

Task 4c. Assess whether persistent priming of neuroinflammation occurs chronically with GW-relevant neurotoxicants and intermittent corticosterone exposure to model the chronic nature of GWI (CDC –100 C57BL/6 mice).

- CDC has completed an initial study of propranolol treatment in the 5 week GWI paradigm. Mice received CORT (200 mg/L) in the drinking water for 7 days followed by a single i.p. injection of DFP (4 mg/kg). Mice then received CORT every other week for 4 additional weeks and an LPS inflammatory challenge (0.5 mg/kg, s.c.) on the last day of the experiment. Mice were sacrificed 6 hours post-LPS exposure and assessed for inflammatory cytokine expression in the cortex.



For the treatment schedule, mice received a single, i.p. injection of propranolol (20 mg/kg) during week 4 (day 24) or 5 (day 31) either outside or during CORT exposure, respectively. Overall, propranolol treatment during CORT exposure significantly reduced the expression of the inflammatory cytokines/chemokines: TNFα, CCL2, LIF, and OSM, as well as the astrocyte marker GFAP. This indicates the potential for propranolol to be an effective treatment for the neuroinflammation associated with GWI. Evaluation of cytokine expression when propranolol was given outside of CORT exposure was slightly less effective, only reducing TNFα, IL-6 and OSM expression.

Previously we have shown acute exposure to the initiating event producing GWI pathobiology (CORT and DFP) did not produce significant glial changes in morphology or neurodegeneration (O’Callaghan et al., 2015). Here, we have used that same initiating event with a systemic inflammatory challenge at 5 weeks with interesting new results.

The astrocyte response to the 5 week GWI phenotype has been previously described. Briefly, the combination of CORT and DFP was able to create a pathology in which a subsequent, systemic low dose LPS challenge was able to produce astrocyte hypertrophy at 24 hours after exposure. Control and challenged GWI phenotype treated astrocytes at high magnification highlight the morphological differences in the astrocytes under these conditions. These results appear to be similar to other animal GWI models showing delayed effects on glia after neurotoxicant exposure (Zakirova et al., 2015; Ojo et al., 2014). Results also could be compatible with blood-brain barrier permeability as suggested by reported increased CNS autoantibodies in GW veterans (Abou Donia et al., 2017).

A protocol for combined CORT/DFP and mild TBI exposure in rats has been submitted to the CDC NIOSH IACUC for approval (November meeting) for the mTBI pilot project.

CDC Accomplishments

One paper was recently published: titled, ‘The neuroinflammatory phenotype in a mouse model of Gulf War Illness is unrelated to brain regional levels of acetylcholine as measured by quantitative HILIC-UPLCMS/MS. Miller JV, LeBouf RF, Kelly KA, Michalovicz LT, Ranpara A, Locker AR, Miller DB, O’Callaghan JP. Toxicol Sci. 2018 May 28. doi:10.1093/toxsci/kfy130’

Plans for pilot treatment studies are underway with IACUC approvals now in place for testing AhR modulators and tubacin in the CDC neuroinflammatory GWI model. The ACURO protocol has been submitted and is approved to begin these pilot studies once new animals can be requisitioned.

Task 4d. Assess the relative contributions of astrocytes and microglia in rodent GWI neuroinflammatory models in order to identify which glial markers will provide the best candidate “druggable” targets (CDC 40 C57BL/6 mice; 40 ALDH1L1 mice; 40 B6.129- Cx3CR1 mice).

ALDH1L1 BAC-TRAP mice have been used for initial studies with the CDC GWI exposure protocol, and we see that DFP exposure produces a similar phenotype in these animals to that seen in C57 exposed animals. The TRAP procedure was then used to isolate actively translating mRNA from astrocytes (ALDH1L1-containing cells) at 6 and 72 hours after DFP exposure with and without CORT. Preliminary data supports the use of this model to understand the enrichment of GWI-relevant molecular signatures and signaling pathways in astrocytes over total tissue expression (mixed cell population), as 110 and 211 significantly altered genes in cortical astrocytes were found to be expressed 10- and 5-fold over total cortex, respectively. Initial functional analysis of the set of 211 genes indicates that these genes are largely involved in immune signaling and show particular enrichment for cytokine and complement factor signaling as well as cancer pathways. This paper has been submitted for publication and is under review. This suggests that treatments targeting astrocytes and microglia may be appropriate for GWI treatment development.

CDC has performed a preliminary experiment exposing CX3CR1 KO mice to a short term GWI paradigm. These animals were exposed to 200 mg/L CORT in the drinking water for 7 days followed by a single injection of DFP at 4 mg/kg, i.p. Two days later, the mice were exposed to a single, s.c. injection of 0.5 mg/kg LPS to elicit an inflammatory response. Elimination of CX3CR1 *prevented* the priming of CORT DFP exposures on the subsequent LPS. This suggests that microglial responses are crucial for CORT priming of neuroinflammation and *supports a neuroimmune mechanism for GWI*.

Dr. John Gerdes from RIO pharmaceuticals and Dr. Henry Van Brocklin from UCSF also submitted a proposal to assess the EAAT2 PET ligand in the CDC animal model that was previously approved by the EAB. This study has now gotten IACUC and ACURO approval. This study will have preliminary results to report regarding astrocyte activation in the rat brain following GW-relevant toxicant exposure on PET imaging. Dr. O'Callaghan travelled to UCSF to assist with training of animal dosing in November to help initiate and oversee the EAAT2 PET studies.

4e. Assess the relationship between behavioral testing of learning and memory and enhanced pain, in rodent GWI neuroinflammatory models by assessing hippocampal functioning with a fear conditioning task (U-Colorado- 120 rats).

- Drs. Maier and Watkins have two pilot studies currently in ongoing/ in preparation to begin for treatment of memory functioning and for treatment of pain with two IL10 based treatments.
- Dr. Watkins will have a larger update with some preliminary results to present in the next quarterly report but some results are presented in the next section.
- Dr. Sullivan, Watkins, O'Callaghan and Grace will prepare a translational manuscript for publication that will include the GWI animal pain model and the GWIC clinical pain and cytokine correlation outcomes.

4f. Compare central and peripheral markers of neuroinflammation in brain tissue and blood samples from 60 rodent GWI neuroinflammatory models (CDC, Nova). -

CDC sent mouse serum samples to NOVA Southeastern University for cytokine analysis following acute exposure to DFP, as well as following the 3-week and 3-month GWI phenotype paradigms. With the longer exposure periods, both brain and serum samples showed a significant inflammatory priming in the CORT DFP exposed samples that are subsequently challenged with LPS. These results will be submitted for publication in a translational paper indicating similarities with the clinical study results.

4g. Compare the effectiveness of several relevant preclinical treatments for GWI in cell and animal studies, including inflammatory glial activation modulators, antioxidants, and neuroprotective peptides (Drexel, Temple, CDC, U-Colorado).-

These important treatment experiments are now well underway and will provide results in the coming months.

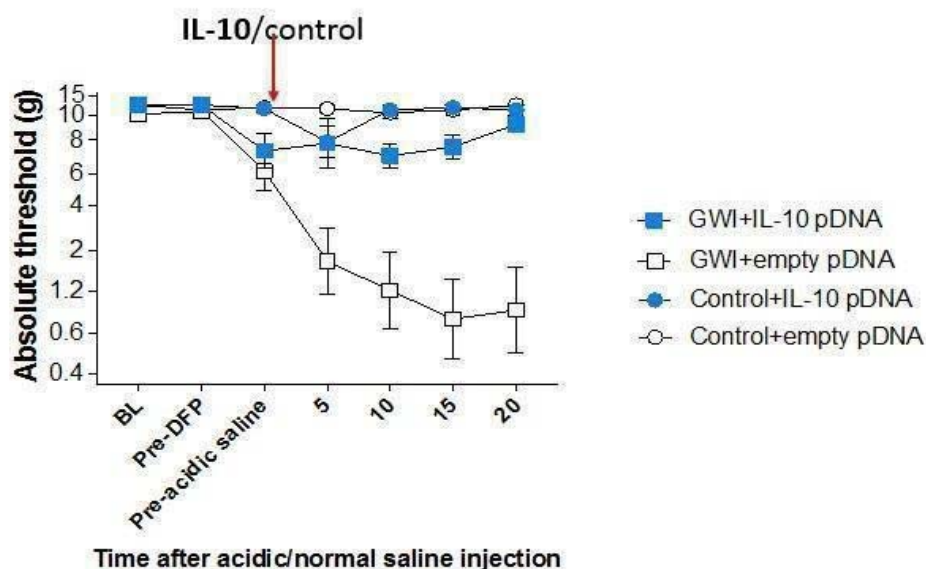
1) As previously mentioned, preparations and initial results for studies using propranolol in chronically symptomatic/exposed animal models have been completed by Dr. O'Callaghan's lab at CDC.

2) Drexel investigators have assessed the role of tubacin on stabilizing microtubules and thus reducing acetylated or unstable microtubules. Using tubacin, an FDA-approved HDAC6 inhibitor, changes after DFP exposure, with or without CORT pretreatment, were stabilized and restored to control levels. Human neurons were also treated with DFP + cortisol and the total acetylation ratio was similar in human cells as with rat neurons. This indicates a potential clinical translational therapeutic strategy for GWI. This manuscript was published in *Traffic* in May 2017. Additional HDAC6 treatments that are FDA approved were submitted in a now funded grant to CDMRP for further high-throughput treatment study with GWIC investigators to further this line of treatment development.

3) CDC ran their Tubacin experiment, AhR modulators and luteolin to study their effectiveness in reducing neuroinflammation. Dr. O'Callaghan is working on subsequent animal protocol approvals.

4) Initial dosing studies for the treatments luteolin, HDAC6 inhibitor: tubastatin, and AhR inhibitors: galangin and alpha-naphthoflavone have been completed and are currently being analyzed to determine an appropriate dose for testing in the 5 week GWI paradigm model.

5) Dr. Linda Watkins at U-Colorado submitted a pilot study to assess a novel pain treatment in rat GWI animal models called by XT150, which is an interleukin-10 based therapy nearing Investigational New Drug status. It has shown great promise in other animal models including in dogs and horses with chronic pain. This pilot study is now underway with initial results shown below indicating that it increases the pain threshold in rats exposed to the GWI animal model. This suggests IL10 therapies should be further explored in animal and clinical studies.



5) Dr. Steve Maier at U-Colorado also submitted a pilot study at the last EAB meeting that was approved for IL10 based therapy for behavioral/memory improvement in GWI animal models. Amendments for IACUC and then ACURO approvals are being prepared for this new pilot treatment study once staffing for this pilot study is established with Dr. Maier.

6) On the clinical side, Drs. Klimas, Sullivan, Krengel and other GWI investigators submitted a grant for a GWI Clinical Trials Consortium where several new Phase I/II treatment trials are planned using the current infrastructure of both the Boston and Miami GWI consortia. This grant was awarded to the group and the kick-off meeting with DOD and the EAB for the GWI Clinical Trials consortium (GWICTIC) was held in Baltimore in November. Thus, treatment development is occurring at the preclinical and clinical sites simultaneously.

TASK 5. SCREENING, RECRUITMENT AND ASSESSMENT OF GULF WAR VETERANS FROM THREE SITES (MONTHS 9-42)

Participant recruitment and screening is ongoing at the 3 study sites. Currently 404 participants have been screened and 175 have been assessed at the Boston, Miami and Houston. The Boston site currently has three additional participants scheduled. Study enrollment and data collection began in October 2017 at the Houston/Baylor College of Medicine (BCM) study site and have continued through the current quarter. As of the report date, a total of 32 veteran

subjects have been evaluated at the BCM site, with 1 additional appointments pending for veterans who have agreed to participate after screening eligible for the study. In addition, there are preliminary indications that MRI scans at the BCM site are showing a number of previously unreported brain tissue changes that have the potential to be clinically significant. Dr. Steele has continued to move forward with activities to expand recruitment for the site. Also as of the report date, enthusiasm for study participation has been high among veterans contacted about the project. See current recruitment Table 1 below. Although below initial estimates, recruitment is steadily increasing and retention is excellent from time of screening to study completion. We have continued to reach out to our veterans' advisory group members. The aim of this group is to have veterans provide advice and assist with recruitment efforts. We have also continued to reach out to veterans through our Facebook page through videos, articles, posters and Ad postings. Many veterans have contacted us through Facebook, showing interest in being a part of our study. We plan on making more videos on GWIC related topics and posters and sharing them with veterans on our Facebook page. We are also working with other GW researchers in a 'PI recruitment network' to share recruitment strategies.

Task 5a. Obtain informed consent from potentially eligible GW veterans

Recruitment has been ongoing this quarter and 424 total subjects have contacted GWIC coordinators through print advertising, free newsletters to VSOs or social media outlets. From these contacts, 266 were found eligible to participate in the studies. Those not screened were largely not GW veterans. Of this group, 193 have been scheduled and 175 (149 case, 25 controls) have completed the study protocols at Boston, Miami or Houston sites. With the Texas site on board with recruitment, we expect to increase recruitment to catch up to the expected recruitment goals of 300 total participants in year 6 of the to-be approved no-cost extension period. Table 1 lists the current demographic breakdown of the cohort.

**GWIC: Gulf War Illness Consortium
Screening and Enrollment Summary Report
12/12/2018**

	Total	Boston	Miami	Texas
Number of Subjects Contacted	424	276	69	79
Number of Subjects Screened	404 (95.3%)	264 (95.7%)	69 (100.0%)	71 (89.9%)
Number of Subjects Eligible	266 (65.8%)	156 (59.1%)	60 (87.0%)	50 (70.4%)
Number of Subjects with Appointments Made	193 (72.6%)	104 (66.7%)	56 (93.3%)	33 (66.0%)
Number of Subjects Assessed	175 (90.7%)	100 (96.2%)	43 (76.8%)	32 (97.0%)

	Total		Boston		Miami		Texas	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	368	36	238	26	62	7	68	3
Number of Subjects Eligible	235	31	134	22	53	7	48	2
Number of Subjects Assessed	149	25	82	18	36	6	31	1

Task 5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, brain imaging and blood draw and saliva

As planned, cognitive assessment data are being analyzed with neuroimaging data to assess for brain-behavior relationships in GWI. Initial demographic outcomes suggest a fairly diverse cohort of veterans in terms of race and gender which is helpful with current gene-exposure and SNP analyses. The most recent results were presented at the June 2018 EAB meeting. Updated results are presented in Table 2 below and show significant differences between groups for exposures during the war (chem/bio weapons, wore pesticide treated uniforms and history of mTBI during or post-GW) when compared by students t-tests or chi-square analyses. Interaction analyses comparing chem/bio exposures and mTBI were also significantly different between the groups ($p=.007$) when compared by MANOVA

(see Figure 1). This indicates that there is significant evidence of increased risk of GWI for those with both chemical weapons (CBW) and mTBI exposure. Also, risk of other medical conditions was compared in the mTBI + CBW group when compared with the nonexposed group. These results were recently published in Janulewicz et al., 2018 (see appendixes). Specifically, the risk of having other medical conditions was between 4 and 22 times higher in the mTBI + CBW exposed group compared with the non-exposed group. Kansas GWI criteria domains also showed significantly higher symptom domain scores in the mTBI x CBW exposed group for all symptom domains. The findings also correspond and expand upon findings from the Ft. Devens cohort published by Drs. Kregel, Sullivan, and Janulewicz (Yee et al., 2015; Yee et al., 2017).

Table 1. GWIC Subject Demographics

Subject Demographics	N=137	N=115	N=22
Demographics	Overall	Cases	Controls
Age at time of study (mean, SD)	52.1 (10.5)	52.0 (11.2)	52.6 (6.3)
Years of education (mean, SD)	15 (2)	15 (2)	15 (2)
Female	15%	17%	5%
Ethnicity			
Caucasian	75%	74%	81%
African American	14%	14%	10%
Other	11%	12%	9%
GW-related Exposures			
Reported PB pill usage	69%	71%	62%
Wore pesticide treated uniforms	53%	56%	33%
Saw pesticides sprayed/fogged	40%	43%	24%
Chemical/bio weapon exposed	47%	53%	14%*
mTBI during the war	36%	43%	0%*
MFI-20 Fatigue Score (sd)	68 (18)	73 (15)	47 (13)*
McGill Pain Score (sd)	30 (15)	33 (15)	14 (9)*

Figure 1. Chi-square analyses of mTBI rates in GWI cases and controls pre, during and post Gulf War.

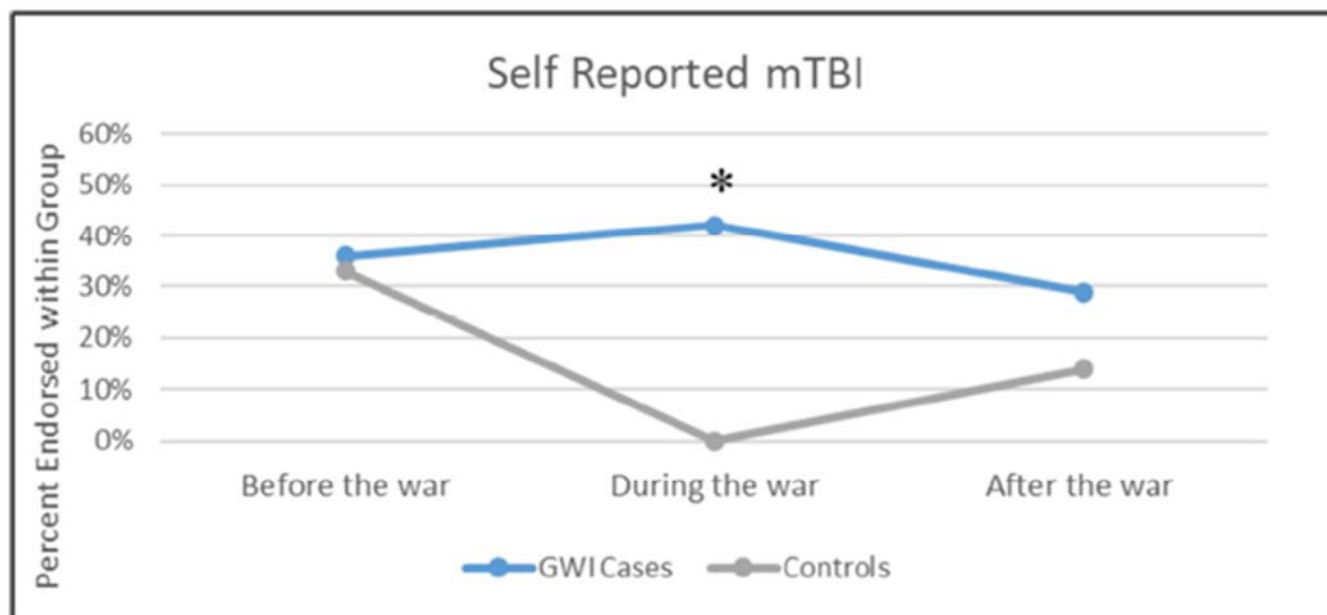


Figure 1. Percent of veterans with self-reported mTBI before, during, and after the war. * $p < 0.005$.

Task 5c. Upload neuroimaging data to BUSPH for post-processing of MR images and for data analysis.

MRI scans were obtained from the first 71 study participants have been post-processed. The Baylor site is transferring their MRI scans electronically to the Center for Biomedical Imaging at Boston University School of Medicine for post-processing. Each scan undergoes quality checking that consists of a visual inspection for the presence of noise or artifact as well as a review of scan parameters to ensure that the appropriate ones were used in the acquisition. Scans that fail the quality check are rejected by the study and remediation discussed with the appropriate site investigator. Scans that pass the quality check enter the post-processing pipeline. The first 71 scans have been through the post-processing pipeline for multivariate statistical analysis and initial correlation results are presented in the sections below. New processing pipelines are also being developed for second generation diffusion MRI index mapping on cortical ROIs, PET data processing and pCASL processing. A doctoral student at BUMC has been identified to assist with the PET and resting state fMRI processing.

MRI Imaging: The scanning session include: 1) Three plane TFSE scout scan, 2) a Sense reference Scan, 3) an accelerated high resolution MPRAGE scan acquired in the sagittal plane, 4) a multi-component T2 imaging sequence acquired in the axial plane, 5) a Diffusion Tensor Scan with 32 directions acquired in the axial plane, 6) a resting state functional magnetic resonance imaging scan, and 7) a pCASL sequence obtained while the participant is at rest and 8) a High Angular Resolution Diffusion Imaging (HARDI DTI) scan.

Task 5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.

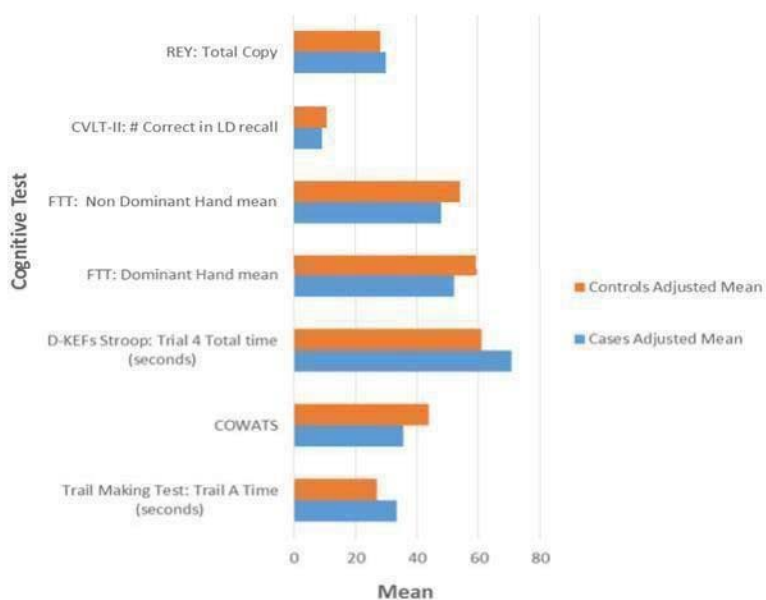
Data from the first 136 participants has been scored and cleaned and basic means (sd) are presented in Table 2. As data is collected, quality control procedures have remained in place including double entry of data collection forms in the REDCap data collection website, built in range checks and quality control audits of all data collection by the Data Coordinating Center staff and the local BU Administrative Core neuropsychologists. Dr. Toomey also conducts biweekly conference calls to review scoring and quality control, as well as regular reviews of data entered and spot checks of any questionable data to ensure data administration and scoring integrity throughout the recruitment period. This has ensured the highest quality data available for analysis.

Task 5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.

Blood and saliva samples have been sent to NOVA Southeastern University for each of the recently completed 175 study participants. The analysis of cytokine and chemokines has been completed for the first 150 samples. Cortisol measurements that will include testing for neuroendocrine and immune alterations and for hypothalamic pituitary adrenal axis abnormalities have also been completed for the initial batched samples. Specifically, blood samples are sent to NOVA Southeastern University for analysis of proinflammatory cytokine and chemokines, and nanostring analysis of mRNA and miRNA of proteins related to TLR4 functioning and glial activation including miR-155, miR-21 and miR-146. Multiplex Quansys ELISA system will be used with an existing cytokine platform created by Dr. Klimas' research laboratory. Dr. Klimas' laboratory currently measures 16 cytokines, chemokines and immune markers in plasma. Gene expression and pathways will also be assessed using an Agilent microarray system and quantitative realtime PCR for validation of differentially expressed genes. Preliminary results of t-tests and univariate analyses of variance controlling for gender comparing cytokine, chemokine, monocyte, glutamate and lymphocytes between cases and controls indicate initial significant differences in multiple cytokines (TNFR1, TNFR2, IL13) and white blood cells between the groups.

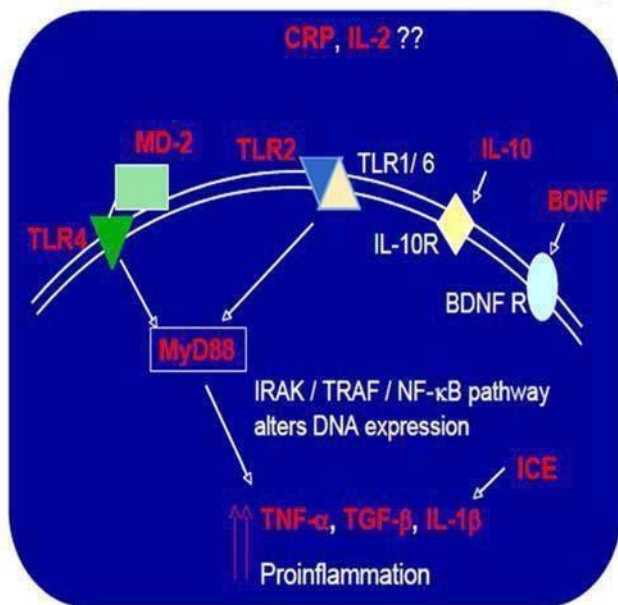
Several other investigators have reported some of the same cytokines to be different between cases and controls (Parkitny et al., 2015; Zhang et al., 1999; Skowera et al., 2004; Smylie et al, 2013; Khaiboullina et al., 2015). Preliminary MANCOVA analyses of cytokines with cognitive outcomes are also shown in Table 6 below. Results to date show significant differences between cases and controls on the Trail Making Test (TMT), Delis Kaplan Executive Function Systems (DKEFS) Color- Word Interference Task, Controlled Oral Word Association Test (COWAT), Finger Tap Test (FTT), California Verbal Learning Test – delayed recall and a trend for the Rey-Osterreith Complex figure test. These tests span the attention and executive system, motor and memory domains. These results are consistent with our recently published meta-analysis of cognitive functioning in multiple studies of GW veterans (Janulewicz et al., 2017).

Significant Neuropsych Assessments - Adjusted for Age, Gender, and Years of Education



Task 5f. Send additional saliva samples to University of Adelaide for genetic polymorphism analysis

Immune Genetic Markers from Saliva Samples



To date, genomic DNA isolated from 117 saliva samples and genotyped for genetic variability for 21 single nucleotide polymorphisms (SNPs) in the following genes: *IL1B*, *IL2*, *IL6*, *IL10*, *TNFFA*, *TGFB*, *ICE*, *IL6R*, *TLR2*, *TLR4*, *MD2*, *MYD88*, *BDNF*, *CRP*, and *OPRM1*. Differences between allele frequencies were compared amongst Caucasian Cases, Controls and population-based data (HapMap) and the impact of genetic variability on peripheral cytokine expression, and on pain, fatigue and CPT scores was examined.

It was observed that the frequency of a TGF-beta (TGFB) allele (rs1800469) frequency in GWI cases was significantly different to HapMap; 38% vs 29% OR = 1.52 (1.02-2.26), P=0.048 suggesting a possible association of this SNP with GWI. Stronger support for this association was shown when comparing GWI cases vs Controls on TGFB allele (rs1800469) frequency where GWI cases were significantly different to controls; 38% vs 15% OR = 3.31 (1.20-9.12), P=0.022. Thus, GW veterans with this allele have 3 times the risk of being a GWI case.

Other analyses with the previously reported differences in *IL-10* variants showed (rs1800896) in Cases was just

below significance compared to HapMap: 44% vs 53% OR = 1.46 (0.99-2.14), $P=0.054$. There was no impact of genetic variability on expression of IL-1b, IL-6, IL-2, or TNFa. However, there was a significant association between *IL-10* (rs1800871) genotype and expression of IL-10 in cases ($p=0.03$) and Controls ($p=0.0012$). Further analyses are being conducted to assess whether greater expression of IL10 in some cases is associated with less severe symptoms compared with other allele types.

There was also an association of genetic variants with pain and CPT scores when examined in IL10 (rs1800896) variant alleles which were significantly associated with pain scores in GWI cases $P=0.007$ but not in controls. IL10 (rs1800871) variant alleles were also significantly associated with CPT reaction time scores in controls $P=0.008$, but not in GWI cases.

These findings suggest that there may be important genetic-susceptibility in GWI cases and symptom severity that warrant further assessment. A grant was submitted by Drs. Collier, Sullivan, Klimas and Steele for a further grant application to study these SNPs in the larger BBRAIN cohort that will be developed. These results also continue to suggest that the pilot studies with IL10 treatments may indeed be important in GWI clinical and animal models.

Task 5g. Conduct preliminary analyses of clinical data

The BUSPH Data Coordinating Center has cleaned all current data and prepared the datasets for statistical analysis from the REDCap data capture web database in direct collaboration with the study biostatistician Dr. Heeren, Dr. Sullivan and the study PIs.

The overall aims of this integrated multidisciplinary consortium scientific focus are to (1) To identify validated markers of GW illness by using state of the art neuroimaging, behavioral, genetic and blood markers of neuroinflammatory activation in both clinical and preclinical models that will elucidate targeted and validated treatment strategies (2) To create a Neuroinflammation Risk Profile for GWI (3) To identify viable mechanistic treatments based on identified pathophysiological pathways of GWI that have been validated in preclinical treatment models.

Results of Pearson correlation coefficient analyses of cognitive testing outcomes compared with cytokines in GWI cases indicates significant correlations between cognitive outcomes and proinflammatory cytokines in both individual cognitive tests within different cognitive domains and by combining tests within a domain for the cognitive domains of memory, attention and executive functions, motor functions, visuospatial skills and mood functioning. For GWI cases the motor domain was correlated with TNF-alpha and the executive function domain was correlated with IL1-b ($p<0.05$).

In terms of health symptoms, the McGill Pain inventory was correlated with IL1-a and IL1-b and Pittsburgh sleep quality index was significantly correlated with TNF-RII levels. When compared with Kansas case criteria symptom domains and cytokine outcomes, IL1-b and TNF-a were significantly correlated with fatigue, pain, neurological and skin domains and IL2 and IL4 and TNF-RI were significantly correlated with the gastrointestinal and respiratory domains. These results again suggest that treatments targeting IL1, TNF-a and other proinflammatory cytokines are viable candidates for reducing pain, fatigue and other symptoms of GWI.

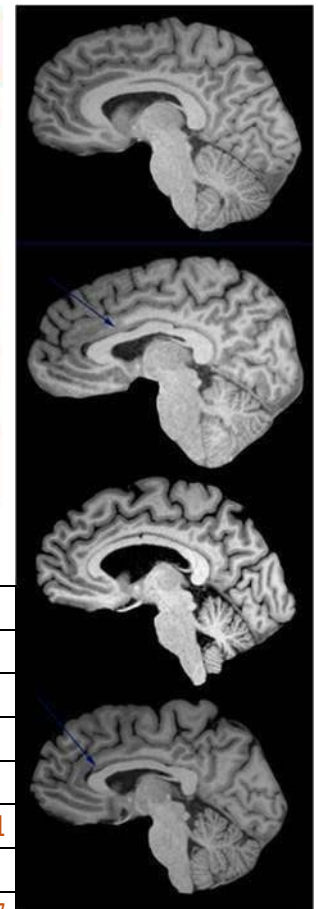
Brain volumetric analysis between cases and controls showed significant differences between MRI cortex volume and precentral gyrus volume ($p<0.05$) and pars-triangularis and superior longitudinal fasciculus ($p<0.01$) when controlling for age, gender, education and total intracranial brain volume in multivariate analyses. In addition, glutamate levels were significantly negatively correlated with many frontal gray and white matter pathways ($p<0.01$) suggesting the potential importance of excitatory neurotransmitter signaling in GWI. White matter HARDI pathways results are presented in table 2 below and show WM microstructural changes in the corpus callosum and the inferior and superior lateral fasciculus (SLF) that correspond to clinical observations clearly seen and marked on MRI scans below. SLF results correspond with those reported previously by Rayhan et al., 2013 and add strong concerns regarding additional loss of brain volumes and atrophy in superior frontal and parietal cortical areas and WM microstructural integrity in the corpus callosum and other structures. Collectively, these results suggest further quantification and validation of these potential objective diagnostic and pathogenic imaging markers of GWI. 32

Table 2. MRI Analyses Adjusted for Age, Gender, Years of Education and Total Intracranial Volume

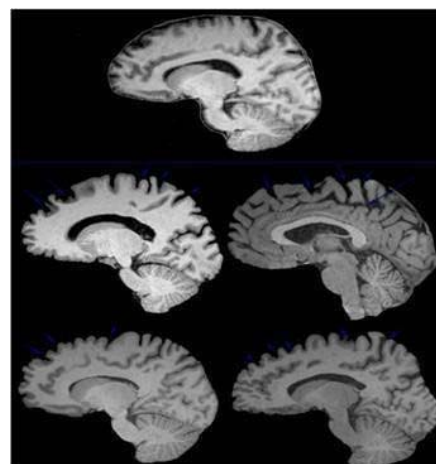
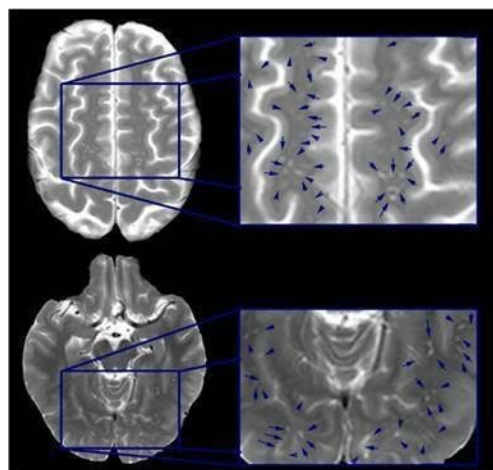
Brain Area	GW case Mean	Control Mean	P-value
Total Gray matter volume	595744	645131	0.01
Total cortex volume*	434566	452208	0.05
Precentral gyrus*	24465	27626	0.02
Caudal middle frontal gyrus	11595	13314	0.01
Pars-opercularis	7602	8207	0.05
Rostral middle frontal gyrus	19208	20989	0.03
Superior frontal gyrus	48851	52995	0.03
Pars-triangularis WM*	6766	6084	0.01
Superior Long. fasciculus WM* parietal endings -rh	1044	1271	0.005

Table 3. T-test Comparisons of WM Microstructural Integrity in GWI Cases vs Controls

Structure	Left FA	Left MD	Left RD	Right FA	Right MD	Right RD	Right AD	Left AD
Anterior Thalamic Radiations	0.98	0.27	0.37	0.10	0.41	0.99	0.01	0.14
Cingulum - Cingulate	0.60	0.63	0.81	0.24	0.34	0.24	0.96	0.32
Cingulum - Angular	0.14	0.12	0.15	0.24	0.34	0.81	0.96	0.14
Corticospinal Tract	0.61	0.52	0.42	0.21	0.26	0.13	0.2	0.23
Inferior Long. Fasciculus	0.48	0.07	0.10	0.78	0.06	0.13	0.02	0.001
Sup. Long. Fas. - Parietal	0.92	0.02	0.03	0.92	0.02	0.03	0.002	0.12
Sup. Long. Fas. - Temporal	0.84	0.03	0.15	0.35	0.03	0.19	0.002	0.007
Uncinate Fasciculus	0.96	0.61	0.75	0.94	0.30	0.45	0.22	0.38
Structure	FA	MD	RD	AD				
Corpus Callosum - minor	0.43	0.21	0.56	0.02				
Corpus Callosum - major	0.05	0.04	0.03	0.16				



Arro



The Boston call-back studies are currently recruiting and in progress. The microbiome pilot study is successfully recruiting GWIC participants with 25 of 30 participants recruited to date. This pilot study provided microbiome pilot data that resulted in two new grant submissions that were recommended for funding with Dr. Saurabh Chatterjee from University of S. Carolina and GWIC investigators. These studies will focus on gut-brain axis as a contributor to GWI and assess sodium butyrate as a potential new treatment for gastrointestinal and other symptoms of GWI. Drs. Chatterjee, Sullivan and Janulewicz have published a paper in Toxicology and Applied Pharmacology (TAAP) which shows reduction of neuroinflammation in GWI mice given oral sodium butyrate with no adverse outcomes in the animals at (Seth et al., 2018). Specifically, results in a GWI-mouse model showed that oral butyrate restored gut homeostasis and increased GPR109A receptor copies in the small intestine (SI). Claudin-2, a protein shown to be upregulated in conditions of leaky gut was significantly decreased following butyrate administration. Butyrate decreased TLR4 and TLR5 expressions in the liver concomitant to a decrease in TLR4 activation.

Specific recruitment numbers, plans and updates for other call back studies are listed below.

GWIC: Gulf War Illness Consortium
Screening, Enrollment, and Call-Back Studies Summary Report
12/12/2018

	Total	Boston	Miami	Texas
Number of Subjects Contacted	424	276	69	79
Number of Subjects Screened	404 (95.3%)	264 (95.7%)	69 (100.0%)	71 (89.9%)
Number of Subjects Eligible	266 (65.8%)	156 (59.1%)	60 (87.0%)	50 (70.4%)
Number of Subjects with Appointments Made	193 (72.6%)	104 (66.7%)	56 (93.3%)	33 (66.0%)
Number of Subjects Assessed	175 (90.7%)	100 (96.2%)	43 (76.8%)	32 (97.0%)
Subjects Participating in Stem Cell Call-Back Study	19	19	0	0
Subjects Participating in LP Call-Back Study	8	8	0	0
Subjects Participating in PET Scan Call-Back Study	23	23	0	0
Subjects Participating in Microbiome Call-Back Study	25	25	0	0

Task 6a. Perform lumbar punctures to obtain cerebrospinal fluid markers of neuroinflammation in 50 GW Veterans

The first eight lumbar punctures have been completed and recruitment efforts for this follow up study will continue in the coming quarters.

Task 6b. Perform positron emission tomography (PET) scanning with novel EAAT2 ligand in partnership with RIO pharmaceuticals in 15 GW veterans.

The PET animal pilot study has received ACURO approval this week and will begin soon. Dr. O'Callaghan has trained the RIO staff on animal dosing procedures and now the animals can be dosed and imaged with PET tracers shortly thereafter.

Task 6c. Perform FDG-PET scan imaging with 30 GW veterans after a computerized CPT cognitive challenge task. - Because many marker differences identified in GWI have been shown after challenge tasks, a FDG-PET imaging pilot study is being conducted after a continuous performance test (CPT) of information processing and sustained attention task 30 GW veterans will be assessed for differences in glucose utilization when compared with GW veteran healthy controls. The first twenty-three participants have been completed and recruitment is

ongoing for this study with several more participants scheduled in the coming quarter to finish this pilot study. Importantly, a new study has suggested that FDG PET signal may actually be driven by astrocytic glutamate transport as a glial contribution to neuroenergetics (Zimmer et al., 2017). Thus, analyses will be done to compare glutamate and GFAP blood levels in participants in this pilot study to assess potential astrocyte contribution to this imaging modality. A new CDMRP grant was also submitted to assess PET imaging ligands including PBR28 (microglial), EAAT2 or deprenyl (astrocyte) and [¹⁸F] FDG PET to use now available radioligands to tease out whether GWI is a predominant microglial or astrocyte induced (or both) chronic neuroinflammatory disorder. If FDG PET overlaps with and is equivalent to the other astrocyte PET ligands, it would be an easily deployable diagnostic test due to its wide availability in military and VA as well as other civilian hospitals.

Task. 7. Interim Analyses, Grant Submission, and Annual Reporting (Months 18-42)

7a. Data entry of all questionnaires, evaluations and quality control measures will be ongoing

Data entry of all questionnaires and evaluations has been ongoing in as close to real time as possible. The Data Coordinating Center also tracks missing and inconsistent data. The latest data integrity report is listed below and shows very few missing or out of range data points.

GWIC: Gulf War Illness Consortium Data Integrity Report Data as of October 4, 2018

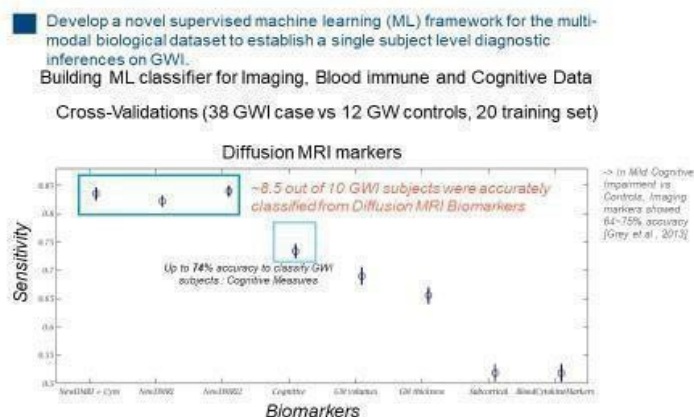
Site	Out of Range Data	Missing Data Fields	Invalid Logic
Boston	0.03% (15 of 52816)	0.08% (136 of 166162)	0.00% (0 of 2838)
Miami	0.00% (0 of 13952)	0.05% (23 of 43792)	0.00% (0 of 748)
Texas	0.01% (1 of 13920)	0.26% (114 of 43793)	0.00% (0 of 748)
Total	0.02% (16 of 80688)	0.11% (273 of 253747)	0.00% (0 of 4334)

7b. Interim Statistical analyses of data obtained from cognitive evaluations, blood markers, neuroimaging and questionnaire data will be performed periodically.

Interim analyses were presented above in Task 6 and additional analyses of machine learning multi-modal approach to brain connectomics is continuing to be conducted. Results to date are very promising and show that diffusion tensor imaging of WM microstructural outcomes predict Kansas GWI cases 85% of the time. Further analyses are being conducted to improve the prediction rate further to closer to 100% GWI case classification. A new CDMRP grant was submitted to include longitudinal MRI and other markers of GWI to further refine and predict GWI

criteria using machine learning and other advanced data analytics. A manuscript of the machine learning outcomes is planned for the next quarter.

Multimodal Image Processing Pipelines

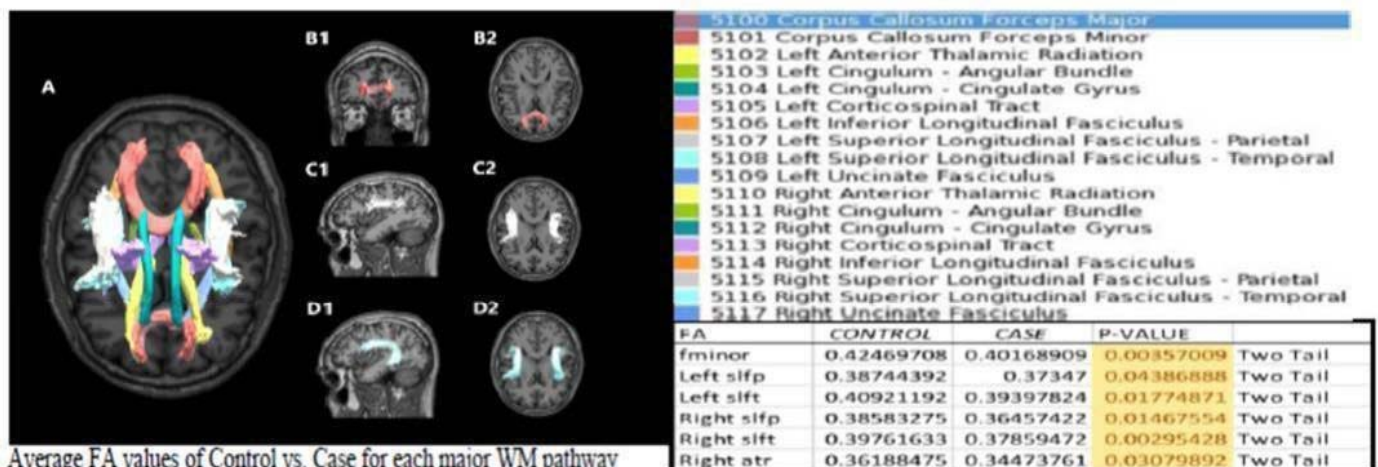


Brain imaging studies have shown significantly lower cortical frontal pathway volumes and worse microstructural integrity of the WM pathways including the superior and inferior lateral fasciculi and the corpus callosum forceps minor that correlate with increased signaling of the excitatory neurotransmitter glutamate (see figure below). GWIC structure-function relationships in GWI cases showed correlations with High Angular Resolution Diffusion Imaging (HARDI) sequences to determine

WM microstructural integrity and behavioral outcomes such that as WM integrity decreased, symptom complaints of pain, fatigue and poor sleep quality increased and cognitive performance on attention/executive system functioning, motor functioning and information processing were adversely affected.

Dr. Deb Little from the GWIC Houston site has observed a number of pathological indicators on the MRI brain scans of the veterans with GWI scanned for the GWIC. Three types of tissue abnormalities were consistently observable on MRI scans of individual veterans with GWI and may have clinical utility and diagnostic potential for ill veterans. Drs. Little and Steele in Houston have collaborated with Drs. Sullivan and Killiany in Boston on a new grant proposal to quantify, validate and further expand these findings and determine their diagnostic utility.

Magnetic Resonance Imaging (MRI) of veterans with GWI have found reduced white matter volumes in the brain. In order to gain a better understanding of the microstructural changes in the remaining white matter, we assessed the major white-matter pathways using diffusion-weighted MRI (DWI). Data were obtained from 12 healthy and 48 veterans with GWI. We used Freesurfer v6 TRActs Constrained by UnderLying Anatomy (TRACULA) function to reconstruct 18 major pathways in the brain of each subject. A single high resolution structural scan and a High Angular Resolution Diffusion-weighted Imaging (HARDI) scan was input into TRACULA. For each pathway, we obtained measures of average Fractional Anisotropy (FA), Radial Diffusivity (RD) and Mean Diffusivity (MD). Independent samples student's t-tests were used to identify differences between the groups. These analyses revealed that the microstructural integrity of the Corpus Callosum [forceps minor], Superior Longitudinal Fasciculus (SLF) [temporal and parietal tracts] along with the Anterior Thalamic Radiation (ATR) was compromised in veterans with GWI. These results add to the increasing evidence indicating that alterations to the white matter of the brain is an essential component of GWI.



Average FA values of Control vs. Case for each major WM pathway present. Values highlighted in yellow represent those showing significance. Figure A represents all 18 tract outputs from TRACULA with each color corresponding to the table to the right. Figure B1 and B2 are coronal and transverse views of the forceps minor, which contain fibers connecting the lateral and medial surfaces of the frontal lobes. Figure C1 and C2 are sagittal and transverse views of the SLF-T, which contain fibers from frontal and temporal lobes. Figure D1 and D2 are sagittal and transverse views of the SLF-P, which contain fibers from frontal and parietal lobes.

7c. Grant submissions to relevant funding agencies for further collaborative studies based on initial results and preliminary data targeted toward treatment strategies will be ongoing.

Nine letters of intent were submitted this quarter primarily from our GWIC senior investigators and collaborators in order to further expand scientific expertise in solving the problem of GWI biomarkers and treatment development. These studies will utilize the GWIC biorepository for further analyses of biomarkers, gene-exposure outcomes and clinical treatments and we also now utilized the recently funded BBRAIN biorepository network of GWI samples.

Newly Submitted Grant Applications:

1. 'Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response'
2. 'Clarifying the Role Played by Microglia and Astrocyte Activation in Gulf War Illness Using Positron Emission Tomography (PET)'
3. 'Immune-genetic Biomarkers of Risk and Resiliency for GWT'
4. 'Identifying Clinically Relevant Objective Brain Imaging Diagnostic Markers of GWT'
5. 'Identifying Objective Diagnostic Markers of Gulf War Illness: Salivary and Plasma Autoantibodies against Neural Proteins validated with Brain Imaging'
6. 'Studying the unique virome signature in Gulf War Illness to treat dysbiosis and symptom persistence by a phage-therapy approach'
7. 'Assessing miRNA, mitochondria and motor proteins in neurons and astrocytes in GWT'
8. 'Investigating systematic pathological mechanisms linked to CNS symptoms of GWI via a human multicellular 3D brain model'
9. 'Mild Traumatic Brain Injury Association with Gulf War Illness: Evaluation with Established Models'

7d. Annual reports of progress will be written. Annual reports have been written for the past 5 years with corresponding quarterly reports in between.

Key Research Accomplishments: Fifteen manuscripts and 30 abstracts have been published. Twenty new studies have been funded to date including two new consortia. This multi-institutional collaboration of highly qualified GWI researchers from public universities, federal agencies, and the private sector, provide an unprecedented opportunity to fully elucidate the underlying pathobiology of GWI in one integrated model that once proven, will lead to focused treatment trials that can be quickly implemented.

The central hypothesis for the pathobiological mechanisms of GWI in this consortium includes chronic neuroinflammation as a result of initial glial activation and then priming of glial responses that cause stronger and longer responses that do not shut off the chemical cascade of proinflammatory cytokines and chemokines that cross-talk between the immune system and the brain. This could result in a lasting multisystem illness affecting many body systems, as seen in GWI.

Improved understanding of the role of glial activation in chronic pain states has given rise to rapidly expanding efforts to identify pharmaceuticals that specifically focus on glial functions. The growing availability of treatments of this type gives particular urgency to our efforts to determine the extent to which glial activation and central cytokine activation explain the symptoms of GWI. In order to specifically address the research gaps outlined by the IOM and the RAC reports with regard to biomarker identification and pathobiology of GWI, this research team is characterizing disease symptoms and validating and improving pathobiological markers based on collective prior clinical and preclinical studies and leveraging longitudinal cohorts and stored blood samples with the ultimate goal of identifying targeted and effective treatments for GWI. Results to date suggest that the consortium animal model of GWI is correlated with behavioral alterations seen in clinical studies including altered memory functioning and that chronic neuroinflammation and microglial and astrocyte activation are present in the DFP + CORT model. Myelin studies show an increase in myelin basic protein at early and later time points that is not associated with new myelination. Calcium signaling is also increased in oligodendrocytes in the GWI model. Microtubule stability and axonal transport are also found in animal and stem cell derived organoid GWI models. These preclinical model results provide clear pathways for objective biomarkers and targeted treatments for GWI.

Conclusion: Several promising therapeutic avenues have been developed to date in the GWIC preclinical studies and several more have been approved for ongoing and planned pilot studies. Preliminary clinical study results suggest brain-immune-behavioral outcome correlations that bode well for the derivation of a neuroinflammatory risk profile of GWI, diagnostic marker development and targeted therapeutic strategies in the very near future. Specific Results show cognitive decrements and brain imaging alterations in white matter (WM) volumes, WM integrity in Corpus Callosum and other key WM pathways and frontal and parietal gray matter volumes. These structure and functional alterations correlate with blood markers including cytokines and glutamate. These provide key areas to focus on for objective biomarkers of GWI and focused treatment targets. GWI.

PUBLICATIONS, ABSTRACTS and PRESENTATIONS

- CDC presented a poster for the 2017 Society for Neuroscience annual Meeting (Washington, D.C.; Nov-2017) and the Gulf War Veteran's Illness Symposium (VA Palo Alto; Sept-2017).
- CDC presented 4 posters related to GWI work at the 46th Annual Society for Neuroscience meeting in San Diego, CA, November 12-16, 2016.
- CDC presented data on GWI as part of a panel discussion on sickness behavior and disease at the 50th Annual Winter Conference on Brain Research in Big Sky, MT, January 28-February 2, 2017.
- CDC presented data on GWI as part of a symposium on sickness behavior and disease at the 56th Annual Society of Toxicology meeting in Baltimore, MD, March 12-16, 2017.
- BUSPH and Duke investigators presented a poster related to the collaborative CNS autoantibody study at the 56th Annual Society of Toxicology meeting in Baltimore, MD, March 12-16, 2017.
- Drexel and BUSPH investigators presented posters related to GWIC studies and were chosen for a media press conference for the Annual Society for Neuroscience meeting in Washington, DC November 11-15, 2017.

Publications (see appendix for full-papers and *for those freely available)

GWIC Published Papers:

1. Janulewicz, Patricia, Maxine Krengel, Emily Quinn, Timothy Heeren, Rosemary Toomey, Ronald Killiany, Clara Zundel, et al. "The Multiple Hit Hypothesis for Gulf War Illness: Self- Reported Chemical/Biological Weapons Exposure and Mild Traumatic Brain Injury." *Brain Sciences* 8, no. 11 (November 13, 2018). <https://doi.org/10.3390/brainsci8110198>.*
2. Kelly, K. A., Michalovicz, L. T., Miller, J. V., Castranova, V., Miller, D. B., & O'Callaghan, J. P. (2018). Prior exposure to corticosterone markedly enhances and prolongs the neuroinflammatory response to systemic challenge with LPS. *PLOS ONE*, 13(1), 0190546. <https://doi.org/10.1371/journal.pone.0190546>*
3. Koo, B.-B., Michalovicz, L. T., Calderazzo, S., Kelly, K. A., Sullivan, K., Killiany, R. J., & O'Callaghan, J. P. (2018). Corticosterone potentiates DFP-induced neuroinflammation and affects high-order diffusion imaging in a rat model of Gulf War Illness. *Brain, Behavior, and Immunity*, 67, 42–46. <https://doi.org/10.1016/j.bbi.2017.08.003>
4. Maule, A. L., Janulewicz, P. A., Sullivan, K. A., Krengel, M. H., Yee, M. K., McClean, M., & White, R. F. (2018). Meta-analysis of self-reported health symptoms in 1990-1991 Gulf War and Gulf War-era veterans. *BMJ Open*, 8(2), e016086. <https://doi.org/10.1136/bmjopen-2017-016086>*
5. Joshi U, Evans JE, Joseph R, Emmerich T, Saltiel N, Lungmus C, Oberlin S, Langlois H, Ojo J, Mouzon B, Paris D, Mullan M, Jin C, Klimas N, Sullivan K, Crawford F, Abdullah L. Oleoylethanolamide treatment reduces neurobehavioral deficits and brain pathology in a mouse model of Gulf War Illness. <https://www.nature.com/articles/s41598-018-31242-7>. Sci Rep. 2018 Aug 27;8(1):12921. doi: 10.1038/s41598-018-31242-7.*

6. Seth, R. K., Kimono, D., Alhasson, F., Sarkar, S., Albadrani, M., Lasley, S. K., ... Chatterjee, S. (2018). Increased butyrate priming in the gut stalls microbiome associated- gastrointestinal inflammation and hepatic metabolic reprogramming in a mouse model of Gulf War Illness. *Toxicology and Applied Pharmacology*, 350, 64–77. <https://doi.org/10.1016/j.taap.2018.05.006>
7. Rao, A. N., Patil, A., Brodnik, Z. D., Qiang, L., Espana, R. A., Sullivan, K. A., ... Baas, P.W. (2017). Pharmacologically increasing microtubule acetylation corrects stress-exacerbated effects of organophosphates on neurons. *Traffic (Copenhagen, Denmark)*, 18(7), 433–441. <https://doi.org/10.1111/tra.12489>
8. Abou-Donia, M. B., Conboy, L. A., Kokkotou, E., Jacobson, E., Elmasry, E. M., Elkafrawy, P., ... Sullivan, K. (2017). Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicology and Teratology*, 61, 36–46. <https://doi.org/10.1016/j.ntt.2017.03.002>
9. Emmerich, T., Zakirova, Z., Klimas, N., Sullivan, K., Shetty, A. K., Evans, J. E., ... Crawford, F. (2017). Phospholipid profiling of plasma from GW veterans and rodent models to identify potential biomarkers of Gulf War Illness. *PLOS ONE*, 12(4), e0176634. <https://doi.org/10.1371/journal.pone.0176634>*
10. Fields, R. D., Dutta, D. J., Belgrad, J., & Robnett, M. (2017). Cholinergic signaling in myelination. *Glia*, 65(5), 687–698. <https://doi.org/10.1002/glia.23101>
11. Janulewicz, P. A., Kregel, M. H., Maule, A., White, R. F., Cirillo, J., Sisson, E., ... Sullivan, K. (2017). Neuropsychological characteristics of Gulf War illness: A meta-analysis. *PLOS ONE*, 12(5), e0177121. <https://doi.org/10.1371/journal.pone.0177121>*
12. Locker, A. R., Michalovicz, L. T., Kelly, K. A., Miller, J. V., Miller, D. B., & O'Callaghan, J. P. (2017). Corticosterone primes the neuroinflammatory response to Gulf War Illness- relevant organophosphates independently of acetylcholinesterase inhibition. *Journal of Neurochemistry*, 142(3), 444–455. <https://doi.org/10.1111/jnc.14071>
13. O'Callaghan, J. P., Michalovicz, L. T., & Kelly, K. A. (2016). Supporting a Neuroimmune Basis of Gulf War Illness. *EBioMedicine*, 13, 5–6. <https://doi.org/10.1016/j.ebiom.2016.10.037>
14. Qiang, L., Rao, A. N., Mostoslavsky, G., James, M. F., Comfort, N., Sullivan, K., & Baas, P. W. (2017). Reprogramming cells from Gulf War veterans into neurons to study Gulf War illness. *Neurology*, 88(20), 1968–1975. <https://doi.org/10.1212/WNL.0000000000003938>
15. Abdullah, L., Evans, J. E., Joshi, U., Crynen, G., Reed, J., Mouzon, B., ... Crawford, F. (2016). Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness. *Toxicology*, 372, 22–33. <https://doi.org/10.1016/j.tox.2016.10.012>

Abstracts

DH Woo, DJ **Dutta**, W Huffman, M Robnett, PR Lee, K **Sullivan**, R Killiany, J O'Callaghan, RD Fields: Role of glia in the pathophysiology of Gulf War Illness. Abstract & Poster. Society for Neuroscience, San Diego, CA Oct 2016.

L. Abdullah, T. Emmerich, JE Evans, U Joshi, J Reed, G. Laco, V. Mathura, **K. Sullivan**, **N. Klimas**, **M. Mullan**, F. Crawford. Application of lipidomics for identifying novel blood biomarkers of Gulf War Illness. Abstract and poster. International Association of Chronic Fatigue Syndrome, Ft. Lauderdale, FL Oct 2016.

M.B. Abou-Donia, **K. Sullivan**, L. Conboy, E. Kokkotou: Serum Autoantibodies to Neural-Specific Proteins as Objective Biomarkers for Gulf War Illness. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016.

Kelly KA, **Locker AR**, Michalovicz LT, Miller DB, O'Callaghan JP: Exploration of the gulf war illness phenotype in a mouse model challenged with LPS at long term time points. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016.

Revitsky AR, **Kelly KA**, Miller DB, Lasley SM, **O'Callaghan JP**: Organophosphate-induced Neuroinflammation, With and Without Corticosterone Pretreatment, Is Not Due to Acetylcholinesterase Inhibition. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Michalovicz LT, **Locker AR**, **Kelly KA**, Miller DB, **O'Callaghan JP**: Corticosterone priming of the neuroinflammatory response to acetylcholinesterase inhibitors results in overexpression of TLR2 and downstream targets, but not activation of the NLRP3 inflammasome. Abstract & Poster. Society of Toxicology, New Orleans, LA March 2016

Yee, M., Seichepine, D., **Janulewicz Lloyd P.**, **Sullivan, K.** & **Krengel, M.** History of Pre-War Brain Injuries Influences Total Current Health Symptoms in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 8.

Seichepine, D., Yee M., **Janulewicz Lloyd P.**, **Sullivan, K.** & **Krengel, M.** Frequency of Traumatic Brain Injuries in a Cohort of 1990-1991 Gulf War Veterans. Abstract. 43rd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, March 2015: 14.

Maule, A., **Janulewicz, P.**, **Krengel, M.**, White, RF, Judd, S., Cirillo, J., **Sullivan, K.** A meta-analysis of Self-reported Neurological and Neuropsychological Symptoms in Gulf War Veterans. Journal of the International Neuropsychological Society, Supplement 1, March 2015: 9.

Seichepine, D., Yee, M., **Janulewicz-Lloyd P.**, **Sullivan, K** & **Krengel, M.** Chronicity of Health Symptoms in the Ft. Devens Cohort. International Neuropsychological Society, 42nd Annual Meeting Abstracts, Journal of the International Neuropsychological Society, Supplement 1, February 2014; 165.

Michalovicz LT, **Locker AR**, **Kelly KA**, Miller DB, **O'Callaghan JP**: Chronic corticosterone primes the brain response to select neuroinflammatory agents by overexpression of toll-like receptor 2 and S100A8: A potential role of microglia. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Locker AR, Kelly KA, Michalovicz LT, Miller DB, O'Callaghan JP: Corticosterone primes the neuroinflammatory responses to Gulf War Illness associated exposures: Effects of irreversible vs reversible acetylcholinesterase inhibitors. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Kelly KA, Locker AR, Michalovicz LT, Miller DB, O'Callaghan JP: Phenotype comparisons of ALDH1L1 BAC-TRAP mice under control and neurotoxic (MPTP) conditions. Abstract & Poster. Society for Neuroscience, Chicago, IL October 2015

Revitsky AR, Kelly KA, Miller DB, Lasley SM, O'Callaghan JP: Pyridostigmine bromide suppresses neuroinflammation induced by DFP. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Kelly KA, Revitsky AR, Miller DB, Lasley SM, O'Callaghan JP: Chronic glucocorticoid and nerve agent DFP exposures produce a neuroinflammatory model of Gulf War Illness without neurodegeneration. Abstract & Poster. Society of Toxicology, San Diego, CA March 2015

Presentations

K. Sullivan. Understanding Gulf War Illness: Brain-Immune Biomarkers, Cognitive Functioning and Treatment Development Strategies 25 Years after the War. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

K. Sullivan. Brain Immune Interactions in Gulf War Illness: Cytokines and Cognition in US Military Veterans. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

K. Sullivan Cytokines, Cognition and Gulf War Illness in a US Military Veteran Cohort. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Golier, J. A Controlled Trial of a Glucocorticoid Receptor Antagonist in Gulf War Veterans with Chronic Multisymptom Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Abou-Donia M. Screening for novel objective central nervous system biomarkers in veterans with Gulf War Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Krengel, M. Exploring the association between cognitive symptoms and exposures in a cohort of 1990-1991 US Gulf War Veterans. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Meggs, W. Double-blinded Placebo-Controlled Cross-over Pilot Trial of Naltrexone to Treat Gulf War Illness. Symposium. International Neuropsychological Society (INS) in London, July 6th- 8th

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston

University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 16, 2016.

Sullivan, K., Klimas, N. Committee and Panel Discussion: 'how to discussion' for GWI Biomarker Research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC, September, 2014.

O'Callaghan, J., Sullivan, K. Committee and Panel Discussion: 'how to discussion' for GWI animal research, Research Advisory Committee on Gulf War Veterans' Illnesses; Spring Meeting, Washington, DC,

April, 2014.

Seichepine, D., Yee M., **Janulewicz Lloyd P., Sullivan, K. & Krengel, M.** Chronicity of Health Symptoms in the Ft. Devens Cohort. International Neuropsychological Society, 42nd Annual Meeting, Seattle, WA, February 2014.

Steele, L. Committee and Panel Discussion: ‘how to discussion’ for GWI Case Criteria Research Advisory Committee on Gulf War Veterans’ Illnesses; Winter Meeting, Washington, DC, January, 2014.

Seichepine, D., Yee, M., **Janulewicz Lloyd P., Sullivan, K., Proctor, S., & Krengel, M.** Traumatic Brain Injury and Health Status of Veterans from the 1990-1991 Gulf War. Boston University Second Annual Joining Forces TBI/PTSD Event, Boston, MA, December, 11, 2013.

Sullivan, K. RAC-GWVI Treatment Development Discussion. Research Advisory Committee on Gulf War Veterans’ Illnesses; Summer Meeting, Washington, DC, June, 2013.

Sullivan, K. Neurotoxicity of Gulf War Deployment: The Neuropsychological and Neuroimaging Correlates. Boston University School of Public Health, Introduction to Toxicology (EH768, guest lecture), Boston, MA, March, 26, 2013.

INVENTIONS, PATENTS AND LICENSES – A

REPORTABLE OUTCOMES

Current/Newly Funded Studies

- Boston Biorepository and Integrative Network for Gulf War Illness (BBRAIN) (PI: Sullivan; GW170055)
- The Gulf War Illness Clinical Trials and Interventions Consortium (GWICTIC) (PI: Klimas; GW170044)
- Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans (PI: Baas; GW170033)
- Novel Combinatorial screening for Neurotrophins, Neurotrophic cytokines, Matrix Metalloproteinases and Complement components in relevance to Neuronal Autoantibodies in the serum and CSF of Veteran with Gulf War illness (PI: Mulugu; GW170103)
- Computer Aided Decoding of Brain-Immune Interactions in Gulf War Illness (GWI): A Joint Embedding on Brain Connectomic and Immunogenetic Markers (PI: Koo; GW160032)
- BChE + PON1 biomarker epidemiological New Investigator proposal (PI: Janulewicz; GW160053)
- Epigenetic DNA methylation study with Naval Research Lab investigators (PI: Malanoski; GW160096)
- B-cell depletion Rituximab treatment trial proposal with NSU investigators (PI: Klimas; GW160123)
- Tau pathology as a contributor to GWI (PI: Qiang; GW160151)
- PET PBR28 study funded with MGH investigators (Loggia: PI; GW130100)
- Human induced pluripotent stem cells (iPSC) stem cell grant funded with Drexel and BU investigators (Baas PI; Sullivan site PI; GW140086)
- D-cycloserine pilot treatment study funded with Boston University investigators (Toomey PI; Sullivan co-I) (GW140069)
- CNS autoantibody grant with Duke investigators (Sullivan Initiating PI; Abou Donia Partnering PI; GW140140)
- CoQ10 Phase III trial, 4 site study submitted to VA with Miami VA, GWIC and other investigators
- Ft Devens cohort cognitive, blood and neuroimaging assessment of brain antioxidant glutathione

- levels (Krengel, Initiating PI; Sullivan Partnering PI; GW150050)
- PON1 study with GWIC investigators and San Francisco VA investigators GW150037)
- Gulf War Women's Health Cohort with Augusta University investigators (GW150116)
- Lipidomics and proteomics study with Roskamp Institute investigators (GW150056)
- +naltrexone pain treatment New Investigator proposal with U-Colorado investigators (GW150187)

Newly Funded Studies

Title: Boston Biorepository, Recruitment and Integrative Network (BBRAIN) for GWI (PI: Sullivan)

Supporting agency: Department of Defense (CDMRP/GWIRP GW170055)

Performance period: 9/01/18 – 8/31/21

Level of funding: 3,278,756

Brief description of project's goals/Specific aims: The primary objective of BBRAIN is to establish a retrospective and prospective biorepository network for GWI research by data mining from existing BBRAIN collaborator specimens and for recruiting 500 additional repository samples. The four prospective recruitment resource sites will include Boston, Miami, Bronx and San Francisco. The BBRAIN structure will provide centralized cataloguing and coordination of retrospective biorepository samples from 10 collaborating institutions who will share existing blood plasma, sera, PBMCs, cerebrospinal fluid, human-induced pluripotent stem cells (hiPSCs), DNA and saliva samples. Corresponding cognitive outcomes, brain imaging, demographics and health symptom surveys will be included in BBRAIN network datasets to allow for the comparison of biomarkers with behavioral outcomes.

Title: The Gulf War Illness Clinical Trials and Interventions Consortium (GWICTIC) (PI: Klimas)

Supporting agency: Department of Defense (CDMRP/GWIRP GW170044)

Performance period: 9/01/18 – 8/31/22

Level of funding: 8,000,000

Brief description of project's goals/Specific aims: This consortium aims to unify the expertise that has been developed through past CDMRP funding of GWICs based at NSU and BU, and build on their integrated research findings to implement early phase clinical trials of interventions targeting neuro-inflammation, previously identified biologic markers of disease activity and mechanisms of homeostatic reset. The infrastructure established in this proposal will thus facilitate a rapid and effective approach to evaluating potential interventions through early-phase studies and identifying promising candidates for phase III study. Specifically, study 1 (phase I) and study 2 (phase II) will evaluate a combination approach using entanercept, an anti-TNF agent, and mifepristone, a synthetic steroid with anti-progesterone and anti-glucocorticosteroid effects. Study 3 and 4 in the phase 1 will compare CoQ10 to glutathione ability to correct CNS oxidative stress, the phase 2 takes the antioxidant with the best CNS effect and combines it with intranasal insulin. Lastly, study 5 will evaluate a nutraceutical, Bacopa, that has been shown to have multiple impacts on inflammatory cytokines and mitochondrial function.

Title: Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans (PI: Baas)

Supporting agency: Department of Defense (CDMRP/GWIRP GW170023)

Performance period: 9/01/18 – 8/31/21

Level of funding: 700,000

Brief description of project's goals/Specific aims: The primary objective of this study is to establish a High throughput treatment development pipeline using hiPSC cell lines derived from Gulf War veterans and using animal models to test HDAC and kinesin 5 inhibitors and the antioxidant Co-Q10.

Title: Novel Combinatorial screening for Neurotrophins, Neuropoietic cytokines, Matrix Metalloproteinases and Complement components in relevance to Neuronal Autoantibodies in the serum and CSF of Veteran with Gulf War illness (PI: Mulugu)

Supporting agency: Department of Defense (CDMRP/GWIRP GW170103)

Performance period: 9/01/18 – 8/31/21

Level of funding: 700,000

Brief description of project's goals/Specific aims: The primary objective of this study is to establish Biomarkers of GWI that build on prior work to generate autoantibodies to CNS proteins in the blood of GW veterans by assessing additional CNS biomarkers in blood samples from GW veterans including neurotrophins, neuropoietic cytokines and matrix metalloproteinases to provide new biomarker avenues for GWI diagnostics and therapeutics.

Title: Examination of Neuroimaging, Cognitive Functioning, and Plasma Markers in a Longitudinal Cohort of Gulf War Deployed Veterans: The Fort Devens Cohort Supporting agency: DoD/CDMRP (GW150050P1)

Performance Period: 09/30/16 – 9/29/19

Level of funding: \$242,662

Brief description of project's goals/ Specific aims: The goal of this study is to develop brain imaging and peripheral blood plasma biomarkers of oxidative stress that correlate with cognitive and health symptom outcomes in the longitudinally followed Ft. Devens cohort of Gulf War veterans. The specific aims are (1) to conduct follow- up longitudinal cognitive evaluations on 150 GW veterans and (2) to determine, in 100 GW veterans, cross- sectional blood and neuroimaging biomarkers of glutathione metabolite (GSH) oxidative stress markers that will be correlated with cognitive and imaging outcomes.

Title: Examination of plasma PON1 paraoxonase activity and genotype in Gulf War Veterans (PI: Chao)

Supporting agency: DoD/CDMRP

(GW150037) Performance period: 09/30/16 – 09/29/19

Level of funding: \$587,011

Brief description of project's goals/ Specific aims: The goal of this study is to evaluate the extent to which paraoxonase (PON1), a human enzyme that can hydrolyze the active metabolites of several organophosphorus (OP) compounds and Gulf War (GW)-related exposure interactions contribute to the risk for developing Gulf War Illness (GWI) in a large (> 800) sample of GW veterans by leveraging existing PON1 paraoxon activity and PON1192 genotype data and GW-related exposure data in 4 independent cohorts of GW veterans.

Title: Gulf War Women's Health Cohort (PI:

Coughlin) Supporting agency: DOD/CDMRP

(GW150116) Performance Period: 09/30/16 – 9/29/19

Level of funding: \$ 1,147,729

Brief description of project's goals/ Specific aims: The goal of this study is to develop a large (>900) cohort of Women Gulf War veterans from prior studies, resurvey them and determine differences bet An in- vivo investigation of Brain Inflammation ween men and women Gulf War veterans' health outcomes.

Title: Examination of Neuroimaging, Cognitive Functioning, and Plasma Markers in a Longitudinal Cohort of

Gulf War Deployed Veterans: The Fort Devens Cohort (Krengel PI; Sullivan PI)

Supporting agency: DoD/CDMRP (GW150050P1) Performance Period: 09/30/16 – 9/29/19

Level of funding: \$242,662

Brief description of project's goals/ Specific aims: The goal of this study is to develop brain imaging and peripheral blood plasma biomarkers of oxidative stress that correlate with cognitive and health symptom outcomes in the longitudinally followed Ft. Devens cohort of Gulf War veterans. The specific aims are (1) to conduct follow-up longitudinal cognitive evaluations on 150 GW veterans and (2) to determine, in 100 GW veterans, cross-sectional blood and neuroimaging biomarkers of glutathione metabolite (GSH) oxidative stress markers that will be correlated

with cognitive and imaging outcomes.

Title: A Randomized, Double-blind Placebo-controlled Phase III Trial of Coenzyme Q10 in Gulf War Illness. (PI: Klimas)

Supporting Agency: Department of Veterans Affairs

Performance Period: 04/1/17 – 1/31/20

Level of funding: \$ 3,200,000

Project Description: The goal of this study is to perform a phase III treatment trial of Co-enzyme Q10 in Gulf War veterans at four sites around the country.

Title: Computer-Aided Decoding of Brain-Immune Interactions in Gulf War Illness (GWI): A Joint Embedding on Brain Connectomic and Immunogenomic Markers (PI: Koo)

Supporting Agency: Department of Defense/CDMRP GW160032

Performance Period: 09/1/17 – 8/31/20

Level of funding: \$ 666,189

Brief description of project goals/specific aims: The purpose of the proposed study is to apply a novel classification framework based on a combination of brain connectomics and immunoproteomics to the GWI Consortium (GWIC) database in order to develop a computerized diagnostic system for GWI. The project's 3 specific aims are (1) to build a unimodal classifier per each biological measure of GWI, including neuroimaging as well as central (CSF) and peripheral (blood) markers; (2) to decode brain-immune interactions based on joint embedding of the multidimensional classification features; and (3) to build multimodality classifiers of subtle symptom clusters in addition to the overall GWI definition (eg, the Kansas definition).

Title: Investigating Gene-Environment Interactions in Multiple Cohorts of 1990-1991 Gulf War Veterans (PI: Janulewicz)

Supporting Agency: Department of Defense/CDMRP GW160053

Performance Period: 09/1/17 – 8/31/20

Level of funding: \$687,016

Brief description of project goals/specific aims: The project's specific aims are to (1) determine associations among GWI, as defined by Kansas and CDC case definitions, butyrylcholinesterase (BChE) genotype, and cholinergic exposures encountered during deployment in 4 independent cohorts of 1990-1991 Gulf War

veterans (GWVs) comprising a total sample of 834 veterans; and (2) determine the association between GWI, as defined by Kansas and CDC case definitions, and exposure to cholinergic compounds in subgroups of veterans defined by specific combinations of BChE and PON1 192 genotypes.

Title: Identification of Epigenetic Signatures as Biomarkers of Gulf War Illness (PI: Malanoski)

Supporting Agency: Department of Defense/CDMRP GW160096

Performance Period: 10/1/17 – 9/30/20

Level of funding: \$1,132,441

Brief description of project goals/specific aims: Specific Aim 1: Identify Lymphocyte (leukocyte) DNA Methylation patterns specific to GWI Specific Aim 2: Discover the changes in microRNA profiles associated with GWI Specific Aim 3: Apply high performance computing bioinformatics to characterize GWI pathogenesis.

Other Achievements.

The consortium website (<http://sites.bu.edu/gwic>) and social media pages are continually updated to disseminate news about new papers and studies related to Gulf War Illness. Multiple news media stories have highlighted GWIC work this past year and are included on the GWIC website.

REFERENCES

1. Abou-Donia MB, Conboy LA, Kokkotou E, Jacobson E, Elmasry EM, Elkafrawy P, Neely M, Bass CR, Sullivan K. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol Teratol*. 2017 May Epub 2017 Mar 9.
2. Abdullah L, Evans JE, Joshi U, Crynen G, Reed J, Mouzon B, Baumann S, Montague H, Zakirova Z, Emmerich T, Bachmeier C, Klimas N, Sullivan K, Mullan M, Ait-Ghezala G, Crawford F. Long title: Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness. *Toxicology*. 2016 Oct 29. pii: S0300-483X(16)30256-6. doi: 10.1016/j.tox.2016.10.012.
3. Banks, C.N., & Lein, P.J. (2012). A review of experimental evidence linking neurotoxic organophosphorus compounds and inflammation. *Neurotoxicology*.
4. G. Broderick, A. Kreitz, J. Fuite, M.A. Fletcher, S.D. Vernon, N. Klimas, A pilot study of immune network remodeling under challenge in Gulf War Illness, *Brain, behavior, and immunity* 25 (2011) 302-13.
5. Chao, L.L., Abadjian, L., Hlavin, J., Meyerhoff, D.J., Weiner, M.W. (2011). Effects of low-level sarin and cyclosarin exposure and Gulf War Illness on brain structure and function: a study at 4T. *Neurotoxicology*, 32, 814-22.
6. Emmerich T, Zakirova Z, Klimas N, Sullivan K, Shetty AK, Evans JE, Ait-Ghezala G, Laco GS, Hattiangady B, Shetty GA, Mullan M, Crynen G, Abdullah L, Crawford F. Phospholipid profiling of plasma from GW veterans and rodent models to identify potential biomarkers of Gulf War Illness. *PLoS One*. 2017 Apr 28;12(4):e0176634. doi: 10.1371/journal.pone.0176634. eCollection 2017. <https://www.ncbi.nlm.nih.gov/pubmed/28453542>
7. Fields RD, Dutta DJ, Belgrad J, Robnett M. Cholinergic signaling in myelination. *Glia*. 2017 May;65(5):687-698. doi: 10.1002/glia.23101. Epub 2017 Jan 19.
8. Heaton KJ, Palumbo CL, Proctor SP, et al. (2007). Quantitative magnetic resonance brain imaging in US army veterans of the 1991 Gulf War potentially exposed to sarin and cyclosarin. *Neurotoxicology*. 28(4):761-769.
9. Hutchinson MR, et al. (2008) Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain, Behavior, and Immunity*. 22(8): 1178-1189.
10. Institute of Medicine. (2010). *Gulf War and Health: Volume 8. Health Effects of Serving in the Gulf War*. Washington, DC: National Academies Press. <http://www.iom.edu/Reports/2010/Gulf-War-and-Health-Volume-8-Health-Effects-of-Serving-in-the-Gulf-War.aspx>
11. Janulewicz PA, Krengel MH, Maule A, White RF, Cirillo J, Sisson E, Heeren T, Sullivan K. Neuropsychological characteristics of Gulf War illness: A meta-analysis. *PLoS One*. 2017 May 17;12(5):e0177121. doi: 10.1371/journal.pone.0177121. eCollection 2017. <https://www.ncbi.nlm.nih.gov/pubmed/28520755>

12. Koo BB, Michalovicz LT, Calderazzo S, Kelly KA, Sullivan K, Killiany RJ, O'Callaghan JP. Corticosterone potentiates DFP-induced neuroinflammation and affects high-order diffusion imaging in a rat model of Gulf War Illness. *Brain Behav Immun*. 2017 Aug 4; pii: S0889-1591(17)30391-4. doi: 10.1016/j.bbi.2017.08.003. [Epub ahead of print]
13. Locker AR, Michalovicz LT, Kelly KA, Miller JV, Miller DB, O'Callaghan JP. Corticosterone primes the neuroinflammatory response to Gulf War Illness-relevant organophosphates independently of acetylcholinesterase inhibition. *J Neurochem*. 2017 Aug;142(3):444-455. doi: 10.1111/jnc.14071. Epub 2017 Jun 14.
14. Milligan, E.D., & Watkins, L.R. (2009). Pathological and protective roles of glia chronic pain. *Nature reviews Neuroscience*, 10, 23-36.
15. Milligan ED., Chacus M., Gazda LS., Armstrong C., Wang H., Tracey KJ., Maier SF., Watkins LR.,(2001) Sciatic inflammatory neuritis (SIN): behavioral allodynia is paralleled by peri-sciatic proinflammatory cytokine and superoxide production. *Journal of Peripheral Nervous System* 6(3):111-29.
15. O'Callaghan, JP., Kelly, KA., Locker AR., Miller DB., Lasley SM. (2015) Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *Journal of Neurochemistry* 133(5):708-21. doi: 10.1111/jnc.13088
16. O'Callaghan, JP, Michalovicz, LT, Kelly, KA. Supporting a Neuroimmune Basis of Gulf War Illness. *EbioMedicine*. 2016 Oct 26. pii: S2352-3964(16)30498-4. doi: 10.1016/j.ebiom.2016.10.037.
17. Qiang L, Rao AN, Mostoslavsky G, James MF, Comfort N, Sullivan K, Baas PW. Reprogramming cells from Gulf War veterans into neurons to study Gulf War illness. *Neurology*. 2017 May 16;88(20):1968-1975. doi: 10.1212/WNL.0000000000003938.
18. Rao AN, Patil A, Brodnik ZD, Qiang L, España RA, Sullivan KA, Black MM, Baas PW. Pharmacologically increasing microtubule acetylation corrects stress-exacerbated effects of organophosphates on neurons. *Traffic*. 2017 Jul;18(7):433-441. doi: 10.1111/tra.12489. Epub 2017 May 25.
19. Rathbone, AT., Tharmaradinam, S., Jiang S, Rathbone MP, Kumbhare DA. (2015) A review of the neuro- and systemic inflammatory responses in post-concussion symptoms: Introduction of the “post-inflammatory brain syndrome” PIBS. *Brain, Behavior and Immunity*. 46; 1-16.
20. Research Advisory Committee on Gulf War Veterans' Illnesses. (2008). *Gulf War illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations*. Washington, DC: US Government Printing Office. http://www1.va.gov/RAC-GWVI/Committee_Documents.asp
21. Rivest, S. (2009). Regulation of innate immune responses in the brain. *Nature Reviews. Immunology*, 9(6), 429-439. doi:[10.1038/nri2565](https://doi.org/10.1038/nri2565)

22. Sullivan K, Krengel M, Proctor SP, Devine S, Heeren T, White RF. (2003). Cognitive functioning in treatment-seeking Gulf War veterans: pyridostigmine bromide use and PTSD. *Journal of Psychopathology and Behavioral Assessment*. 25(2):95-103.
23. Spradling, K.D., Lumley, L.A., Robison, C.L., Meyerhoff, J.L., Dillman, J.F. (2011). Transcriptional responses of the nerve agent-sensitive brain regions amygdala, hippocampus, piriform cortex, septum, and thalamus following exposure to the organophosphonate anticholinesterase sarin. *Journal of neuroinflammation*, 8, 84.
24. Toomey R, Alpern R, Vasterling JJ, Baker DG, Reda DJ, et al. (2009). Neuropsychological functioning of U.S. Gulf War veterans 10 years after the war. *J Int Neuropsychol Soc*. 15(5):717-29. doi:10.1017/S1355617709990294
25. Turnbridge, EM., Weickert CS., Kleinman JE., Herman MM., Chen J., Kolachana BS., Harrison PJ., Weinberger DR. (2006) Catechol-o-methyltransferase enzyme activity and protein expression in human prefrontal cortex across the postnatal lifespan. *Cerebral Cortex* 17(5):1206-12.
26. Watkins, L. R., Hutchinson, M. R., Ledebor, A., Wieseler-Frank, J., Milligan, E. D., & Maier, S. F. (2007). Norman Cousins Lecture. Glia as the "bad guys": implications for improving clinical pain control and the clinical utility of opioids. *Brain, Behavior, and Immunity*, 21(2), 131-146. doi:[10.1016/j.bbi.2006.10.011](https://doi.org/10.1016/j.bbi.2006.10.011)
27. Watkins, L. R., Hutchinson, M. R., Rice, K. C., & Maier, S. F. (2009). The "toll" of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. *Trends in Pharmacological Sciences*, 30(11), 581-591. doi:[10.1016/j.tips.2009.08.002](https://doi.org/10.1016/j.tips.2009.08.002)
28. Whistler, T., Fletcher, MA., Lonergan, W., Zeng, XR., Lin, JM., LaPierre, A., Vernon, S. & Klimas, N. (2009). Impaired Immune Function in Gulf War Illness. *BMC Medical Genomics*, 2-12. <http://www.biomedcentral.com/1755-8794/2/12>
29. Yee MK, Janulewicz PA, Seichepine DR, Sullivan KA, Proctor SP, Krengel MH. Multiple Mild Traumatic Brain Injuries Are Associated with Increased Rates of Health Symptoms and Gulf War Illness in a Cohort of 1990-1991 Gulf War Veterans. *Brain Sci*. 2017 Jul 9;7(7). pii: E79. doi: 10.3390/brainsci7070079. <https://www.ncbi.nlm.nih.gov/pubmed/28698487>
30. Yee, M., Seichepine, D., Janulewicz Lloyd P., Sullivan, K., Proctor, SP & Krengel, M. Traumatic brain injury, health and rate of chronic multisymptom illness in veterans from the 1990-1991 Gulf War. *Journal of Head Trauma Rehabilitation*. Aug 19, 2015 (pub ahead of print).
31. Zhang, D., Xiaoming, H., Qang, L., O'Callaghan, JP, Hong, JS. (2010). Astroglialosis in CNS Pathologies: Is there a role for microglia? *Mol. Neurobiology*, doi:[10.1007/s12035-010-8098-4](https://doi.org/10.1007/s12035-010-8098-4).
32. Zhang, F., Liu, J., & Shi, J. (2010). Anti-inflammatory activities of resveratrol in the brain: role of resveratrol in microglial activation. *European Journal of Pharmacology*, 636(1-3), 1-7. doi:[10.1016/j.ejphar.2010.03.043](https://doi.org/10.1016/j.ejphar.2010.03.043)
33. Zhang, Q., Zhou, X.D., Denny, T., Ottenweller, J.E., Lange, G., LaManca, J.J., Laviates, M.H., Poller, C., Gause, W.C., Natelson, B.H. (1999). Changes in immune parameters seen in Gulf War veterans but not in civilian with chronic fatigue syndrome. *Clinical and diagnostic laboratory immunology*, 6, 6-13.

APPENDICES

Appendix A – Quad Chart

Appendix B. Recent GWIC publications.

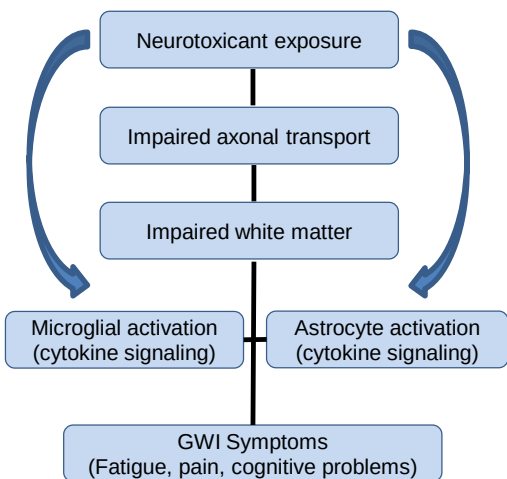
Brain Immune Interactions as the Basis of Gulf War Illness: Gulf War Illness Consortium (GWIC)

Award Number: GW120037 / W81XWH-13-2-0072

PI: Dr. Kimberly Sullivan

Org: Boston University Medical Campus

Award Amount: \$4,888,851

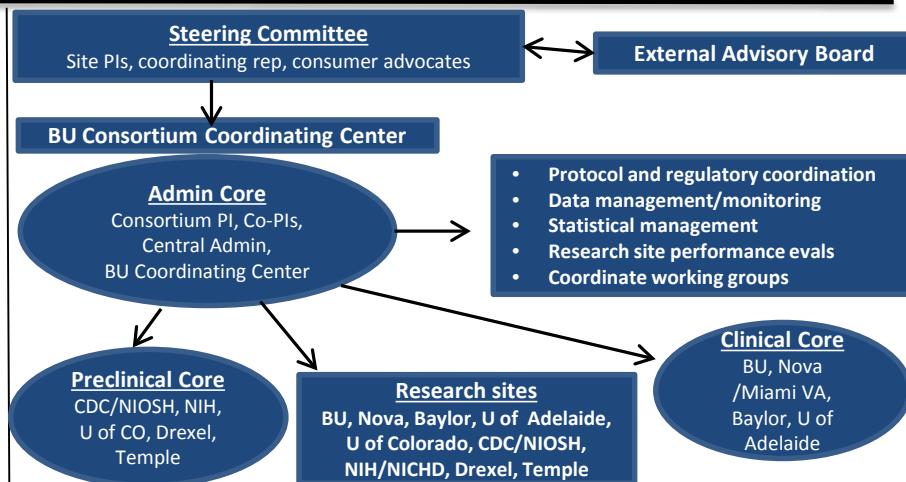


Hypothesized GWI Mechanisms

Approach

A series of clinical and preclinical studies to test whether GWI is related to chronic brain-immune activation and chronic inflammation.

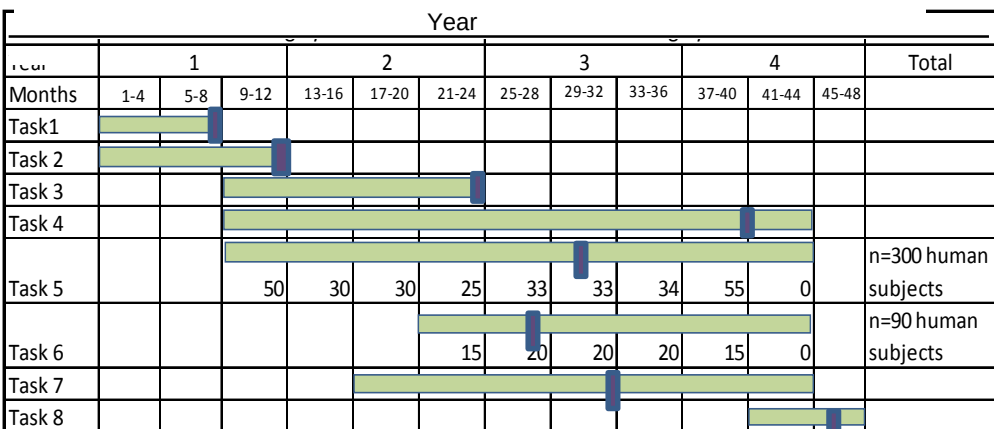
- Clinical case-control studies will be conducted in parallel at 3 sites — Boston, Miami, and Central Texas and will include a total of 300 Gulf War veterans.
- Markers in blood, cerebrospinal fluid, brain imaging (advanced MRI, PET scans) and memory testing will be examined.
- Parallel preclinical studies will evaluate persistent effects of GW neurotoxins in *in vitro* and rodent models of GWI.



Accomplishments: 1- IACUC and ACURO approvals are in place for all sites 2- IRB and HRPO approval obtained from BU, Baylor, Miami VA and NOVA. 3- Data and tracking systems, websites finalized 4- Laboratory methods established for immunologic assays. 5- Preclinical studies ongoing at all sites, have initial results and pilot treatments started. 6- Subject recruitment ongoing and first 175 subjects completed. 7- Twenty additional grant applications were funded for further collaborative research efforts. 8 -Conference symposia, 30 abstracts and 15 manuscripts published, multiple news stories published.

Sept 2013 Start

Timeline



Goals/Milestones

FY13 Goal – Obtain necessary authorization prior to human/animal studies and preparation for consortium clinical/preclinical studies

- ✓ Protocol preparation and initiation of approvals for animal/human use (Task 1)
- ✓ Creation of databases/manuals and data use agreements (Task 2)
- ✓ Prepare rodent dosing models and *in vitro* cell models (Task 3)

FY14 Goal –

- ✓ Perform preclinical cell/animal studies (Task 4)
- ✓ Screening, recruitment, assessment of GW veterans at 3 sites (Task 5)

FY15 Goal – Recruitment and assessment for Boston CSF/PET studies

- ✓ (Task 6-7)
- ✓ **FY16/17 Goal** – Statistical and validation analysis (Task 7-8)
- ✓ **FY17/18 Goal** - Publications and grants submissions (Task 7-8)

Next External Advisory Board meeting scheduled for May 2018

Article

The Multiple Hit Hypothesis for Gulf War Illness: Self-Reported Chemical/Biological Weapons Exposure and Mild Traumatic Brain Injury

Patricia Janulewicz ^{1,*}, Maxine Krengel ^{2,3}, Emily Quinn ⁴, Timothy Heeren ⁵, Rosemary Toomey ⁶, Ronald Killiany ⁷, Clara Zundel ⁸, Joy Ajama ¹, James O'Callaghan ⁹, Lea Steele ¹⁰, Nancy Klimas ^{11,12} and Kimberly Sullivan ¹ 

¹ Department of Environmental Health, Boston University School of Public Health, Boston, MA 02118, USA; ajamajoy@bu.edu (J.A.); tty@bu.edu (K.S.)

² Research Service, VA Boston Healthcare System, Boston, MA 02130, USA; mhk@bu.edu

³ Department of Neurology, Boston University School of Medicine, Boston, MA 02118, USA

⁴ Biostatistics and Epidemiology Data Analytics Center, Boston University School of Public Health, Boston, MA 02118, USA; eq@bu.edu

⁵ Department of Biostatistics, Boston University School of Public Health, Boston, MA 02118, USA; tch@bu.edu

⁶ Department of Psychological and Brain Sciences, Boston University, Boston, MA 02215, USA; toomey@bu.edu

⁷ Department of Anatomy and Neurobiology, Boston University School of Medicine, Boston, MA 02118, USA; killiany@bu.edu

⁸ Department of Behavioral Neuroscience, Boston University School of Medicine, Boston, MA 02118, USA; cgzundel@bu.edu

⁹ Centers for Disease Control and Prevention-NIOSH, Morgantown, WV 26505, USA; jdo5@cdc.gov

¹⁰ Department of Psychiatry and Behavioral Sciences, Division of Neuropsychiatry, Baylor College of Medicine, Houston, TX 77030, USA; Lea.Steele@bcm.edu

¹¹ Department of Medicine, Miami VA Healthcare System, Miami, FL 33125, USA; nklimas@nova.edu

¹² Department of Clinical Immunology, Nova Southeastern University, Miami, FL 33314, USA

* Correspondence: paj@bu.edu; Tel.: +1-617-638-4620

Received: 15 October 2018; Accepted: 12 November 2018; Published: 13 November 2018



Abstract: The Gulf War Illness Consortium (GWIC) was designed to identify objective biomarkers of Gulf War Illness (GWI) in 1991 Gulf War veterans. The symptoms of GWI include fatigue, pain, cognitive problems, gastrointestinal, respiratory, and skin problems. Neurotoxicant exposures during deployment, such as pesticides, sarin, and pyridostigmine bromide pills have been identified as contributors to GWI. We have also found an association between mild traumatic brain injury (mTBI) and increased rates of GWI. However, the combined impact of these physical and chemical exposures has not yet been explored in GWI. The objective of this study was to examine both self-reported mTBI and exposure to chemical/biological weapons (CBW) as a multiple or two hit model for increased risk of GWI and other chronic health conditions. The study population included 125 Gulf War (GW) veterans from the Boston GWIC. Exposure to CBW was reported in 47.2% of the study population, and 35.2% reported sustaining a mTBI during the war. Results confirmed that those with both exposures (mTBI and CBW) had higher rates of comorbid chronic health conditions while rates of GWI were equivalent for mTBI and CBW or mTBI alone. The timing of exposure to mTBI was found to be strikingly different between those with GWI and those without it. Correspondingly, 42.3% of GWI cases reported experiencing a mTBI during military service while none of the controls did ($p = 0.0002$). Rates of mTBI before and after the war did not differ between the cases and controls. In addition, 54% of cases compared to 14.3% of controls ($p = <0.001$) reported being exposed to CBW during military service. The current study examined the relation of the separate and combined effects of exposure to mTBI and CBW in 1991 GW veterans. The findings from this study suggest that both

exposure to mTBI and CBW are associated with the development of GWI and multiple chronic health conditions and that combined exposure appears to lead to higher risk of chronic health effects.

Keywords: Gulf War Illness; Gulf War; mild traumatic brain injury; chemical weapons; neurotoxicant exposures

1. Introduction

Exposure to chemical mixtures has long been reported as a potential cause for Gulf War (GW) veterans' chronic health symptoms [1–5]. It has been found in numerous studies that GW veterans with exposure to neurotoxicants, including pesticides, pyridostigmine bromide pills, and chemical/biological weapons (CBW), have higher rates of chronic symptoms than those without such exposures [6]. In fact, a recent study found a strong inverse correlation with veterans reporting total number of chemical alarm sounds heard during the war and total cortical gray matter brain volumes [7]. Additionally, GW veterans with an increased reporting of CBW exposures have more frequently met criteria for the current GW Illness case definitions [8], including chronic multi-symptom illness (CMI) [9] and Kansas GWI criteria [10], than those without such exposures [11–13]. The symptoms that have most often been found to correlate with CBW exposures have been fatigue, muscle aches, irritability, cognitive impairment, and a depressed and anxious mood. These symptoms notably engulf multiple organ systems that are reflective of central nervous system (CNS) impairment [2,6,14,15].

Mild traumatic brain injury (mTBI) has been reported as another less intuitive potential factor in the development of chronic health symptoms experienced by GW veterans that can also affect CNS functioning [16,17]. Correspondingly, GW veterans with any history of mTBI in the large, longitudinally followed Ft. Devens cohort (FDC) have been found to have significantly higher rates of GWI (Kansas and CMI criteria) than those without mTBI [16,17]. In addition, the total number of health symptoms reported by the Ft. Devens cohort was positively correlated with total number of mTBIs. These symptoms included CNS and non-CNS body systems. Correspondingly, FDC studies also have shown differences in veterans with mTBI versus no mTBI in reported health symptoms across multiple functional domains, including cardiac, dermatological, gastrointestinal, musculoskeletal, neurological, neuropsychological, and pulmonary [17]. In another smaller cohort, Chao et al. [18] reported that a history of mTBI was not associated with higher rates of GWI when Kansas criteria or severe CMI criteria were used, but rather only when mild or moderate CMI criteria were used. This study also found that the highest rates of mTBI were reported before the war and after the war, with the lowest rates occurring during the war. However, neither the FDC nor the Chao study assessed the combined impact of mTBI and CBW exposures during the war on the development of chronic GWI and other medical outcomes. The results of these two sets of studies raise important questions about the impact of timing of the mTBI on chronic health symptoms and the development of chronic GWI symptoms.

Given that both mTBI and CBW have the capacity to influence CNS function, it is possible that those who have experienced both “exposures” may have higher rates of chronic symptoms than those with only one or neither as part of a “two hit” or “multiple hit” scenario. What has been lacking in the literature on GWI is the relative impact of these multiple exposures (e.g., TBI and CBW) on health symptoms and the documentation as to whether or not there is an interaction of the two in terms of chronicity of health outcomes. Therefore, the purpose of this study was to document a “multiple hit hypothesis” in GWI. Multiple hit hypotheses investigate the effects of multiple risk factors or insults, additive or interactive, on an outcome. Numerous studies have utilized the multiple hit hypothesis to investigate nervous system insults on various outcome measures [19–23]. The current study compared multiple GW exposures (mTBI and CBW) in a more representative sample of GW veterans than those mentioned above from different parts of the country, representing all branches of the military and with varying occupational specialties during the war.

It was hypothesized that GW veterans who had both a history of mTBI and CBW exposures would have increased rates of GWI and other chronic health outcomes compared to GW veterans who had only a history of mTBI or only CBW toxicant exposure. GW veterans with multiple “hits” during the war would be at significantly greater risk for illness than those with no “hits” during the war. It was also hypothesized that GW veterans who had both a history of mTBI and CBW exposures (“two hits”) would have increased rates of health outcomes compared to GW veterans who had only a history of mTBI or only CBW exposure. To our knowledge, this is the first paper to assess the impact of CBW exposure and mTBI during the war on GWI and other chronic health outcomes in GW veterans in a new more representative sample of GW veterans recruited as part of the Boston multi-site Gulf War Illness Consortium (GWIC).

2. Materials and Methods

2.1. Participants

The study population included 125 GW veterans from the Boston University Gulf War Illness Consortium (GWIC). The GWIC is a multi-site consortium with three clinical sites around the country (Boston, Miami, and Houston). The inclusion criteria required deployment to the Gulf War between August 1990 and July 1991. The exclusion criteria for the study included diagnoses that could otherwise account for the symptoms reported by the veterans. This included autoimmune, central nervous system, or major psychiatric disorders that may affect brain and immune functions (e.g., epilepsy, stroke, severe head injury, brain tumor, multiple sclerosis, Parkinson’s disease, Alzheimer’s disease, schizophrenia, bipolar disorder, and autoimmune disorders). The Kansas GWI case criteria required endorsement of at least 3 out of 6 symptom domains (fatigue, pain, neurological, skin, gastrointestinal, and respiratory) of at least moderate severity. GW veterans not meeting Kansas or exclusionary criteria were considered controls. Once enrolled, subject data were obtained between 2013 and 2018.

2.2. Self-Reported Mild Traumatic Brain Injury (mTBI)

To assess the occurrence of mTBI, participants were provided with a definition of concussion consistent with current guidelines from the American Academy of Neurology, which has been used in several recent publications [24–26]. This definition clarifies what constitutes a mTBI and provides examples of common symptoms [24]. The following description was provided to the participants;

“Some people have the misconception that mild traumatic brain injury (also known as ‘concussion’) only happens when you lose consciousness after being hit on the head or when the symptoms last for a long time. However, a mild traumatic brain injury occurs anytime you have an impact to the head that causes symptoms for any amount of time (e.g., seconds or longer). These symptoms include: sensitivity to light or noise, headache, dizziness, balance problems, nausea, vomiting, trouble sleeping, fatigue, confusion, difficulty remembering, difficulty concentrating, or loss of consciousness.”

Participants were asked to report whether they had experienced any mTBIs during three time-periods: before the war, during the war, and after the war. In addition, they were asked to self-report how many mTBIs they sustained during each of those periods given this definition and to be sure to include those from participation in sports.

2.3. Demographics, Chemical/Biological Weapon (CBW) Exposure, and Health Outcome Surveys

GWIC subjects were administered a general demographic information and medical conditions questionnaire, the Kansas Gulf War and Health Questionnaire and Kansas Gulf War Experiences and Exposure Questionnaire, and the Structured Neurotoxicant Assessment Checklist (SNAC) [6,10,27]. Self-reported exposures were obtained from the SNAC [6]. For the purpose of this study, we analyzed the SNAC self-reported exposure to CBWs by asking whether or not they were exposed during military service. Health outcomes were measured by a medical conditions questionnaire in which the

participant endorsed whether or not they had a confirmed diagnosis of any of the 20 queried health outcomes, including asthma, diabetes, respiratory problems, hypertension, memory loss, chronic fatigue syndrome, and irritable bowel syndrome [6].

2.4. Clinical Interview

The Clinician Administered Posttraumatic Stress Disorder Scale (CAPS) [28] and Structured Clinical Interview for Diagnostic and Statistical Manual 4 Diagnosis (SCID) [29] were administered to each participant by a trained examiner. The CAPS for Diagnostic and Statistical Manual 4th edition (DSM-IV) asked the participant to describe any traumatic event and to answer direct questions about symptoms relating to intrusions, avoidance, and cognitive and mood alterations secondary to the event. The SCID was also an interview-based survey enquiring about specific symptoms relating to psychiatric diagnoses. For the purpose of this study, the utilized modules included those for major depression, bipolar disorder, and anxiety disorders other than post-traumatic stress disorder (PTSD).

2.5. Data Analysis

We evaluated the association between mTBI and CBW exposure and demographic and health outcomes in 125 GWIC study participants. Chi-square and one-way ANOVA tests were used, as appropriate, to compare exposure groups for descriptive purposes. Separate, unadjusted logistic regressions were performed to determine the effect of mTBI, CBW exposure, and simultaneous mTBI and CBW exposure during military service on health outcomes. Unadjusted Odds Ratios (OR) and 95% confidence intervals (CI) from regression models are reported. Analyses were conducted using 2-sided tests and an alpha level of 0.05. All analyses were conducted using SAS 9.4 (Statistical Analysis Systems, Cary, NC, USA).

3. Results

3.1. Participant Characteristics

The first 125 GWIC participants from three parts of the country made up the study sample. The study sample was 84.8% male with a mean age of 51.5 years. The majority of participants were white (74.4%) and highly educated, with 82.4% of the population having at least a two-year college degree (Table 1). The study sample was representative of all branches of the military. Army was the most common branch of service (67.2%), followed by Navy (16.0%), Marines (10.4%), and then Air Force (6.4%). In this sample of GW veterans, 34 had a current diagnosis of PTSD as determined by the CAPS criteria while 83.2% of the cohort met the Kansas GWI case criteria.

Table 1. Population characteristics.

Total Sample = 125	
Age, years: Mean (SD)	51.5 (6.0)
Females: <i>n</i> (%)	19 (15.2%)
Race: <i>n</i> (%)	
Black/African America <i>n</i>	18 (14.4%)
White/Caucasian	93 (74.4%)
Other/Multiracial	14 (11.2%)
Hispanic or Latino: <i>n</i> (%)	12 (9.6%)
Education: <i>n</i> (%)	
Grade 12	8 (6.4%)
GED	1 (0.8%)
HS plus technical training	13 (10.4%)
Associate/2 years college	29 (23.2%)
Some college	17 (13.6%)
Bachelors	22 (17.6%)
Advanced degree	35 (28.0%)

Table 1. Cont.

Total Sample = 125	
Military Branch: <i>n</i> (%)	
Army	84 (67.2%)
Marines	13 (10.4%)
Navy	20 (16.0%)
Air Force	8 (6.4%)
Current PTSD: <i>n</i> (%) [<i>n</i> = 91]	34 (37.4%)
GWIC Case (Kansas): <i>n</i> (%)	104 (83.2%)
Self-reported mTBI, during deployment: <i>n</i> (%)	44 (35.2%) [Mean SD among those with: 2.0 (1.4)]
Self-reported mTBI, prior to deployment: <i>n</i> (%)	46 (36.8%) [Mean SD among those with: 2.5 (2.3)]
Self-reported mTBI, after deployment: <i>n</i> (%)	33 (26.4%) [Mean SD among those with: 2.4 (2.0)]
Self-reported chemical/biological exposure: <i>n</i> (%)	59 (47.2%)

Note: GED = General Equivalency Degree, HS = high school, PTSD = post-traumatic stress disorder, mTBI = mild traumatic brain injury, GWIC = Gulf War Illness.

3.2. CBW and mTBI Self-Reported Exposures in GWIC Cohort

CBW exposure during military service was reported in 47.2% of the GWIC study population and 35.2% reported sustaining a mTBI during the war. Subjects were also asked to report mTBI during their lifetime including before, during, and after the war. In the overall study population, 36.8% of veterans reported having a mTBI before the war, 35.2% reported experiencing a mTBI during the war, and 26.4% reported having a mTBI after the war (Table 1).

3.3. Results of mTBI and CBW Self-Reported Exposure by GWIC Case Status

Statistically significant differences were found between the veterans who self-reported mTBI and CBW exposure during military service by GWIC case status. GW veterans with GWIC were much more likely to have reported experiencing CBW exposure during military service. Fifty-four percent of cases compared to 14.3% of controls ($p = <0.001$) reported being exposed to CBW. In addition, the time-period of exposure was found to be of great importance when examining whether the veterans with GWIC experienced any mTBIs. Correspondingly, 42.3% of GWIC cases reported experiencing a mTBI during the war while none of the controls reported experiencing a mTBI during the war ($p = 0.0002$) (Figure 1). Thirty-eight percent of GWIC cases reported experiencing a mTBI before the war compared to 33.3% of controls ($p = 0.72$). Self-reported mTBI after the war was not found to be statistically different between the two groups (28.9% of cases vs. 14.3% of controls, $p = 0.17$).

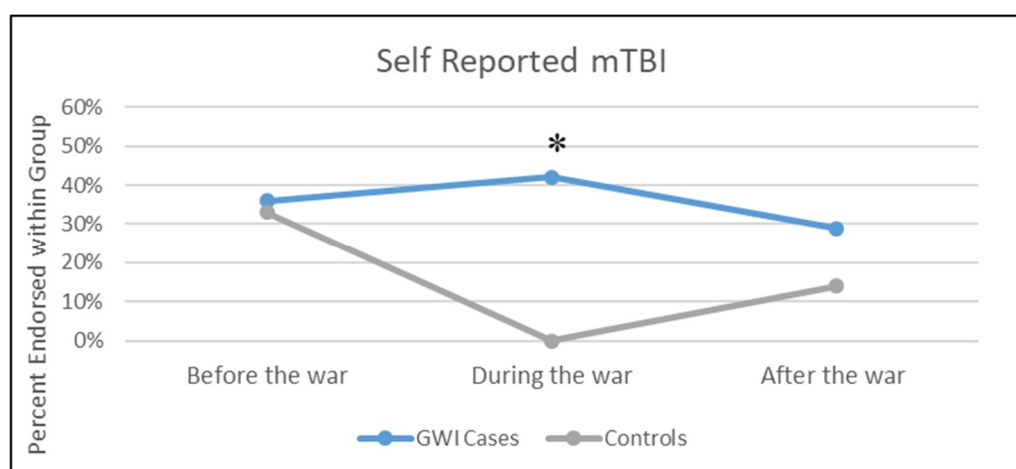


Figure 1. Percent of veterans with self-reported mTBI before, during, and after the war. * $p < 0.005$.

3.4. Participant Characteristics by Exposure Categories

The study population was classified into four exposure groups based on the following definitions: mTBI and CBW exposure (yes/yes), mTBI exposure and no CBW exposure (yes/no), no mTBI exposure and CBW exposure (no/yes), and lastly no mTBI and no CBW exposure (no/no). Examining the groups in this way allowed us to examine the effect of individual exposure (CBW only and mTBI only) and the multiple exposure (CBW and mTBI) categories. As a result of small numbers, the mTBI variable used in the analysis for this paper was limited to self-reported mTBI during the war (yes/no) and the number of mTBIs was not examined. The number of subjects with GWI significantly differed by mTBI and CBW groups but was not higher for the combined groups compared with mTBI only (Table 2). Specifically, 100% of the mTBI/CBW and mTBI-only groups, 90.9% of the CBW-only group, and 62.5% of the no mTBI/CBW group met the criteria for GWI ($p < 0.001$). In terms of demographic comparisons of the four groups, there was a statistically significant difference between the groups in the number of subjects of Hispanic or Latino descent but no other demographic outcomes differed.

Table 2. Demographic characteristics by mTBI and chemical/biological weapon (CBW) exposure categories.

Variable	<i>n</i> Mean (SD)	mTBI & CBW	mTBI & No CBW	No mTBI & CBW	No mTBI & No CBW	<i>p</i> -Value
Age	<i>n</i> Mean (SD)	26 50.1 (6.0)	18 51.3 (5.1)	33 51.4 (4.9)	48 52.4 (7.0)	0.50
Gender	Female, <i>n</i> (%)	5 (19.2%)	1 (5.6%)	9 (27.3%)	4 (8.3%)	0.07
	Male, <i>n</i> (%)	21 (80.8%)	17 (94.4%)	24 (72.7%)	44 (91.7%)	
Race	Black/African American, <i>n</i> (%)	4 (15.4%)	5 (27.8%)	2 (6.1%)	7 (14.6%)	0.49
	White/Caucasian, <i>n</i> (%)	18 (69.2%)	11 (61.1%)	28 (84.8%)	36 (75.0%)	
	Other/Multiracial, <i>n</i> (%)	4 (15.4%)	2 (11.1%)	3 (9.1%)	5 (10.4%)	
Hispanic or Latino	Yes, <i>n</i> (%)	6 (23.1%)	1 (5.6%)	0 (0.0%)	5 (10.4%)	0.03
	No, <i>n</i> (%)	20 (76.9%)	17 (94.4%)	33 (100.0%)	43 (89.6%)	
Education	Grade 12, <i>n</i> (%)	0 (0.0%)	2 (11.1%)	0 (0.0%)	6 (12.5%)	0.50
	GED, <i>n</i> (%)	0 (0.0%)	0 (0.0%)	1 (3.0%)	0 (0.0%)	
	HS plus technical training, <i>n</i> (%)	3 (11.5%)	4 (22.2%)	2 (6.1%)	4 (8.3%)	
	Associate/2 years college, <i>n</i> (%)	6 (23.1%)	5 (27.8%)	9 (27.3%)	9 (18.8%)	
	Some college, <i>n</i> (%)	5 (19.2%)	2 (11.1%)	4 (12.1%)	6 (12.5%)	
	Bachelors, <i>n</i> (%)	6 (23.1%)	2 (11.1%)	6 (18.2%)	8 (16.7%)	
	Advanced degree, <i>n</i> (%)	6 (23.1%)	3 (16.7%)	11 (33.3%)	15 (31.3%)	
Current PTSD (<i>n</i> = 91)	Yes, <i>n</i> (%)	10 (50.0%)	2 (22.2%)	8 (33.3%)	14 (36.8%)	0.49
	No, <i>n</i> (%)	10 (50.0%)	7 (77.8%)	16 (66.7%)	24 (63.2%)	
GWI case (Kansas)	Yes, <i>n</i> (%)	26 (100.0%)	18 (100.0%)	30 (90.9%)	30 (62.5%)	<0.01
	No, <i>n</i> (%)	0 (0.0%)	0 (0.0%)	3 (9.1%)	18 (37.5%)	
Military Branch	Army, <i>n</i> (%)	18 (69.2%)	11 (61.1%)	26 (78.8%)	29 (60.4%)	0.68
	Marines, <i>n</i> (%)	3 (11.5%)	2 (11.1%)	4 (12.1%)	4 (8.3%)	
	Navy, <i>n</i> (%)	3 (11.5%)	4 (22.2%)	2 (6.1%)	11 (22.9%)	
	Air Force, <i>n</i> (%)	2 (7.7%)	1 (5.6%)	1 (3.0%)	4 (8.3%)	

mTBI = mild traumatic brain injury; CBW = chemical/biological weapons, GED = General Equivalency Diploma, HS = high school, PTSD = post-traumatic stress disorder, GWI = Gulf War Illness.

3.5. Kansas Criteria by Exposure Group

As described above, all of the veterans with both mTBI and CBW exposure met the criteria for GWI, as defined by the Kansas criteria (Table 2). Upon further examination, it was evident that those veterans with both exposures (mTBI and CBW) endorsed symptoms in all six symptom domains (fatigue, pain, neurological, skin, gastrointestinal, and respiratory) and significantly more symptoms in each domain (Figure 2). This profile was also seen in the exposure group with CBW only. The exposure group defined as mTBI-only endorsed a significantly higher percent of symptoms in the fatigue, neurological, and gastrointestinal symptom domains compared to the unexposed group.

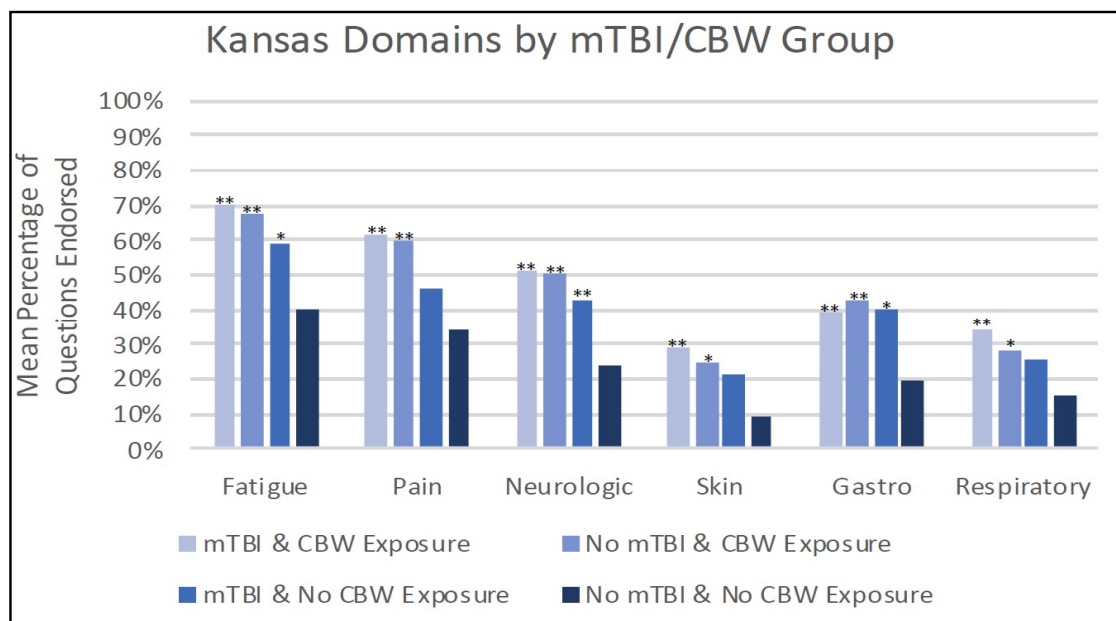


Figure 2. Kansas symptom domains by mTBI and CBW groups. * $p < 0.05$, ** $p < 0.005$ for comparison with reference group (no mTBI & no CBW exposure), mTBI = mild traumatic brain injury, CBW = chemical/ biological weapons.

3.6. Association between Exposure Groups and Chronic Health Conditions

Elevated unadjusted odds ratios, reaching statistical significance, were found in four chronic conditions and across all three exposure groups when compared with the non-exposed group (Table 3). Increased odds of having respiratory allergies or sinus problems, repeated trouble with neck, back or spine, memory loss, and depression was seen in the CBW exposure vs. no exposure groups, the mTBI exposure vs. no exposure, and the dual mTBI and CBW exposure vs. no exposure group. Two additional chronic conditions had statistically significant elevated odds ratios in both the CBW exposure vs. no exposure and the dual mTBI and CBW exposure vs. no exposure groups. These two chronic conditions were chronic fatigue syndrome and irritable bowel syndrome. The remaining eight health outcomes with statistically significant elevated odds ratios were only seen in the dual mTBI and CBW exposure groups and included asthma, high sugar or diabetes, blindness or trouble seeing with either eye, chronic lung disease, chronic skin conditions, deafness or trouble hearing in either ear, chemical sensitivity, and “other” gastrointestinal disorders. In summary, a total of 12 chronic conditions had statistically significant elevated odds ratios in the dual mTBI and CBW exposure group, six chronic conditions for the group exposure to CBW exposures only, and four chronic conditions for those with mTBI exposures only during the war.

Table 3. Unadjusted logistic regression analysis examining CBW exposure, mTBI exposure, and mTBI + CBW.

Variable	CBW Exposure Only vs. Neither	mTBI Exposure Only vs. Neither	mTBI + CBW Exposure vs. Neither
	OR (95% CI)	OR (95% CI)	OR (95% CI)
High Blood Pressure	0.80 (0.32,1.99)	2.80 (0.90,8.72)	0.88 (0.33,2.32)
Heart Attack or MI	1.47 (0.09,24.35)	2.76 (0.16,46.70)	1.88 (0.11,31.35)
Asthma ¹	5.11 (0.96,27.13)	2.87 (0.37,22.13)	6.90 (1.28,37.18) *
High sugar/diabetes ¹	2.07 (0.43,9.92)	4.28 (0.85,21.49)	4.50 (1.02,19.81) *
Respiratory allergies or sinus problems ^{1,2,3}	5.88 (2.16,16.00) *	5.42 (1.67,17.61) *	6.93 (2.37,20.26) *
Arthritis, rheumatism or gout	1.54 (0.59,3.99)	2.69 (0.88,8.27)	1.68 (0.61,4.64)
Repeated trouble with neck, back or spine ^{1,2,3}	3.30 (1.30,8.35) *	4.86 (1.52,15.51) *	10.20 (3.21,32.44) *
Blindness or trouble seeing with either eye ¹	3.33 (0.77,14.44)	4.29 (0.85,21.50)	7.94 (1.92,32.88) *
Chronic lung disease ¹	4.70 (0.47,47.30)	9.40 (0.91,97.25)	20.89 (2.44,179.09) *
Chronic skin conditions ¹	2.93 (0.99,8.62)	2.25 (0.61,8.32)	6.83 (2.25,20.78) *
Memory loss ^{1,2,3}	5.67 (2.05,15.65) *	3.97 (1.22,12.95) *	8.39 (2.50,28.21) *
Deafness or trouble hearing in either ear ¹	1.62 (0.57,4.67)	2.76 (0.84,9.09)	4.33 (1.51,12.46) *
Depression ^{1,2,3}	2.64 (1.05,6.61) *	4.40 (1.39,13.96) *	4.16 (1.51,11.44) *
Current PTSD [N = 91]	0.86 (0.29, 2.51)	0.49 (0.09, 2.69)	1.71 (0.57, 5.13)
Chemical sensitivity ¹	2.62 (0.83,8.27)	3.50 (0.95,12.85)	8.17 (2.58,25.83) *
Chronic Fatigue Syndrome ^{1,2}	5.17 (1.96,13.65) *	2.69 (0.85,8.48)	9.13 (3.05,27.35) *
Irritable Bowel Syndrome ^{1,2}	4.71 (1.82,12.22) *	1.71 (0.55,5.37)	4.31 (1.56,11.88) *
Other GI disorders ¹	1.46 (0.55,3.87)	1.12 (0.33,3.81)	3.40 (1.24,9.36) *

* = statistically significant. ¹ = statistically significant difference between multiple exposure (mTBI + CBW) and no exposure. ² = statistically significant difference between chemical/biological-weapons-only exposure and no exposure. ³ = statistically significant difference between mTBI-only exposure and no exposure. mTBI = mild traumatic brain injury; CBW = chemical/biological weapons; MI = myocardial infarction; GI = gastrointestinal.

4. Discussion

The current study examined a multiple hit hypothesis (mTBI and CBW) in 1990–1991 GW veterans in relation to GWI and chronic medical conditions. To our knowledge, this is the first paper to examine exposure to both mTBI and a neurotoxicant (CBW) in relation to GWI. In our previous studies, we investigated the multiple hit hypothesis using two neurotoxicants known to be frequent in the GW, pesticides, and pyridostigmine bromide (PB) pills. We found a strong synergistic effect of these mixed exposures on GWI and cognitive outcomes [1]. Given the brevity of the GW ground war, mTBI has not been investigated as a primary factor of GWI until recently. In our recent work with the Ft. Devens Cohort, veterans with mTBI had increased rates of GWI as determined by Centers for Disease Control (CDC) and Kansas GWI criteria [16,17]. These studies also showed that the increased number of mTBIs was associated with higher number of total symptoms reported [16]. However, the timing of the mTBI was not available for the Ft. Devens Cohort studies, leaving open the question of whether the proximity of the mTBI to CBW exposure had an impact on the rates of GWI and comorbid chronic health conditions.

Findings from the current study identified a surprisingly strong effect of timing of mTBI during the war and GWI in that those who experienced a mTBI during the war were statistically significantly more likely to endorse symptoms consistent with GWI than those who did not report mTBI during the war. Conversely, rates of mTBI in the GWI cases and controls did not differ before or after the war, indicating that the timing of the mTBI insult appeared to be critical to development of chronic health symptoms. These results add an important concept to consider when doing GWI research. This suggests that brain sequelae of mTBI as well as neurotoxicant induced brain changes, including white matter (WM) macro and microstructural integrity alterations [30–32], should be considered as prime candidates in the chronic CNS pathobiology of GWI.

The only other study reporting on mTBI rates in GW veterans found remarkably similar rates to ours with 47% of their GW veteran cohort and 46% of our GWIC cohort reporting a history of ever having an mTBI [18]. Chao also reported that only 7% of her sample as compared with 36% of our sample reported mTBIs sustained during the war. Chao did not find an association between the history of mTBI during the war and GWI, however the study did find that those with mTBI during the war had increased chronic symptoms. The lack of association with GWI could be influenced by the small sample size of veterans who sustained a mTBI during the war ($n = 7$). However, our data showed an association between mTBI such that nearly half of our cases and none of our controls reported a mTBI during the war. The differences in respective rates of mTBIs during the war may reflect the military occupational specialties (MOS) of our cohort. Our multi-site GWI consortium was more representative of the whole GW veteran cohort because participants were recruited from three sites across the country (Boston, Houston, and Miami), had varied MOS, and represented every branch of military service, making them representative of all deployed GW veterans.

The most relevant findings of this study included the replication of results from our previous work that mTBI is associated with increased rates of GWI. In relation to comorbid health conditions, findings emerged that confirm our hypothesis of a synergistic effect, with increased odds ratios for those with both mTBI and CBW on 12 of the total medical conditions. In essence, the “two hit” group showed more than double the reporting of chronic conditions than the “one hit” groups. Correspondingly, in this study, we report that GW veterans with both mTBI and CBW had significantly increased health symptoms across all six Kansas GWI criteria domains, including fatigue, pain, neurologic, skin, gastrointestinal, and respiratory symptoms [10]. These results suggest that more than the CNS is affected in these groups.

However, confirmation of our original hypothesis that multiple exposures in this two hit model (mTBI and CBW) are consistent with increased rates of GWI was not found in that the rate of GWI did not differ between those with multiple versus single hits. The difference in the rates of GWI may have been obscured because individuals with mTBI and no CBW may have had other exposures,

including sarin, pesticides, and PB pills that were not accounted for in these analyses. These additional neurotoxicant exposures could have influenced the rates of GWI in those without CBW.

While the current study cannot confirm the etiology for GWI symptoms, several studies suggest that the neurotoxicant exposures experienced in war can lead to an increased immune response, not only in the brain, but throughout the body [33–36]. Likewise, TBIs can also lead to an increased neuroinflammatory state by priming the immune cells of the brain—microglia and astrocytes—to respond longer and stronger after each additional encounter with foreign or “danger” associated molecular patterns (DAMPs) from damaged or apoptotic neural cells [37–41]. This chronic inflammatory overactivation has now been termed post-inflammatory brain syndrome (PIBS) [42]. GWI sequelae fits this profile and can be considered a result of PIBS depending on the individual veterans’ “danger” exposure history (i.e., multiple toxicant or mTBI exposures or both).

Consistent with the literature on multiple hits, when more than one exposure occurs, more CNS dysfunction and other body systems are affected [16,43]. In this scenario, damaged or apoptotic cells from the brain can signal into the periphery and vice-versa from a leaky blood brain barrier (BBB) or gut-brain axis [36,44,45]. Future studies should further investigate the pathobiology of this multiple hit hypothesis.

Like all studies, this study had several limitations worth mentioning. First, the use of a relatively small cohort of GW veterans who self-reported exposures during the war suggests that further study is warranted in larger study samples. Second, the mTBI and CBW exposures were self-reported and the chronic conditions were not confirmed by medical records. However, the veterans did endorse that their chronic conditions had been diagnosed by a physician. Although the sample size of the groups was modest, it was more representative than other cohorts reporting on neurotoxicant and mTBI outcomes published to date in that it represented GW veterans from all branches of the military and three study sites around the country. Third, we examined associations on 18 outcome measures across four exposure groups, and, given our limited sample size, we did not control for multiple comparisons. However, we found elevated risks for 12 out of 18 outcomes in the multiple hit exposure group, which was far more than would be expected by chance. There remain questions about the timing of the mTBI and CBW exposures that could not be answered in this study, such as whether mTBI exposure increases CBW toxicity. It was also unclear whether mTBI is specific to the multiple hits needed for chronic conditions or if it represented a proxy for other exposure combinations during deployment.

5. Conclusions

The current study examined the relation of the separate and combined effects of exposure to mTBI and CBW in 1991 GW veterans. The findings from this study suggest that both exposure to mTBI and CBW are associated with the development of GWI and multiple chronic health conditions and that combined exposure appears to lead to higher risk of chronic health effects.

Author Contributions: Conceptualization, P.J., M.K., T.H., J.O., N.K. and K.S.; Data curation, E.Q., T.H., J.A. and K.S.; Formal analysis, E.Q. and T.H.; Funding acquisition, K.S.; Investigation, P.J., M.K., C.Z., L.S. and K.S.; Methodology, P.J., T.H., R.T., R.K. and L.S.; Project administration, P.J. and J.A.; Validation, P.J.; Visualization, C.Z.; Writing—original draft, P.J.; Writing—review & editing, P.J., M.K., R.K., C.Z., J.O., L.S. and K.S.

Funding: This work was supported by the Congressionally Directed Medical Research Program (CDMRP) through the Gulf War Illness Research Program (GWIRP) under Award No. W81XWH-13-2-0072 to Kimberly Sullivan.

Acknowledgments: Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense or the Centers for Disease Control. The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office for this work. The authors would like to thank all of the Gulf War veterans who participated in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Sullivan, K.; Krengel, M.; Bradford, W.; Stone, C.; Thompson, T.A.; Heeren, T.; White, R.F. Neuropsychological functioning in military pesticide applicators from the Gulf War: Effects on information processing speed, attention and visual memory. *Neurotoxicol. Teratol.* **2018**, *65*, 1–13. [[CrossRef](#)] [[PubMed](#)]
2. White, R.F.; Steele, L.; O'Callaghan, J.P.; Sullivan, K.; Binns, J.H.; Golomb, B.A.; Bloom, F.E.; Bunker, J.A.; Crawford, F.; Graves, J.C.; et al. Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: Effects of toxicant exposures during deployment. *Cortex* **2016**, *74*, 449–475. [[CrossRef](#)] [[PubMed](#)]
3. Research Advisory Committee on Gulf War Veterans' Illnesses. *Gulf War Illness and the Health of Gulf War Veterans: Research update and recommendations, 2009–2013*; Updated Scientific Findings and Recommendations; US Government Printing Office: Washington, DC, USA, 2014.
4. Research Advisory Committee on Gulf War Veterans' Illnesses. *Gulf War Illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations*; US Government Printing Office: Washington, DC, USA, 2008.
5. Sullivan, K.; Krengel, M.; Proctor, S.; Devine, S.; Heeren, T.; White, R. Cognitive Functioning in Treatment-Seeking Gulf War Veterans: Pyridostigmine Bromide Use and PTSD. *J. Psychopathol. Behav. Assess.* **2003**, *25*, 95–103. [[CrossRef](#)]
6. Proctor, S.P.; Heeren, T.; White, R.F.; Wolfe, J.; Borgos, M.S.; Davis, J.D.; Pepper, L.; Clapp, R.; Sutker, P.B.; Vasterling, J.J.; et al. Health status of Persian Gulf War veterans: Self-reported symptoms, environmental exposures and the effect of stress. *Int. J. Epidemiol.* **1998**, *27*, 1000–1010. [[CrossRef](#)] [[PubMed](#)]
7. Chao, L.L.; Reeb, R.; Esparza, I.L.; Abadjian, L.R. Associations between the self-reported frequency of hearing chemical alarms in theater and regional brain volume in Gulf War Veterans. *NeuroToxicology* **2016**, *53*, 246–256. [[CrossRef](#)] [[PubMed](#)]
8. Institute of Medicine. *Chronic Multisymptom Illness in Gulf War Veterans: Case Definitions Reexamined*; The National Academies Press: Washington, DC, USA, 2014.
9. Fukuda, K.; Nisenbaum, R.; Stewart, G.; Thompson, W.W.; Robin, L.; Washko, R.M.; Noah, D.L.; Barrett, D.H.; Randall, B.; Herwaldt, B.L.; et al. Chronic multisymptom illness affecting Air Force veterans of the Gulf War. *JAMA* **1998**, *280*, 981–988. [[CrossRef](#)] [[PubMed](#)]
10. Steele, L. Prevalence and Patterns of Gulf War Illness in Kansas Veterans: Association of Symptoms with Characteristics of Person, Place, and Time of Military Service. *Am. J. Epidemiol.* **2000**, *152*, 992–1002. [[CrossRef](#)] [[PubMed](#)]
11. Haley, R.W.; Tuite, J.J. Epidemiologic Evidence of Health Effects from Long-Distance Transit of Chemical Weapons Fallout from Bombing Early in the 1991 Persian Gulf War. *Neuroepidemiology* **2013**, *40*, 178–189. [[CrossRef](#)] [[PubMed](#)]
12. Steele, L.; Sastre, A.; Gerkovich, M.M.; Cook, M.R. Complex Factors in the Etiology of Gulf War Illness: Wartime Exposures and Risk Factors in Veteran Subgroups. *Environ. Health Perspect.* **2011**, *120*, 112–118. [[CrossRef](#)] [[PubMed](#)]
13. Wolfe, J.; Proctor, S.P.; Erickson, D.J.; Hu, H. Risk factors for multisymptom illness in US Army veterans of the Gulf War. *J. Occup. Environ. Med.* **2002**, *44*, 271–281. [[CrossRef](#)] [[PubMed](#)]
14. Proctor, S.P.; Heaton, K.J.; Heeren, T.; White, R.F. Effects of sarin and cyclosarin exposure during the 1991 Gulf War on neurobehavioral functioning in US army veterans. *NeuroToxicology* **2006**, *27*, 931–939. [[CrossRef](#)] [[PubMed](#)]
15. White, R.F.; Proctor, S.P.; Heeren, T.; Wolfe, J.; Krengel, M.; Vasterling, J.; Ozonoff, D.M. Neuropsychological function in Gulf War veterans: Relationships to self-reported toxicant exposures. *Am. J. Ind. Med.* **2001**, *40*, 42–54. [[CrossRef](#)] [[PubMed](#)]
16. Yee, M.; Janulewicz, P.; Seichepine, D.; Sullivan, K.; Proctor, S.; Krengel, M. Multiple Mild Traumatic Brain Injuries Are Associated with Increased Rates of Health Symptoms and Gulf War Illness in a Cohort of 1990–1991 Gulf War Veterans. *Brain Sci.* **2017**, *7*, 79. [[CrossRef](#)] [[PubMed](#)]
17. Yee, M.K.; Seichepine, D.R.; Janulewicz, P.A.; Sullivan, K.A.; Proctor, S.P.; Krengel, M.H. Self-Reported Traumatic Brain Injury, Health and Rate of Chronic Multisymptom Illness in Veterans from the 1990–1991 Gulf War. *J. Head Trauma Rehabil.* **2016**, *31*, 320–328. [[CrossRef](#)] [[PubMed](#)]

18. Chao, L.L. The Relationship Between Traumatic Brain Injury and Rates of Chronic Symptomatic Illness in 202 Gulf War Veterans. *Mil. Med.* **2018**. [CrossRef] [PubMed]
19. Feng, L. Alpha-Synuclein and the Multiple Hit Hypothesis of Parkinson's Disease. Ph.D. Thesis, Georgetown University, Washington, DC, USA, 2012.
20. Barnett, M.L.; Tusor, N.; Ball, G.; Chew, A.; Falconer, S.; Aljabar, P.; Counsell, S.J. Exploring the multiple-hit hypothesis of preterm white matter damage using diffusion MRI. *NeuroImage Clin.* **2018**, *17*, 596–606. [CrossRef] [PubMed]
21. Sulzer, D. Multiple hit hypotheses for dopamine neuron loss in Parkinson's disease. *Trends Neurosci.* **2007**, *30*, 244–250. [CrossRef] [PubMed]
22. Smith, M.A.; Raina, A.K.; Perry, G.; Zhu, X. S4-02-01 Alzheimer disease: The two hit hypothesis. *Neurobiol. Aging* **2004**, *25*, S68. [CrossRef]
23. Feigenson, K.A.; Kusnecov, A.W.; Silverstein, S.M. Inflammation and the two-hit hypothesis of schizophrenia. *Neurosci. Biobehav. Rev.* **2014**, *38*, 72–93. [CrossRef] [PubMed]
24. Vynorius, K.C.; Paquin, A.M.; Seichepine, D.R. Lifetime Multiple Mild Traumatic Brain Injuries Are Associated with Cognitive and Mood Symptoms in Young Healthy College Students. *Front. Neurol.* **2016**, *7*. [CrossRef] [PubMed]
25. Robbins, C.A.; Daneshvar, D.H.; Picano, J.D.; Gevett, B.E.; Baugh, C.M.; Riley, D.O.; Nowinski, C.J.; McKee, A.C.; Cantu, R.C.; Stern, R.A. Self-reported concussion history: Impact of providing a definition of concussion. *Open Access J. Sports Med.* **2014**, *7*, 99–103. [CrossRef] [PubMed]
26. Seichepine, D.R.; Stamm, J.M.; Daneshvar, D.H.; Riley, D.O.; Baugh, C.M.; Gavett, B.E.; Tripodis, Y.; Martin, B.; Chaisson, C.; McKee, A.C.; et al. Profile of self-reported problems with executive functioning in college and professional football players. *J. Neurotrauma* **2013**, *30*, 1299–1304. [CrossRef] [PubMed]
27. Proctor, S.P. Development of a Structured Neurotoxicant Assessment Checklist (SNAC) for Clinical Use in Veteran Populations. Department of Veterans Affairs, VA Boston Healthcare System, 2006. Available online: <https://www.va.gov/RAC-GWVI/meetings/sep2014/Presentation7KimberlySullivanNancyKlimas.pdf> (accessed on 11 November 2018).
28. Blake, D.D.; Weathers, F.W.; Nagy, L.M.; Kaloupek, D.G.; Gusman, F.D.; Charney, D.S.; Keane, T.M. The development of a clinician-administered PTSD scale. *J. Trauma. Stress* **1995**, *8*, 75–90. [CrossRef] [PubMed]
29. First, M.B.; Spitzer, R.L.; Gibbon, M.; Williams, J.B. Structured Clinical Interview for the DSM-IV Axis, I. Disorders. 1996. Available online: <https://www.ptsd.va.gov/professional/assessment/adult-int/scid-ptsd-module.asp#obtain> (accessed on 11 November 2018).
30. Van Riper, S.M.; Alexander, A.L.; Koltyn, K.F.; Stegner, A.J.; Ellingson, L.D.; Destiche, D.J.; Cook, D.B. Cerebral white matter structure is disrupted in Gulf War Veterans with chronic musculoskeletal pain. *Pain* **2017**, *158*, 2364–2375. [CrossRef] [PubMed]
31. Chao, L.L.; Zhang, Y.; Buckley, S. Effects of low-level sarin and cyclosarin exposure on white matter integrity in Gulf War Veterans. *NeuroToxicology* **2015**, *48*, 239–248. [CrossRef] [PubMed]
32. Rayhan, R.U.; Stevens, B.W.; Timbol, C.R.; Adewuyi, O.; Walitt, B.; VanMeter, J.W.; Baraniuk, J.N. Increased Brain White Matter Axial Diffusivity Associated with Fatigue, Pain and Hyperalgesia in Gulf War Illness. *PLoS ONE* **2013**, *8*, e58493. [CrossRef] [PubMed]
33. O'Callaghan, J.P.; Michalovicz, L.T.; Kelly, K.A. Supporting a Neuroimmune Basis of Gulf War Illness. *EBioMedicine* **2016**, *13*, 5–6. [CrossRef] [PubMed]
34. Georgopoulos, A.P.; James, L.M.; Carpenter, A.F.; Engdahl, B.E.; Leuthold, A.C.; Lewis, S.M. Gulf War illness (GWI) as a neuroimmune disease. *Exp. Brain Res.* **2017**, *10*, 3217–3225. [CrossRef] [PubMed]
35. Banks, C.N.; Lein, P.J. A review of experimental evidence linking neurotoxic organophosphorus compounds and inflammation. *Neurotoxicology* **2012**, *33*, 575–584. [CrossRef] [PubMed]
36. Abou-Donia, M.B.; Conboy, L.A.; Kokkotou, E.; Jacobson, E.; Elmasry, E.M.; Elkafrawy, P.; Neely, M.; Bass, C.R.; Sullivan, K. Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol. Teratol.* **2017**, *61*, 36–46. [CrossRef] [PubMed]
37. Matzinger, P. An innate sense of danger. *Ann. N. Y. Acad. Sci.* **2002**, *961*, 341–342. [CrossRef] [PubMed]
38. Matzinger, P. The danger model: A renewed sense of self. *Science* **2002**, *296*, 301–305. [CrossRef] [PubMed]
39. Seong, S.Y.; Matzinger, P. Hydrophobicity: An ancient damage-associated molecular pattern that initiates innate immune responses. *Nat. Rev. Immunol.* **2004**, *4*, 469–478. [CrossRef] [PubMed]

40. Corrigan, F.; Mander, K.A.; Leonard, A.V.; Vink, R. Neurogenic inflammation after traumatic brain injury and its potentiation of classical inflammation. *J. Neuroinflamm.* **2016**, *13*. [[CrossRef](#)] [[PubMed](#)]
41. Borlongan, C.; Acosta, S.; De la Pena, I.; Tajiri, N.; Kaneko, Y.; Lozano, D.; Gonzales-Portillo, G. Neuroinflammatory responses to traumatic brain injury: Etiology, clinical consequences, and therapeutic opportunities. *Neuropsychiatr. Dis. Treat.* **2015**, *11*, 97–106. [[CrossRef](#)] [[PubMed](#)]
42. Rathbone, A.T.; Tharmaradinam, S.; Jiang, S.; Rathbone, M.P.; Kumbhare, D.A. A review of the neuro- and systemic inflammatory responses in post-concussion symptoms: Introduction of the “post-inflammatory brain syndrome” PIBS. *Brain Behav. Immun.* **2015**, *46*, 1–16. [[CrossRef](#)] [[PubMed](#)]
43. Miller, K.J.; Ivins, B.J.; Schwab, K.A. Self-reported mild TBI and postconcussive symptoms in a peacetime active duty military population: Effect of multiple TBI history versus single mild TBI. *J. Head. Trauma. Rehabil.* **2013**, *28*, 31–38. [[CrossRef](#)] [[PubMed](#)]
44. Alhasson, F.; Seth, R.K.; Sarkar, S.; Kimono, D.A.; Albadrani, M.S.; Dattaroy, D.; Chandrashekar, V.; Scott, G.I.; Raychoudhury, S.; Nagarkatti, M.; et al. High circulatory leptin mediated NOX-2-peroxynitrite-miR21 axis activate mesangial cells and promotes renal inflammatory pathology in nonalcoholic fatty liver disease. *Redox Biol.* **2018**, *17*, 1–15. [[CrossRef](#)] [[PubMed](#)]
45. Seth, R.K.; Kimono, D.; Alhasson, F.; Sarkar, S.; Albadrani, M.; Lasley, S.K.; Horner, R.; Janulewicz, P.; Nagarkatti, M.; Nagarkatti, P.; et al. Increased butyrate priming in the gut stalls microbiome associated-gastrointestinal inflammation and hepatic metabolic reprogramming in a mouse model of Gulf War Illness. *Toxicol. Appl. Pharmacol.* **2018**, *350*, 64–77. [[CrossRef](#)] [[PubMed](#)]



© 2018 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

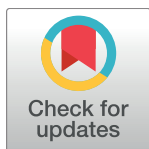
RESEARCH ARTICLE

Prior exposure to corticosterone markedly enhances and prolongs the neuroinflammatory response to systemic challenge with LPS

Kimberly A. Kelly¹, Lindsay T. Michalovicz¹, Julie V. Miller¹, Vincent Castranova², Diane B. Miller¹, James P. O'Callaghan^{1*}

1 Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, Morgantown, West Virginia, **2** Department of Pharmaceutical Sciences, School of Pharmacy, West Virginia University, Morgantown, West Virginia

* Jdo5@cdc.gov



OPEN ACCESS

Citation: Kelly KA, Michalovicz LT, Miller JV, Castranova V, Miller DB, O'Callaghan JP (2018) Prior exposure to corticosterone markedly enhances and prolongs the neuroinflammatory response to systemic challenge with LPS. PLoS ONE 13(1): e0190546. <https://doi.org/10.1371/journal.pone.0190546>

Editor: Cristoforo Scavone, Universidade de Sao Paulo, BRAZIL

Received: May 4, 2017

Accepted: December 15, 2017

Published: January 5, 2018

Copyright: This is an open access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the [Creative Commons CC0](https://creativecommons.org/licenses/by/4.0/) public domain dedication.

Data Availability Statement: Data is available from figshare at: <https://figshare.com/s/f72e4ca76360409631fc>.

Funding: This work was supported by the Intramural funds from the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health and the Congressionally Directed Medical Research Programs: Gulf War Illness Research Program Grants (GW080150, GW120037, GW120045). The

Abstract

Systemic exposure to the inflammagen and bacterial endotoxin lipopolysaccharide (LPS) has been widely used to evaluate inflammation and sickness behavior. While many inflammatory conditions occur in the periphery, it is well established that peripheral inflammation can affect the brain. Neuroinflammation, the elaboration of proinflammatory mediators in the CNS, commonly is associated with behavioral symptoms (e.g., lethargy, anhedonia, anorexia, depression, etc.) termed sickness behavior. Stressors have been shown to interact with and alter neuroinflammatory responses and associated behaviors. Here, we examined the effects of the stress hormone, corticosterone (CORT), as a stressor mimic, on neuroinflammation induced with a single injection (2mg/kg, s.c.) or inhalation exposure (7.5 µg/m³) of LPS or polyinosinic:polycytidylic acid (PIC; 12mg/kg, i.p.) in adult male C57BL/6J mice. CORT was given in the drinking water (200 mg/L) for 1 week or every other week for 90 days followed by LPS. Proinflammatory cytokine expression (TNFα, IL-6, CCL2, IL-1β, LIF, and OSM) was measured by qPCR. The activation of the neuroinflammation downstream signaling activator, STAT3, was assessed by immunoblot of pSTAT3^{Tyr705}. The presence of astrogliosis was assessed by immunoassay of GFAP. Acute exposure to LPS caused brain-wide neuroinflammation without producing astrogliosis; exposure to CORT for 1 week caused marked exacerbation of the LPS-induced neuroinflammation. This neuroinflammatory “priming” by CORT was so pronounced that sub-neuroinflammatory exposures by inhalation instigated neuroinflammation when paired with prior CORT exposure. This effect also was extended to another common inflammagen, PIC (a viral mimic). Furthermore, a single week of CORT exposure maintained the potential for priming for 30 days, while intermittent exposure to CORT for up to 90 days synergistically primed the LPS-induced neuroinflammatory response. These findings highlight the possibility for an isolated inflammatory event to be exacerbated by a temporally distant stressful stimulus and demonstrates the potential for recurrent stress to greatly aggravate chronic inflammatory disorders.

fundings had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: CB, Cerebellum; CCL2, Chemokine (C-C motif) Ligand 2; CORT, Corticosterone; CTX, Cortex; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GFAP, Glial Fibrillary Acidic Protein; HIP, Hippocampus; HYPO, Hypothalamus; IL-1 β , Interleukin 1 beta; IL-6, Interleukin 6; JAK2, Janus Kinase 2; LIF, Leukemia Inhibitor Factor; LPS, Lipopolysaccharide; OB, Olfactory Bulb; OSM, Oncostatin M; PIC, Polyinosinic:Polycytidylic Acid; s.c., Sub cutaneous; SDS, Sodium Dodecyl Sulfate; STAT3, Signal Transducer and Activator of Transcription 3; STR, Striatum; TLR3/TLR4, Toll-like Receptor 3 or 4; TNF α , Tumor Necrosis Factor Alpha.

Introduction

Inflammation can result from acute exposures to pathogens, chemicals, physical damage, irritants, and chronic illnesses. As a component of the innate immune response, inflammation is characterized by the activation of macrophages and their subsequent release of cytokines. While many inflammatory conditions occur in the periphery, it has been well established that peripheral inflammation can exert effects on the brain, as reflected in the enhanced expression of proinflammatory mediators. Anti-inflammatory compounds can ameliorate these neuroinflammatory responses, as well as systemic inflammation. For example, therapy with the classic anti-inflammatory rodent glucocorticoid, corticosterone (CORT), can suppress neuroinflammation and neuroinflammatory signaling caused by exposure to neurotoxins or the bacterial endotoxin, lipopolysaccharide (LPS) [1]. Paradoxically, prior treatment with CORT can enhance rather than suppress the neuroinflammatory response to neurotoxicants [2], acetylcholinesterase inhibitors [3,4], and LPS [5,6]. These results suggest that glucocorticoids can either ameliorate or potentiate the response to a neuroinflammagen depending on whether the treatment occurs before or after an inflammatory exposure.

Increases in plasma cortisol in humans or of CORT in rodents result from stressor exposures. Serum CORT levels frequently are used as indicators of a stress response; accordingly, exogenous CORT administration can be used to mimic stressor exposures in rodents. Precedent exists for a proinflammatory effect of stress hormones [7,8]. The influence of stressors on inflammation vary depending on the duration and frequency of the stressor response. Thus, exposure to acute stressors can either be protective or detrimental to the immune system. However, repeated exposure to a stressor has been shown to profoundly exacerbate inflammation [7–10]. Several studies utilizing various stressors (such as restraint, social defeat, forced swim and isolation) in rodent models show exacerbation of the inflammatory response to LPS after stressor exposure [11–16]. These findings suggest that prolonged or repeated elevations of CORT may predispose the CNS to a heightened or prolonged neuroinflammatory response.

In this study, we sought to determine if chronic exposure to CORT could produce persistent, LPS-induced neuroinflammation. While several studies have examined the immediate effects of CORT exposure on neuroinflammation [17–19], here we investigated the effects of a single, week-long exposure to CORT, as well as recurring exposures to CORT over several months, on LPS-induced neuroinflammation. These CORT exposure regimens induce a persistent and escalating “priming” of the neuroinflammatory response to LPS, findings suggestive of a role for repeated exposures to stressors in the development of chronic neuroinflammatory disorders.

Materials and methods

Materials

The following drugs and chemicals were provided by or obtained from the sources indicated: LPS from *Escherichia coli* serotype 055:B5 (Cat. Number L2880, Sigma-Aldrich, St. Louis, MO, USA), polyinosinic:polycytidylic acid (PIC; Invivogen, San Diego, CA, USA), CORT (Stereoids, Inc., Newport, RI, USA). The materials used in the glial fibrillary acidic protein (GFAP) assay have been described in detail [20,21]. All other reagents and materials were of at least analytical grade and were obtained from a variety of commercial sources.

Animals

Male C57BL/6J mice (n = 5 mice per group), 4–6 weeks of age were purchased from Jackson Labs (Bar Harbor, ME, USA). All procedures were performed within 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 colony was certified by the Association for Assessment and Accreditation of Laboratory Animal Care. Upon receipt, mice were housed individually in a temperature-controlled ($21 \pm 1^\circ\text{C}$) and humidity-controlled ($50 \pm 10\%$) colony room maintained under filtered positive-pressure ventilation on a 12h light/12h dark cycle beginning at 0600. The plastic tub cages were 46 cm in length by 25 cm in width by 15 cm in height; cage bedding consisted of heat-treated pine shavings spread at a depth of approximately 4 cm. Food (Harlan 7913 irradiated NIH-31 modified 6% rodent chow) and water were available ad libitum. The mice in this study were individually housed in order to correlate with other previously studies evaluating CORT priming of neuroinflammation [2,3,4].

Dosing

For short-term exposures, CORT was given in the drinking water (200 mg/L in 0.6% EtOH) for 1 week prior to LPS, PIC, or vehicle treatment on day 8. This regimen of CORT was chosen because of its ability to achieve high circulating levels of this hormone, similar to those achieved with repeated stress [22], and because it was capable of producing significant immunosuppression as evidenced by involution of the thymus (e.g., see O'Callaghan *et al.*, 1991 [23]). LPS was administered in one of two ways: 1) injected subcutaneously (s.c.) at 2 mg/kg where vehicle was 0.9% saline; or 2) whole body inhalation exposure [24] to aerosolized LPS ($7.5 \mu\text{g}/\text{m}^3$) ($\sim 7.5 \times 10^4$ Endotoxin Units/ m^3) for 3 hours where vehicle was water. PIC was administered intraperitoneally at 12 mg/kg (0.9% saline vehicle). For 30 or 90 day exposures, CORT was given in the drinking water (200 mg/L in 0.6% EtOH) for 1 week prior to LPS or vehicle injection on day 37 or 97, respectively. However, for the 90 day CORT +++ exposure, CORT was given in the drinking water (200 mg/L in 0.6% EtOH) every other week for 13 weeks prior to LPS exposure on day 97. Mice were killed by decapitation or focused microwave irradiation (see below) at 2, 6, 12, or 72 hours post-LPS or vehicle exposure and 6 hours post-PIC exposure.

Brain dissection and tissue preparation

Immediately after decapitation, whole brains were removed from the skull with the aid of blunt curved forceps. Striatum, hippocampus, cortex, cerebellum, olfactory bulb, and hypothalamus were dissected free hand on a thermoelectric cold plate (Model TCP-2, Aldrich Chemical Co., Milwaukee, WI, USA) using a pair of fine curved forceps (Roboz, Washington, DC, USA). Brain regions from one side of the brain were frozen at -85°C and used for subsequent isolation of total RNA; brain regions from the other side of the brain were used for total and specific protein analysis. These regions were weighed and homogenized with a sonic probe (model XL-2005, Heat Systems, Farmingdale, NY, USA) in 10 volumes of hot ($90\text{--}95^\circ\text{C}$) 1% sodium dodecyl sulfate (SDS), and stored frozen at -70°C before total protein assay and immunoassay of GFAP. A separate set of mice were used for phosphorylated signal transducer and activator of transcription 3 at tyrosine 705 (pSTAT3^{Tyr705}) analysis and were sacrificed by focused microwave irradiation (Muromachi Kikai, Inc., Tokyo, Japan; Model TMW-4012C, 3.5 KW applied power, 0.90 sec) to preserve steady-state phosphorylation for analysis using phospho-state-specific antibodies [25]. Microwave-fixed brain regions were processed for protein analysis as described above.

RNA isolation, cDNA synthesis, and real-time PCR amplification

The detailed protocols can be found at protocols.io ([DX.DOI.ORG/10.17504/PROTOCOLS.IO.HTRB6M6](https://doi.org/10.17504/PROTOCOLS.IO.HTRB6M6); [DX.DOI.ORG/10.17504/PROTOCOLS.IO.HTVB6N6](https://doi.org/10.17504/PROTOCOLS.IO.HTVB6N6); [DX.DOI.ORG/10.17504/PROTOCOLS.IO.HTWB6PE](https://doi.org/10.17504/PROTOCOLS.IO.HTWB6PE)). Total RNA from striatum, hippocampus, cortex,

cerebellum, olfactory bulb, and hypothalamus was isolated using Trizol[®] reagent (Thermo Fisher Scientific, Waltham, MA, USA) and Phase-lock heavy gel (Eppendorf AG, Hamburg, Germany). The RNA was further cleaned using RNeasy mini spin columns (Qiagen, Valencia, CA, USA). Total RNA (1 µg) was reverse transcribed to cDNA using SuperScript[™] III and oligo (dT)₁₂₋₁₈ primers (Thermo Fisher Scientific, Waltham, MA, USA) in a 20 µL reaction. Real-time PCR analysis of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and of the proinflammatory mediators, TNFα, IL-6, CCL2, IL-1β, leukemia inhibitor factor (LIF), and oncostatin M (OSM) was performed in an ABI7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) in combination with TaqMan[®] chemistry. Specific primers and dual-labeled internal fluorogenic (FAM/TAMRA) probe sets (TaqMan[®] Gene Expression Assays) for these genes were procured from Thermo Fisher Scientific (Waltham, MA, USA) and used according to the manufacturer's recommendations. All PCR amplifications (40 cycles) were performed in a total volume of 50 µL, containing 1 µL cDNA, 2.5 µL of the specific assay of demand primer/probe mix, and 25 µL of Taqman[®] Universal master mix. Relative quantification of gene expression was performed using the comparative threshold ($\Delta\Delta C_T$) method. Changes in mRNA expression levels were calculated after normalization to GAPDH. The ratios obtained after normalization are expressed as fold change over corresponding saline-treated controls.

Protein assay

A detailed protocol can be found at protocols.io ([DX.DOI.ORG/10.17504/PROTOCOLS.IO.HSWB6FE](https://doi.org/10.17504/PROTOCOLS.IO.HSWB6FE)). Total protein was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), per the manufacturer's instructions. Absolute absorbance was measured using the Spectra Max Plus microplate reader and analyzed using Soft Max Pro Plus software (Molecular Devices, Sunnyvale, CA, USA).

GFAP immunoassay

A detailed protocol can be found at protocols.io ([DX.DOI.ORG/10.17504/PROTOCOLS.IO.HSUB6EW](https://doi.org/10.17504/PROTOCOLS.IO.HSUB6EW)). GFAP was assayed in accordance with a previously described procedure [20,21]. In brief, a rabbit polyclonal antibody to GFAP (1:400; DAKO, Carpinteria, CA, USA) was coated on the wells of Immulon-2 microtiter plates (Thermo Labsystems, Franklin, MA). The SDS homogenates and standards were diluted in phosphate-buffered saline (pH 7.4) containing 0.5% Triton X-100. Standards consisted of SDS homogenates of hippocampus with known concentration of GFAP and were prepared the same way as the samples. After blocking non-specific binding with 5% non-fat dry milk, aliquots of the homogenate and standards were added to the wells and incubated. Following washes, a mouse monoclonal antibody to GFAP (1:250; Sigma Chemical Co., St. Louis, MO, USA) was added to 'sandwich' the GFAP between the two antibodies. An alkaline phosphatase-conjugated antibody directed against mouse IgG (1:2000; Jackson ImmunoResearch Labs, West Grove, PA, USA) was then added and a colored reaction product was obtained by subsequent addition of the enzyme substrate p-nitrophenol. Quantification was achieved by spectrophotometry of the colored reaction product at 405 nm in the microplate reader (see above), and analyzed with the same software. The amount of GFAP in the samples was calculated as micrograms of GFAP per milligram total protein.

pSTAT3 immunoblot analysis

A detailed protocol can be found at protocols.io ([DX.DOI.ORG/10.17504/PROTOCOLS.IO.HT4B6QW](https://doi.org/10.17504/PROTOCOLS.IO.HT4B6QW)). Activation of the JAK-STAT3 neuroinflammation effector pathway [1] was assessed by quantifying pSTAT3^{Tyr705} from immunoblots with detection of fluorescent signals

using an infrared fluorescence scanner (Licor Biosciences; Lincoln, NE, USA) as described previously [1,25–27]. Briefly, following incubation with primary antibodies (rabbit anti-phospho-STAT3^{Tyr705} [1:500]; Cell Signaling, Danvers, MA, USA), blots were washed with phosphate buffered saline with 0.1% Tween-20 and incubated with anti-rabbit fluorescent-labeled secondary antibody (1:2500) for 1h prior to scanning by Licor.

Statistics

Statistical analysis of data was performed utilizing SigmaPlot (v. 11.0). The test of significance was performed using either one-way ANOVA (Saline x LPS) or two-way ANOVA (pretreatment [Water or CORT] x exposure [Saline or LPS/PIC]) on log transformed values followed by multiple pairwise comparison analysis using Fisher least significant difference (LSD) post hoc test with statistical significance at 5% ($p < 0.05$). Graphical representations are mean $\pm$ SEM.

Results

Systemic exposure to LPS is neuroinflammatory

Systemic exposure to LPS is known to result in a neuroinflammatory response [1,28–30]. Accordingly, exposure to LPS (2 mg/kg, s.c.) caused a time-dependent increase in expression of neuroinflammatory cytokines and chemokines in cortex (Fig 1A). Neuroinflammation was seen with a peak as early as 2 hours post-LPS. In some cases, neuroinflammation persisted to 12 hours after LPS exposure (e.g., TNF α and IL-1 β). Overall, the response to LPS occurred throughout the brain (see Fig 2 below).

Systemic exposure to LPS activates the STAT3 pathway without causing astrogliosis

IL-6, LIF, and OSM have been shown to bind to the gp130 receptor, with crosstalk input from the other cytokines/chemokines, and to subsequently activate the janus kinase 2 (JAK2)/STAT3 pathway (e.g., see O’Callaghan *et al.*, 2014 [1]). Therefore, we evaluated the phosphorylation state of STAT3 at Tyr705 as an index of the downstream activation of the cytokine/JAK2/STAT3 signaling cascade. In agreement with our prior findings for effects of LPS in striatum [1], pSTAT3^{Tyr705} was significantly increased 6 hours after LPS exposure in all brain areas; cortex data are shown as representative of other areas (Fig 1B). This time point corresponds to the period after the onset of enhanced expression of cytokine and chemokine mRNA (Fig 1A), consistent with activation of STAT3 as a downstream effector of cytokine/chemokine signaling.

The close association of neuronal damage and neuroinflammation has made it difficult to separate the two conditions, with many publications inferring that neuroinflammation causes damage, or *vice versa*. Astrogliosis, as evidenced by an enhanced expression of GFAP, is a hallmark of toxicant-induced neuronal damage [31]. Previously, we have shown that neuroinflammation precedes the activation of the STAT3 pathway in astrocytes and the subsequent increase in expression of GFAP that is associated with toxicant-induced damage to dopaminergic nerve terminals [1]. In contrast, LPS-induced neuroinflammation, while activating the striatal STAT3 pathway, does not result in astrogliosis [1]. In agreement with these latter findings, LPS failed to increase GFAP in cortex (Fig 1C).

Chronic exposure to CORT exacerbates LPS-induced neuroinflammation

Previously, we showed that week-long exposure to CORT enhanced both neuroinflammation and neurodegeneration in mice exposed to a neurotoxic regimen of methamphetamine [2].

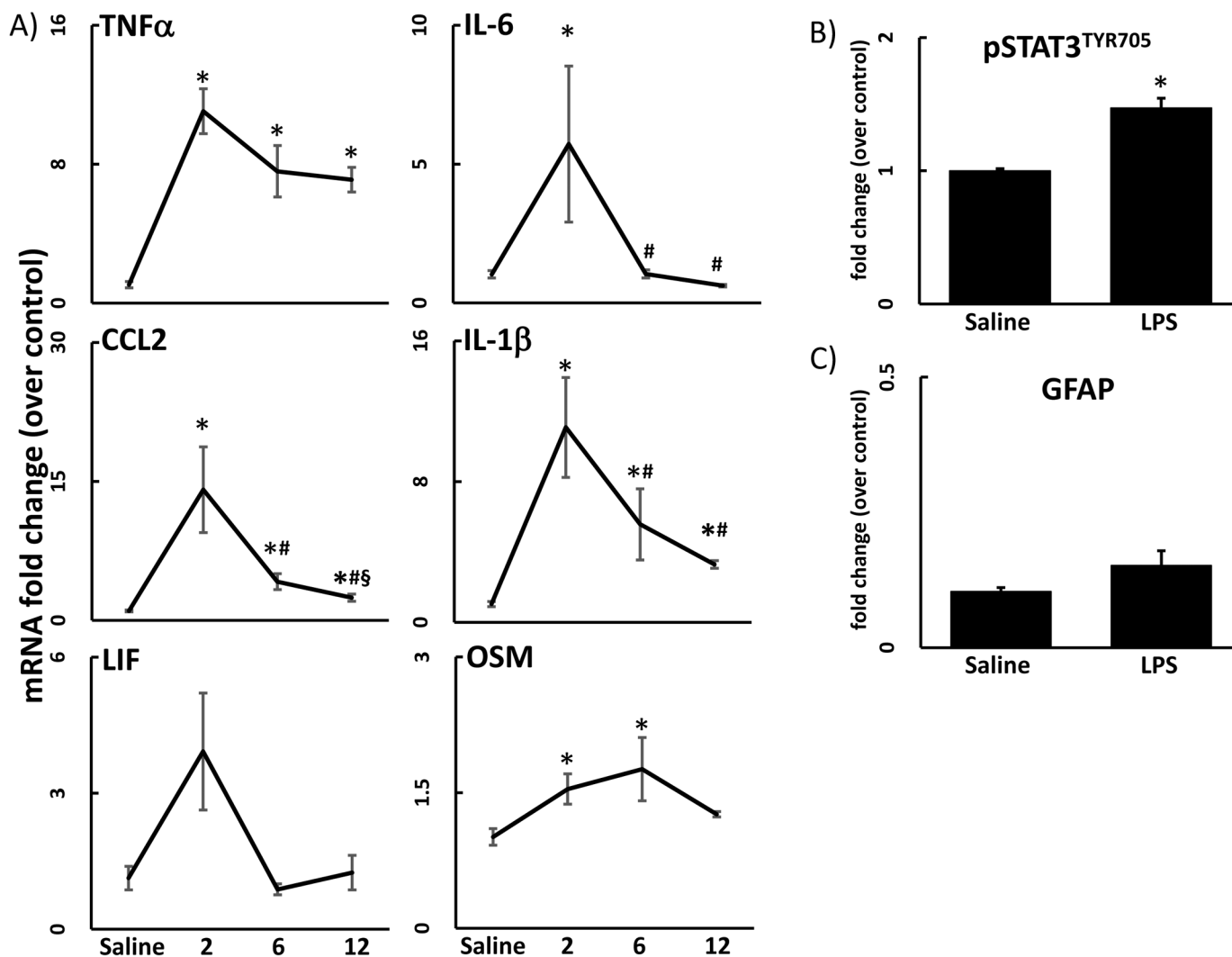


Fig 1. Systemic LPS exposure is neuroinflammatory but does not induce astrogliosis. A) The effects of LPS (2 mg/kg, s.c.) on TNFα, IL-6, CCL2, IL-1β, LIF, and OSM mRNA levels in samples prepared from cortex were determined at 2, 6, and 12 hours after LPS exposure. Data represents mean ± SEM (n = 5 mice/group). Statistical significance of at least p<0.05 is denoted by * as compared to control, # as compared to 2h LPS, and § as compared to 6h LPS. B & C) The effects of LPS exposure (2 mg/kg, s.c.) on activation of the STAT3 signaling module was assessed by immunoblots of pSTAT3^{TYR705} and astrogliosis was assessed by immunoassay of GFAP. Cortex samples were analyzed for pSTAT3^{TYR705} (6 hours) and GFAP (72 hours) after LPS exposure. Data represents mean ± SEM (n = 5 mice/group). Statistical significance of at least p<0.05 is denoted by * as compared to control.

<https://doi.org/10.1371/journal.pone.0190546.g001>

Here, we again administered CORT in the drinking water for 1 week and found that the neuroinflammatory response to LPS was markedly exacerbated by CORT; this enhanced neuroinflammatory response to LPS occurred in several brain regions (Fig 2). The exacerbation of neuroinflammation induced by CORT pretreatment resulted in a longer lasting and more accentuated response as evidenced by significantly heightened neuroinflammation at 6 and 12 hours after LPS exposure in cortex (Fig 3A) compared to LPS exposure alone (LPS data from Fig 1A included for statistical comparison).

While prior CORT can enhance neurodegenerative responses seen with a neurotoxicant such as methamphetamine [2], an effect associated with increased activation of STAT3 and accompanying astrogliosis, these effects were not found with constant exposure to CORT in the drinking water prior to LPS (Fig 3B & 3C; saline and LPS data from Fig 1B & 1C included

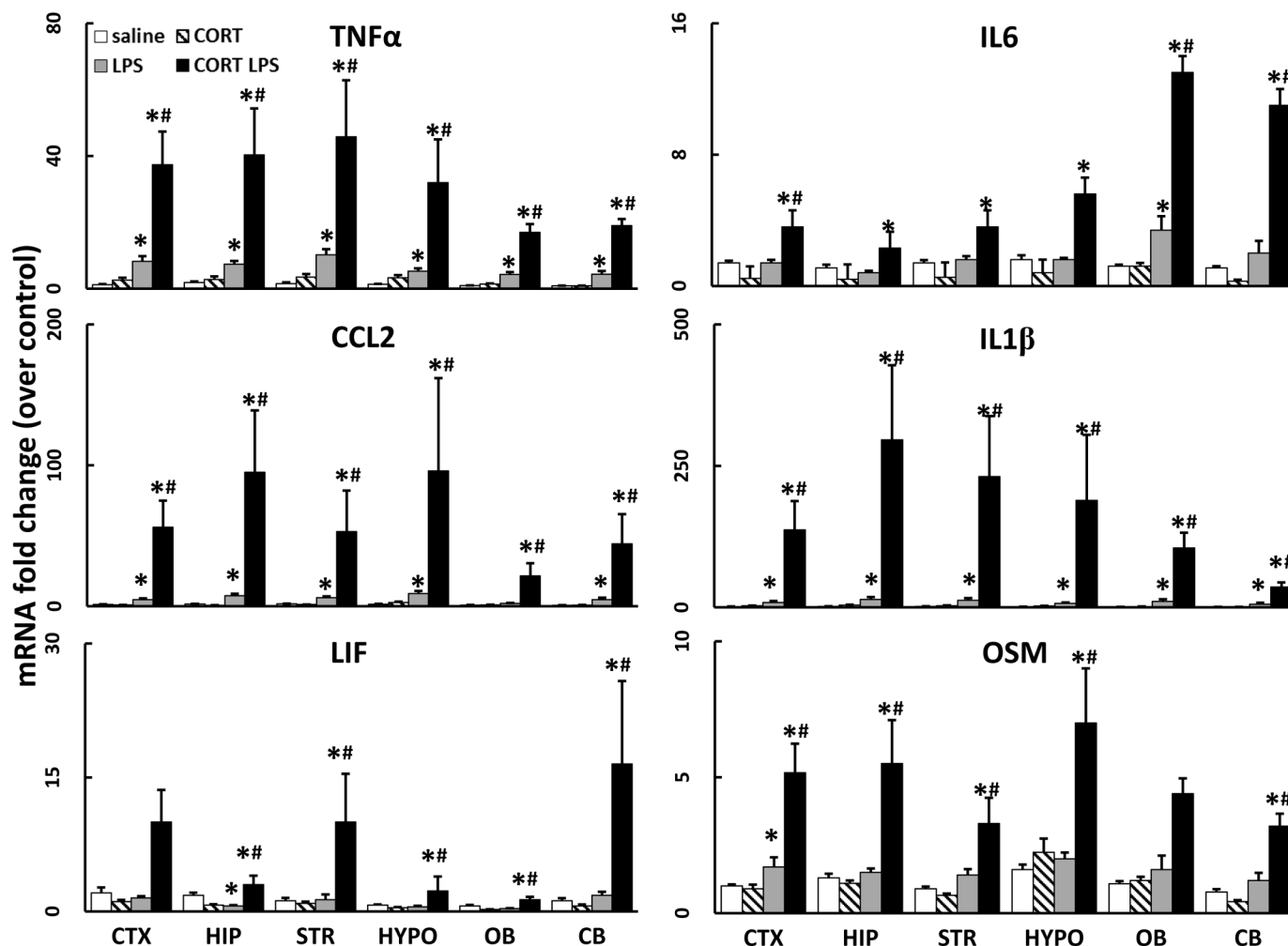


Fig 2. Chronic CORT exacerbates LPS-induced neuroinflammation brain wide. The effects of chronic CORT pretreatment (200 mg/L 0.6% EtOH in drinking water for one week) on LPS (2 mg/kg, s.c.)-induced TNF α , IL-6, CCL2, IL-1 β , LIF, and OSM mRNA levels were measured in samples prepared from cortex (CTX), hippocampus (HIP), striatum (STR), hypothalamus (HYPO), olfactory bulb (OB), and cerebellum (CB) 6 hours after LPS exposure. Data represents mean $\pm$ SEM (n = 5 mice/group). Statistical significance of at least $p < 0.05$ is denoted by * as compared to control, # as compared to LPS alone.

<https://doi.org/10.1371/journal.pone.0190546.g002>

for statistical comparison). These observations are consistent with our prior data for LPS [1] where activation of STAT3 likely was not associated with activation of astrocytes, as we did not observe an accompanying increase in GFAP (Fig 3B; saline and LPS data from Fig 1B included for statistical comparison).

Chronic exposure to CORT exacerbates neuroinflammation induced by another route of LPS exposure

Mice treated with CORT in the drinking water for 1 week were exposed to aerosolized LPS for 3 hours to determine if CORT affected the neuroinflammatory response to LPS via an alternate route of exposure. Inhalation exposure to LPS alone did not affect expression of neuroinflammatory cytokines, but the prior exposure to CORT resulted in a marked increase in all cytokines, except IL-6 (Fig 4). Interestingly, mice that inhaled aerosolized water after a week of CORT had a significantly elevated mRNA expression level of TNF α and slightly reduced IL-6 mRNA.

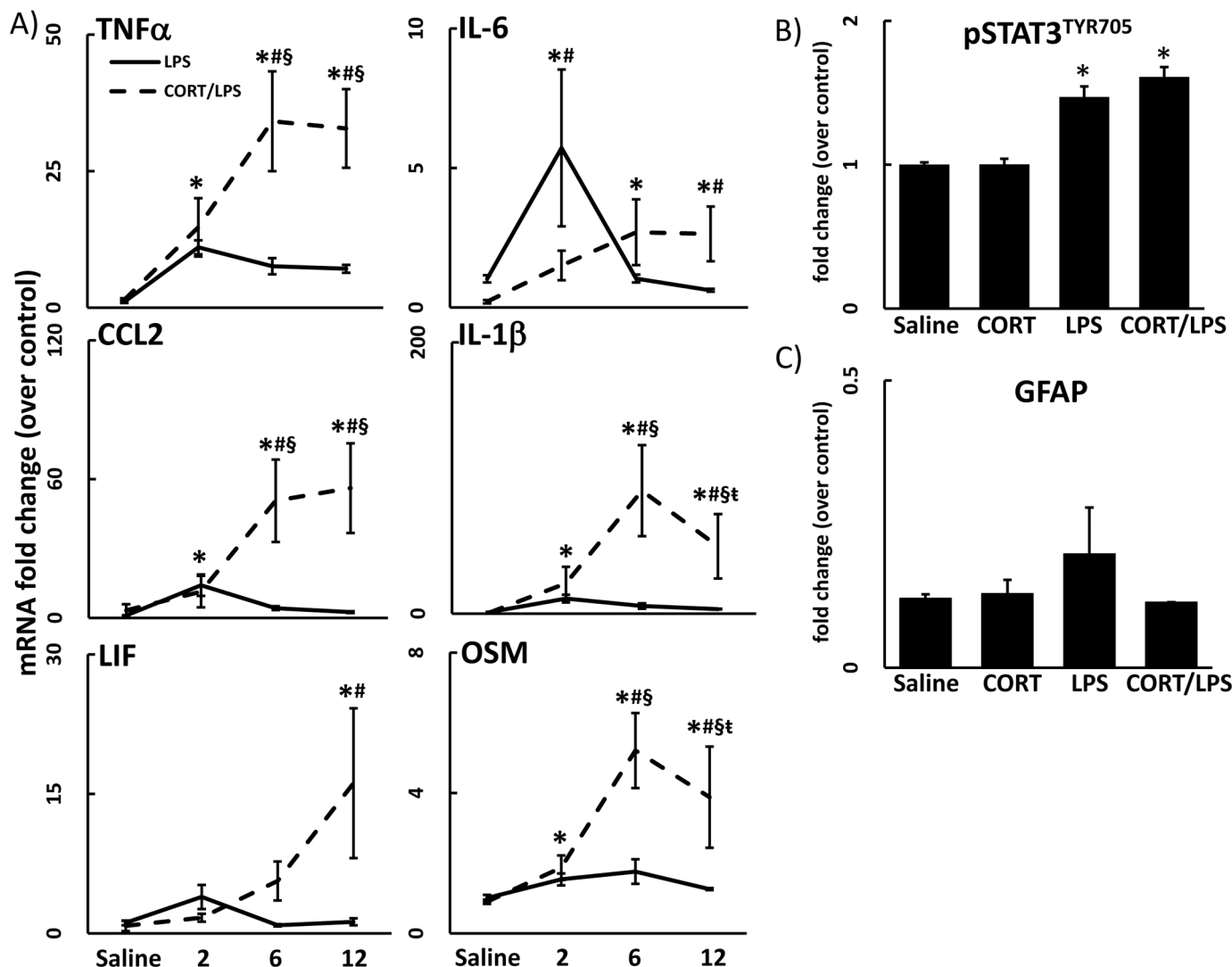


Fig 3. Chronic CORT pretreatment exacerbates and prolongs LPS-induced neuroinflammation but has no effect on activation of STAT3 and does not cause astrogliosis. A) A time course of LPS (2 mg/kg, s.c.)-induced neuroinflammation with and without CORT pretreatment (200 mg/L 0.6% EtOH in drinking water for one week) is shown (saline, 2, 6 and 12 hours). TNF α , IL-6, CCL2, IL-1 β , LIF, and OSM mRNA levels were measured in samples prepared from cortex. Data represents mean $\pm$ SEM (n = 5 mice/group). CTX data for LPS alone treated groups are included from Fig 1A for statistical comparison. Statistical significance of at least $p < 0.05$ is denoted by * as compared to control, # as compared to LPS alone, § as compared to 2h CORT/LPS, and ¶ as compared to 6h CORT/LPS. B & C) Effects of chronic CORT pretreatment (200 mg/L 0.6% EtOH in drinking water for one week) on LPS induced activation of the STAT3 signaling module was assessed by immunoblots of pSTAT3^{TYR705} and astrogliosis was assessed by immunoassay of GFAP. Cortex samples were analyzed for pSTAT3^{TYR705} (6 hours) and GFAP protein (72 hours) after LPS exposure of CORT pretreated animals. Data represent mean $\pm$ SEM (n = 5 mice/group). Saline and LPS alone treated groups are included from Fig 1B & 1C for statistical comparison. Statistical significance of at least $p < 0.05$ is denoted by * as compared to control, # as compared to LPS alone.

<https://doi.org/10.1371/journal.pone.0190546.g003>

Chronic exposure to CORT also exacerbates neuroinflammation induced by the inflammagen, PIC

Similar to LPS, peripheral exposure to the viral mimic, PIC, is known to result in neuroinflammation throughout the brain [32–34]. As such, exposure to PIC alone caused a significant neuroinflammatory response at 6 hours post-exposure (Fig 5). Exposure to CORT in the drinking water for 7 days significantly exacerbated the PIC-induced expression of cytokines and chemokines (Fig 5).

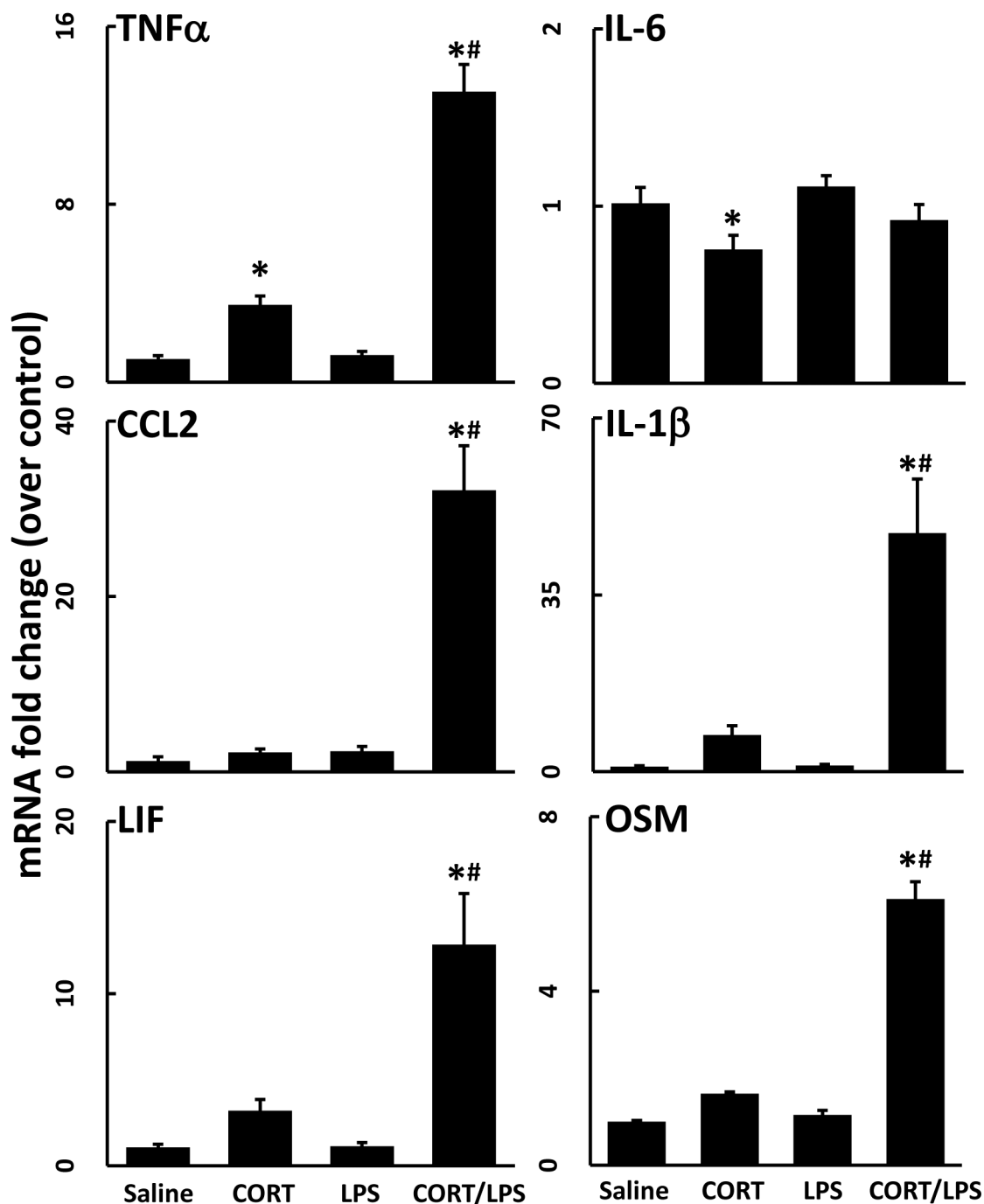


Fig 4. Chronic CORT sensitizes the CNS to a sub-neuroinflammatory inhalation dose of LPS. Mice pretreated with CORT (200 mg/L 0.6% EtOH in drinking water for one week) were subjected to 3 hours of LPS (7.5 $\mu\text{g}/\text{m}^3$) by inhalation. TNF α , IL-6, CCL2, IL-1 β , LIF, and OSM mRNA levels were measured in samples prepared from cortex collected immediately following LPS inhalation exposure. Data represent mean $\pm$ SEM (n = 5 mice/group). Statistical significance of at least P < 0.05 is denoted by * as compared to respective control and # as compared to LPS alone exposed group.

<https://doi.org/10.1371/journal.pone.0190546.g004>

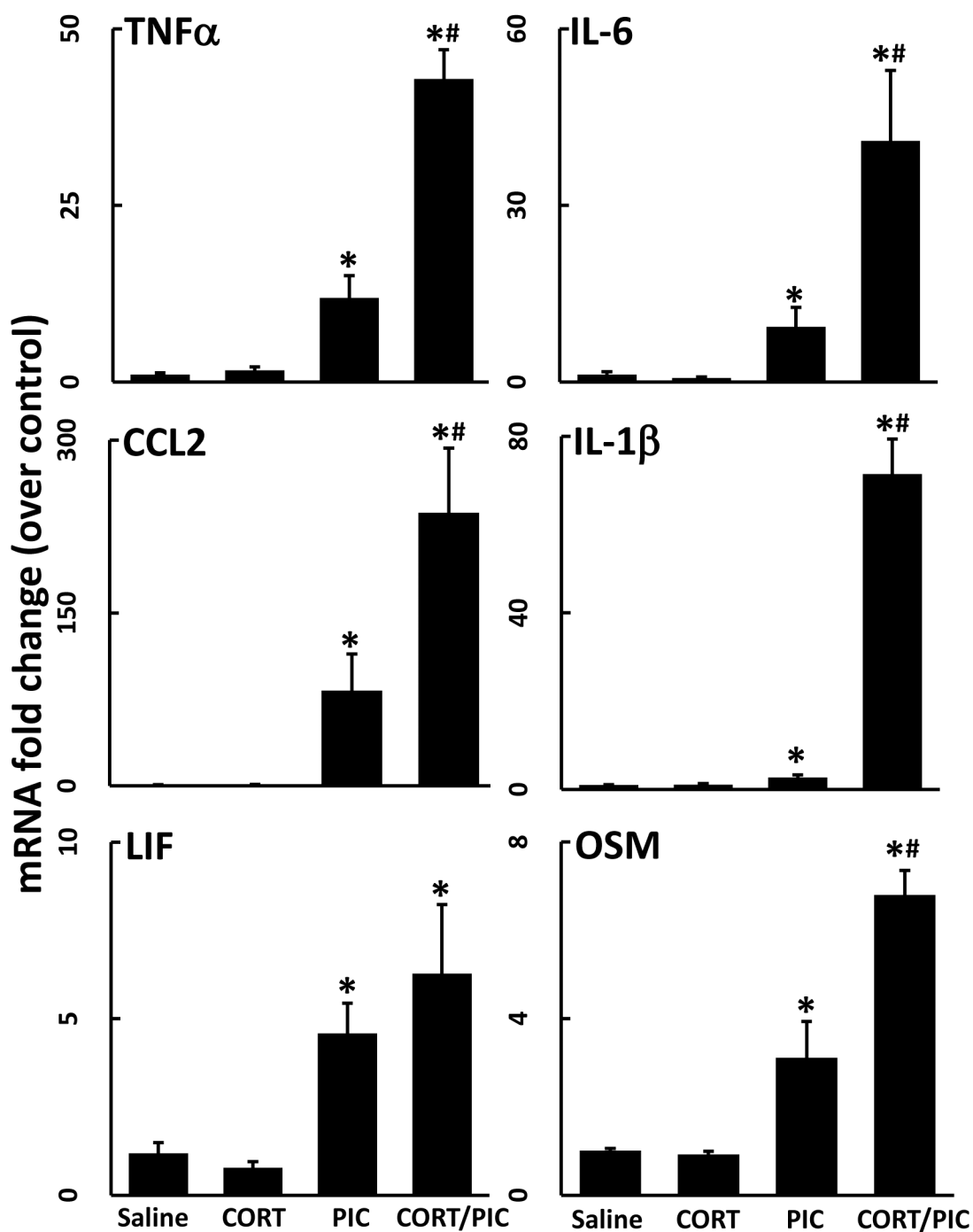


Fig 5. Chronic CORT exacerbates PIC-induced neuroinflammation. Mice were pretreated with CORT (200 mg/L 0.6% EtOH in drinking water for one week). TNFα, IL-1β, LIF, and OSM mRNA levels were measured in samples prepared from cortex collected 6 hours after PIC exposure (12 mg/kg, i.p.). Data represent mean ± SEM (n = 5 mice/group). Statistical significance of at least P < 0.05 is denoted by * as compared to respective control group.

<https://doi.org/10.1371/journal.pone.0190546.g005>

Single and recurring week-long exposures to CORT result in protracted neuroinflammatory priming

While we have shown that a week-long exposure to high physiological levels of CORT in the drinking water serves to potentiate the neuroinflammatory response to systemic LPS administration, the persistence and duration of this effect are unknown. To further define the effects of CORT priming on the neuroinflammatory response to LPS, mice were exposed to CORT in the drinking water for 1 week, but then not exposed to LPS until 30 days later. Even 30 days after the cessation of exposure to CORT, the neuroinflammatory response remained primed to exacerbate LPS-induced neuroinflammation (TNF α , CCL2, IL-1 β) (Fig 6) (compare magnitude of mRNA changes in this figure with those shown in Fig 3). However, an exacerbated neuroinflammatory response to LPS had dissipated by 90 days (Fig 7; CORT/LPS) after CORT treatment, as evidenced by the similarity in mRNA expression between CORT/LPS and LPS alone treated groups. When mice were exposed to multiple, successive waves of 1-week exposures to CORT over 90 days, however, the neuroinflammatory response to LPS was potentiated up to 10-fold greater than the response after a single 1-week CORT exposure prior to LPS (Fig 7; CORT+++/LPS).

Discussion

We have demonstrated that exposure to CORT in the drinking water, at levels designed to mimic high physiological stress levels of this hormone, markedly enhances the neuroinflammatory response to LPS and the viral mimic, PIC. This neuroinflammatory “priming” by CORT is so effective that previously sub-neuroinflammatory exposures to LPS by inhalation instigate significant neuroinflammation when combined with prior exposure to CORT. Furthermore, a single week of exposure to CORT in the drinking water maintains the potential for priming the neuroinflammatory response to LPS for 30 days. Most remarkably, intermittent exposure to 7 days of CORT for up to 90 days serves to markedly escalate the LPS-induced neuroinflammatory response.

Neuroinflammation initiated by both neural damage and LPS is propagated through the STAT3 signaling cascade [1,28]. However, while CORT primed the LPS-induced neuroinflammation, we found that the activation of STAT3 (pSTAT3^{Tyr705}) was unaffected by prior CORT exposure (Fig 3B). STAT3 activation due to neural damage is associated with astrogliosis and subsequent elevations in the expression of the astrocyte intermediate filament protein, GFAP. STAT3 activation caused by inflammagens such as LPS is not necessarily associated with neural damage [1] and, therefore, is not accompanied by an increase in GFAP (Figs 1 and 3). Thus, the lack of an increase in pSTAT3^{Tyr705} or GFAP with CORT given prior to LPS suggests that the CORT-induced exacerbation of LPS-induced neuroinflammation is not accompanied by neural damage. The multiple cytokine pathways affected implicate involvement of microglia, to be explored in the future by more detailed analyses of components of the inflammasome.

Previous studies have demonstrated a glucocorticoid-induced enhancement of neuroinflammation. An investigation that used CORT in the drinking water for a longer duration (10 days vs. the 7 days used here) evaluated the response of cultured primary microglia to LPS exposure and found that microglia secreted more IL-1 β , IL-6, and TNF α with longer CORT pretreatment [17]. Using CORT pellets to mimic mild stress, adult adrenalectomized rats also exhibited increased brain expression of TNF α and IL-1 β , along with NF- κ B activation [18]. In a model of neuropathic pain, intrathecal administration of LPS 24 hours following a single subcutaneous injection of CORT resulted in exacerbated LPS-induced allodynia and increased expression of IL-1 β and IL-6 in the spinal cord [19]. These studies combined with the work

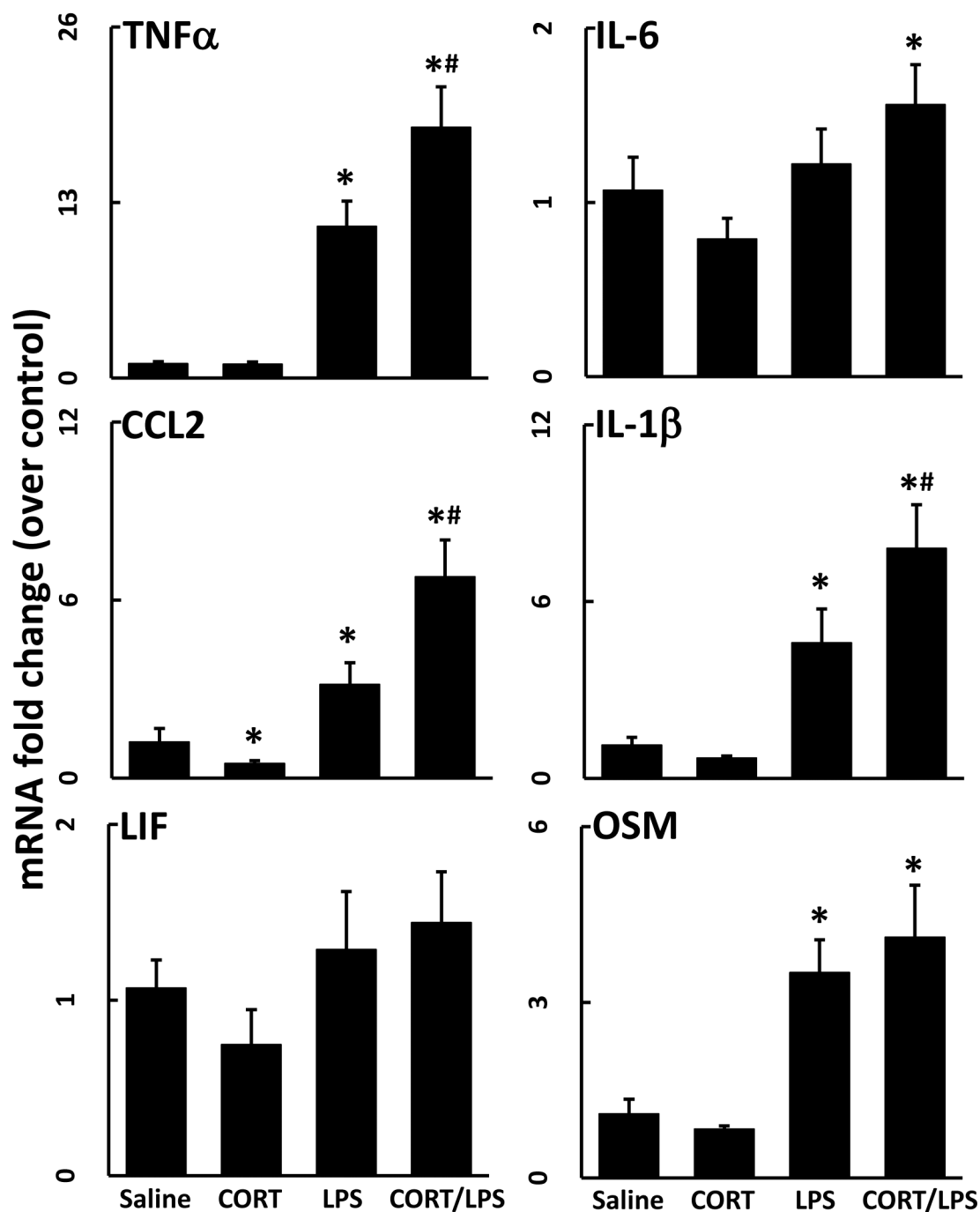


Fig 6. Chronic CORT pretreatment primes the neuroinflammatory response for 30 days. Mice were pretreated with CORT (200 mg/L 0.6% EtOH in drinking water for one week). Thirty days after the cessation of CORT, mice were administered LPS (2 mg/kg, s.c.). TNFα, IL-6, CCL2, IL-1β, LIF, and OSM mRNA levels were measured in samples prepared from cortex 6 hours after LPS exposure. Data represent mean ± SEM (n = 5 mice/group). Statistical significance of at least $P < 0.05$ is denoted by * as compared to appropriate control and # as compared to LPS alone.

<https://doi.org/10.1371/journal.pone.0190546.g006>

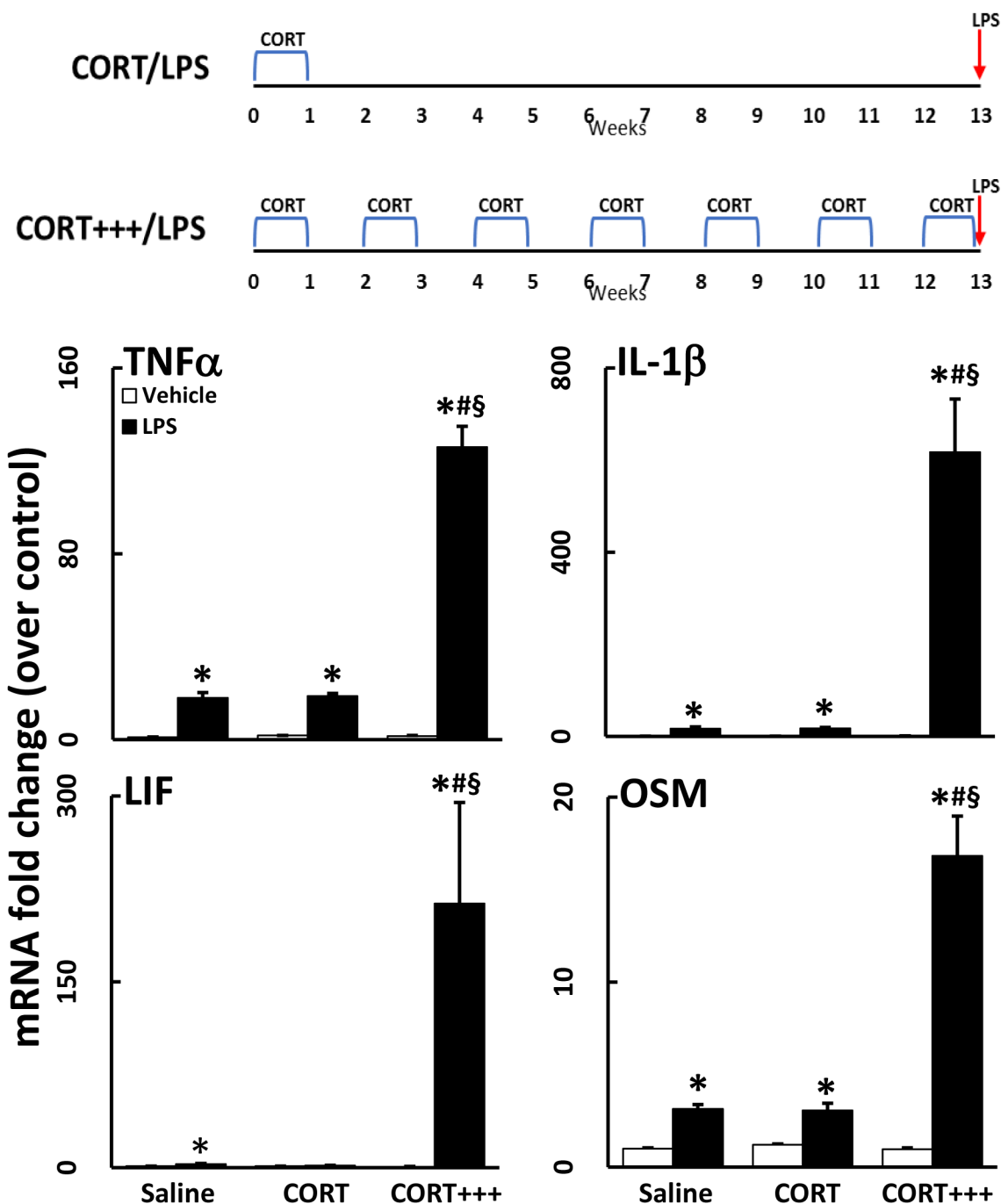


Fig 7. Successive waves of CORT for 90 days greatly exacerbates LPS-induced neuroinflammation. Mice were treated with CORT (200 mg/L 0.6% EtOH) for 7 days in the drinking water followed either by 90 days without (CORT) or 90 days of bimonthly, 7 day CORT exposures (CORT+++). LPS (2 mg/kg, s.c.) was administered on day 97. TNFα, IL-1β, LIF, and OSM mRNA levels were measured in samples prepared from cortex collected 6 hours after LPS exposure. Data represent mean ± SEM (n = 5 mice/group). Statistical significance of at least $P < 0.05$ is denoted by * as compared to appropriate control, # as compared to LPS alone, and § as compared to CORT/LPS.

<https://doi.org/10.1371/journal.pone.0190546.g007>

presented here illustrate that CORT exposure has the potential to exacerbate LPS-induced neuroinflammation both acutely and for a prolonged period (i.e., for 1–3 months, Figs 6 and 7). We have expanded upon the results of previous studies by broadening the number of neuroinflammatory cytokines and chemokines examined, as well as by showing the altered kinetic expression profiles of these genes following CORT pretreatment as well as the brain-wide nature of the CORT-primed neuroinflammatory response. Additionally, our findings appear to be the first to show an escalating priming effect on neuroinflammation at long-term time points after episodic CORT exposures.

While chronic CORT exposure was used in this study to mimic high levels of physiological stress, several others have shown that behavioral stress paradigms can also be pro-inflammatory. For example, a 2 hour repeated social defeat paradigm used in rats increased plasma IL-1 β levels in response to LPS administration [14]. Gibb *et al.* [35] found that both acute (social disruption) or chronic (rotating stressor) stress paradigms prior to systemic LPS exposure resulted in protracted sickness behavior and increased expression of cytokine mRNA in the brain. Evaluation of these studies reveals that the repeated social defeat protocol used by Carobrez and colleagues [14] and the chronic stress paradigm used by Gibb and colleagues [35] result in a greater increase in plasma CORT compared to other paradigms that were not found to be proinflammatory [15,16]. These observations combined with our results clearly indicate that the conditions of CORT exposure are a crucial factor in determining its pro-inflammatory consequences. Furthermore, the variability of the responses to inflammatory challenge following stressor paradigms highlights the utility of our exogenous CORT exposure regimen, which should, theoretically, produce a more uniform stress hormone response.

LPS stimulates the bacterial acute phase response of innate immunity, which is characterized by the release of inflammatory cytokines. Similarly, the synthetic dsRNA PIC instigates the viral acute phase response. As the results of combining a stressor with PIC are mixed [36–38], we exposed CORT-treated mice to PIC to test whether the CORT priming effect was generalizable to another inflammagen. Similar to LPS, the neuroinflammatory response instigated by peripheral PIC exposure was exacerbated by prior exposure to CORT (Fig 5). This is particularly interesting because LPS and PIC have slightly different effects when administered peripherally. First, LPS is a lipophilic compound that readily enters the bloodstream, gaining access to the CNS to activate central neuroinflammation by binding to LPS receptors in the brain [39]. However, intraperitoneally-administered PIC does not enter blood circulation and, conversely, instigates neuroinflammation through peripherally-generated inflammatory mediators [40]. While it is possible that the mechanisms by which CORT pretreatment affects the neuroinflammatory response to these two inflammagens may diverge, it is likely that CORT exposure acts upon the overlapping signaling mechanisms induced by the peripherally-generated cytokines and chemokines following LPS or PIC exposure. This could be achieved through modulation of neural cytokine/chemokine receptors or changes in the transport of peripheral inflammatory mediators across the blood brain barrier by CORT. Second, LPS and PIC signal through different receptors, with LPS signaling through Toll-like receptor 4 (TLR4) and PIC signaling through Toll-like receptor 3 (TLR3), retinoic acid-inducible gene 1 (RIG1), and melanoma differentiation-associated protein 5 (MDA5). The convergence on toll-like receptor signaling between both of these inflammagens makes TLR signaling a viable candidate for further investigation into how CORT exposure instigates inflammatory priming. This may result from effects on the LPS and PIC receptors directly, or indirectly through modulation of shared aspects of the inflammatory cytokine expression profiles instigated by these inflammagens. Overall, the observation that CORT exacerbates LPS-induced and PIC-induced neuroinflammation, as well as our prior observations with neurotoxicant exposures [1–4], helps to build a library of disparate inflammatory conditions comprised of numerous signaling

cascades which can be utilized and manipulated to discover a convergent mechanism by which CORT exposure primes neuroinflammation.

Conclusion

Our findings confirm that prior exposure to CORT resembles the effects of behavioral stressors, several of which markedly increase neuroinflammation associated with a subsequent innate immune system challenge with LPS. When this week-long prior exposure to CORT is extended to multiple weekly episodes of CORT over three months, a dramatic escalation of the neuroinflammatory response to LPS is seen, suggestive of the potential for chronic stressors to cause long-lasting and severe neuroinflammation. Neuroinflammatory signaling serves as the molecular basis for sickness behavior, symptoms of which are associated with chronic neurological disease states such as depression, chemobrain, neuropathic pain and Gulf War Illness [3,4,41–43]. We conclude that there is the potential for recurrent physiological stressors to contribute to and greatly exacerbate such chronic inflammatory disorders.

Acknowledgments

We appreciate the excellent technical assistance provided by Brenda K. Billig, Christopher M. Felton, Ali Yilmaz, and Walter McKinney. Disclaimer: The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

Author Contributions

Conceptualization: Kimberly A. Kelly, Lindsay T. Michalovicz, Vincent Castranova, Diane B. Miller, James P. O’Callaghan.

Data curation: Kimberly A. Kelly, Lindsay T. Michalovicz.

Formal analysis: Kimberly A. Kelly, Lindsay T. Michalovicz.

Funding acquisition: James P. O’Callaghan.

Investigation: Kimberly A. Kelly, Lindsay T. Michalovicz.

Methodology: Kimberly A. Kelly, Lindsay T. Michalovicz, Vincent Castranova, Diane B. Miller, James P. O’Callaghan.

Project administration: Kimberly A. Kelly, Lindsay T. Michalovicz, Vincent Castranova, Diane B. Miller, James P. O’Callaghan.

Resources: Vincent Castranova, Diane B. Miller, James P. O’Callaghan.

Supervision: Kimberly A. Kelly, Lindsay T. Michalovicz, Diane B. Miller, James P. O’Callaghan.

Validation: Kimberly A. Kelly, Lindsay T. Michalovicz, Diane B. Miller, James P. O’Callaghan.

Visualization: Kimberly A. Kelly, Lindsay T. Michalovicz, Diane B. Miller, James P. O’Callaghan.

Writing – original draft: Kimberly A. Kelly, Lindsay T. Michalovicz, Julie V. Miller, Diane B. Miller, James P. O’Callaghan.

Writing – review & editing: Kimberly A. Kelly, Lindsay T. Michalovicz, Julie V. Miller, Vincent Castranova, Diane B. Miller, James P. O’Callaghan.

References

1. O'Callaghan JP, Kelly KA, VanGilder RL, Sofroniew M V, Miller DB. Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity. *PLoS One*. 2014; 9:e102003. <https://doi.org/10.1371/journal.pone.0102003> PMID: 25025494
2. Kelly KA, Miller DB, Bowyer JF, O'Callaghan JP. Chronic exposure to corticosterone enhances the neuroinflammatory and neurotoxic responses to methamphetamine. *J. Neurochem*. 2012; 122:995–1009. <https://doi.org/10.1111/j.1471-4159.2012.07864.x> PMID: 22776046
3. O'Callaghan JP, Kelly KA, Locker AR, Miller DB, Lasley SM. Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *J. Neurochem*. 2015; 133:1–14.
4. Locker AR, Michalovicz LT, Kelly KA, Miller JV, Miller DB, O'Callaghan JP. Corticosterone primes the neuroinflammatory response to Gulf War Illness-relevant organophosphates independently of acetylcholinesterase inhibition. *J. Neurochem*. 2017; 142:444–455. <https://doi.org/10.1111/jnc.14071> PMID: 28500787
5. Frank MG, Baratta M V, Sprunger DB, Watkins LR, Maier SF. Microglia serve as a neuroimmune substrate for stress-induced potentiation of CNS pro-inflammatory cytokine responses. *Brain. Behav. Immun*. 2007; 21:47–59. <https://doi.org/10.1016/j.bbi.2006.03.005> PMID: 16647243
6. Frank MG, Hershman SA, Weber MD, Watkins LR, Maier SF. Chronic exposure to exogenous glucocorticoids primes microglia to pro-inflammatory stimuli and induces NLRP3 mRNA in the hippocampus. *Psychoneuroendocrinology*. 2014; 40:191–200. <https://doi.org/10.1016/j.psyneuen.2013.11.006> PMID: 24485491
7. Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM. The stressed CNS: when glucocorticoids aggravate inflammation. *Neuron*. 2009; 64:33–9. <https://doi.org/10.1016/j.neuron.2009.09.032> PMID: 19840546
8. Sorrells SF, Sapolsky RM. An inflammatory review of glucocorticoid actions in the CNS. *Brain. Behav. Immun*. 2007; 21:259–72. PMID: 17194565
9. Johnson EA, O'Callaghan JP, Miller DB. Chronic treatment with supraphysiological levels of corticosterone enhances D-MDMA-induced dopaminergic neurotoxicity in the C57BL/6J female mouse. *Brain Res*. 2002; 933:130–8. PMID: 11931857
10. Wohleb ES, Hanke ML, Corona AW, Powell ND, Stiner LM, Bailey MT, et al. β -Adrenergic receptor antagonism prevents anxiety-like behavior and microglial reactivity induced by repeated social defeat. *J. Neurosci*. 2011; 31:6277–88. <https://doi.org/10.1523/JNEUROSCI.0450-11.2011> PMID: 21525267
11. Browne CA, O'Brien FE, Connor TJ, Dinan TG, Cryan JF. Differential lipopolysaccharide-induced immune alterations in the hippocampus of two mouse strains: Effects of stress. *Neuroscience*. 2012; 225:237–48. <https://doi.org/10.1016/j.neuroscience.2012.08.031> PMID: 22917616
12. Espinosa-Oliva AM, de Pablos RM, Villarán RF, Argüelles S, Venero JL, Machado A, et al. Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. *Neurobiol. Aging*. 2011; 32:85–102. <https://doi.org/10.1016/j.neurobiolaging.2009.01.012> PMID: 19286276
13. Nowacka MM, Paul-Samojedny M, Bielecka AM, Obuchowicz E. Chronic social instability stress enhances vulnerability of BDNF response to LPS in the limbic structures of female rats: A protective role of antidepressants. *Neurosci. Res*. 2014; 88:74–83. <https://doi.org/10.1016/j.neures.2014.08.008> PMID: 25173454
14. Carobrez SG, Gasparotto OC, Buwalda B, Bohus B. Long-term consequences of social stress on corticosterone and IL-1 β levels in endotoxin-challenged rats. *Physiol. Behav*. 2002; 76:99–105. PMID: 12175593
15. Gibb J, Hayley S, Gandhi R, Poulter MO, Anisman H. Synergistic and additive actions of a psychosocial stressor and endotoxin challenge: Circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. *Brain. Behav. Immun*. 2008; 22:573–89. <https://doi.org/10.1016/j.bbi.2007.12.001> PMID: 18191534
16. Audet MC, Jacobson-Pick S, Wann BP, Anisman H. Social defeat promotes specific cytokine variations within the prefrontal cortex upon subsequent aggressive or endotoxin challenges. *Brain. Behav. Immun*. 2011; 25:1197–205. <https://doi.org/10.1016/j.bbi.2011.03.010> PMID: 21435391
17. Frank MG, Miguel ZD, Watkins LR, Maier SF. Prior exposure to glucocorticoids sensitizes the neuroinflammatory and peripheral inflammatory responses to *E. coli* lipopolysaccharide. *Brain. Behav. Immun*. 2010; 24:19–30. <https://doi.org/10.1016/j.bbi.2009.07.008> PMID: 19647070
18. Munhoz CD, Sorrells SF, Caso JR, Scavone C, Sapolsky RM. Glucocorticoids exacerbate lipopolysaccharide-induced signaling in the frontal cortex and hippocampus in a dose-dependent manner. *J. Neurosci*. 2010; 30:13690–8. <https://doi.org/10.1523/JNEUROSCI.0303-09.2010> PMID: 20943909

19. Loram LC, Taylor FR, Strand KA, Frank MG, Sholar P, Harrison J a, et al. Prior exposure to glucocorticoids potentiates lipopolysaccharide induced mechanical allodynia and spinal neuroinflammation. *Brain. Behav. Immun.* 2011; 25:1408–15. <https://doi.org/10.1016/j.bbi.2011.04.013> PMID: 21536123
20. O'Callaghan JP, Miller DB. The concentration of glial fibrillary acidic protein increases with age in the mouse and rat brain. *Neurobiol. Aging.* 1991; 12:171–4. PMID: 1904995
21. O'Callaghan JP. Measurement of glial fibrillary acidic protein. *Curr. Protoc. Toxicol.* 2002; Chapter 12: Unit12.8. <https://doi.org/10.1002/0471140856.tx1208s11> PMID: 23045073
22. Ganon MN, McEwen BS. Calmodulin involvement in stress- and corticosterone-induced down-regulation of cyclic AMP-generating systems in brain. *J. Neurochem.* 1990; 55:276–284. PMID: 2162378
23. O'Callaghan JP, Brinton RE, McEwen BS. Glucocorticoids regulate the synthesis of glial fibrillary acidic protein in intact and adrenalectomized rats but do not affect its expression following brain injury. *J. Neurochem.* 1991; 57:860–9. PMID: 1677678
24. Huffman LJ, Judy DJ, Rao KMK, Frazer DG, Goldsmith WT. Lung responses to hypothyroidism, hyperthyroidism, and lipopolysaccharide challenge in rats. *J. Toxicology and Environmental Health, Part A.* 2000; 61:623–639.
25. O'Callaghan JP, Sriram K. Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins. *J. Neurosci. Methods.* 2004; 135:159–68. <https://doi.org/10.1016/j.jneumeth.2003.12.006> PMID: 15020100
26. Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for ast. *J. Biol. Chem.* 2004; 279:19936–47. <https://doi.org/10.1074/jbc.M309304200> PMID: 14996842
27. Dinapoli V A, Benkovic SA, Li X, Kelly KA., Miller DB, Rosen CL, et al. Age exaggerates proinflammatory cytokine signaling and truncates signal transducers and activators of transcription 3 signaling following ischemic stroke in the rat. *Neuroscience.* 2010; 170:633–44. <https://doi.org/10.1016/j.neuroscience.2010.07.011> PMID: 20633608
28. Beurel E, Jope RS. Lipopolysaccharide-induced interleukin-6 production is controlled by glycogen synthase kinase-3 and STAT3 in the brain. *J. Neuroinflammation.* 2009; 6:9. <https://doi.org/10.1186/1742-2094-6-9> PMID: 19284588
29. Dantzer R, O'Connor JC, Freund GG, Johnson RW, Kelley KW. From inflammation to sickness and depression: when the immune system subjugates the brain. *Nat. Rev. Neurosci* 2008; 9:46–56. <https://doi.org/10.1038/nrn2297> PMID: 18073775
30. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong J, et al. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia.* 2007; 55:453–62. <https://doi.org/10.1002/glia.20467> PMID: 17203472
31. O'Callaghan JP, Sriram K. Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert Opin. Drug Saf.* 2005; 4:433–42. <https://doi.org/10.1517/14740338.4.3.433> PMID: 15934851
32. Cunningham C, Champion S, Teeling J, Felton L, Perry VH. The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I: C). *Brain. Behav. Immun.* 2007; 21:490–502. <https://doi.org/10.1016/j.bbi.2006.12.007> PMID: 17321719
33. Konat GW, Borysiewicz E, Fil D, James I. Peripheral challenge with double-stranded RNA elicits global up-regulation of cytokine gene expression in the brain. *J. Neurosci. Res.* 2009; 87:1381–8. <https://doi.org/10.1002/jnr.21958> PMID: 19115408
34. Michalovicz LT, Lally B, Konat GW. Peripheral challenge with a viral mimic upregulates expression of the complement genes in the hippocampus. *J. Neuroimmunol.* 2015; 285:137–42. <https://doi.org/10.1016/j.jneuroim.2015.06.003> PMID: 26198930
35. Gibb J, Al-Yawer F, Anisman H. Synergistic and antagonistic actions of acute or chronic social stressors and an endotoxin challenge vary over time following the challenge. *Brain. Behav. Immun.* 2013; 28:149–58. <https://doi.org/10.1016/j.bbi.2012.11.004> PMID: 23164949
36. Gibb J, Hayley S, Poulter MO, Anisman H. Effects of stressors and immune activating agents on peripheral and central cytokines in mouse strains that differ in stressor responsivity. *Brain. Behav. Immun.* 2011; 25:468–82. <https://doi.org/10.1016/j.bbi.2010.11.008> PMID: 21093579
37. Chijiwa T, Oka T, Lkhagvasuren B, Yoshihara K, Sudo N. Prior chronic stress induces persistent poly(I: C)-induced allodynia and depressive-like behavior in rats: Possible involvement of glucocorticoids and microglia. *Physiol. Behav.* 2015; 147:264–73. <https://doi.org/10.1016/j.physbeh.2015.04.050> PMID: 25936823

38. Gandhi R, Hayley S, Gibb J, Merali Z, Anisman H. Influence of poly I:C on sickness behaviors, plasma cytokines, corticosterone and central monoamine activity: moderation by social stressors. *Brain. Behav. Immun.* 2007; 21:477–89. <https://doi.org/10.1016/j.bbi.2006.12.005> PMID: 17267173
39. Lenczowski MJ, Van Dam AM, Poole S, Larrick JW, Tilders FJ. Role of circulating endotoxin and interleukin-6 in the ACTH and corticosterone response to intraperitoneal LPS. *Am. J. Physiol.* 1997; 273: R1870–7. PMID: 9435639
40. Fil D, Borysiewicz E, Konat GW. A broad upregulation of cerebral chemokine genes by peripherally-generated inflammatory mediators. *Metab. Brain Dis.* 2011; 26:49–59. <https://doi.org/10.1007/s11011-010-9231-9> PMID: 21258854
41. Maes M, Berk M, Goehler L, Song C, Anderson G, Galecki P, et al. Depression and sickness behavior are Janus-faced responses to shared inflammatory pathways. *BMC Med.* 2012; 10:66. <https://doi.org/10.1186/1741-7015-10-66> PMID: 22747645
42. Vichaya EG, Chiu GS, Krukowski K, Lacourt TE, Kavelaars A, Dantzer R, et al. Mechanisms of chemotherapy-induced behavioral toxicities. *Front. Neurosci.* 2015; 9:1–17.
43. Strouse TB. The relationship between cytokines and pain/depression: A review and current status. *Curr. Pain Headache Rep.* 2007. p. 98–103. PMID: 17367587

SCIENTIFIC REPORTS

OPEN

Oleoylethanolamide treatment reduces neurobehavioral deficits and brain pathology in a mouse model of Gulf War Illness

Utsav Joshi^{1,2,3}, James E. Evans^{1,3}, Ross Joseph^{1,3}, Tanja Emmerich^{1,3}, Nicole Saltiel^{1,3}, Carlyn Lungmus^{1,3}, Sarah Oberlin¹, Heather Langlois^{1,3}, Joseph Ojo^{1,2}, Benoit Mouzon^{1,2,3}, Daniel Paris^{1,2,3}, Michael Mullan^{1,2,3}, Chao Jin¹, Nancy Klimas^{4,5}, Kimberly Sullivan⁶, Fiona Crawford^{1,2,3} & Laila Abdullah^{1,2,3}

There are nearly 250,000 Gulf War (GW) veterans who suffer from Gulf War Illness (GWI), a multi-symptom condition that remains untreatable. The main objective was to determine if targeting peroxisomal function could be of therapeutic value in GWI. We performed a pilot study that showed accumulation of very long chain fatty acids (VLCFA), which are metabolized in peroxisomes, in plasma from veterans with GWI. We then examined if targeting peroxisomal β -oxidation with oleoylethanolamide (OEA) restores these lipids to the normal levels and mitigates neuroinflammation and neurobehavioral deficits in a well-established mouse model of GWI. In GWI mice, treatment with OEA corresponded with cognitive benefits and reduced fatigue and disinhibition-like behavior in GWI mice. Biochemical and molecular analysis of the brain tissue showed reduced astroglia and microglia staining, decreased levels of chemokines and cytokines, and decreased NF κ B phosphorylation. Treatment with OEA reduced accumulation of peroxisome specific VLCFA in the brains of GWI mice. These studies further support the translational value of targeting peroxisomes. We expect that OEA may be a potential therapy for treating neurobehavioral symptoms and the underlying lipid dysfunction and neuroinflammation associated with GWI. Oleoylethanolamide is available as a dietary supplement, making it appealing for human translational studies.

Gulf War Illness (GWI) affects nearly a third of the US military personnel deployed in the 1991 Gulf War (GW), who continue to experience multiple symptoms including chronic pain, fatigue, and memory impairment¹. To date, GWI remains difficult to understand but is thought to have a central nervous system (CNS) component characterized by damage to the brain regions involved in storing and processing memory and the axonal fibers transmitting sensations of pain and fatigue^{1,2}. Animal studies suggest a pathogenic mechanism involving activation of the brain astroglia and microglia cells in promoting neuroinflammation that corresponds with metabolic disturbances following exposure to GW chemicals¹. It is therefore important to explore treatment strategies that are geared toward targeting biological disturbances in the brain to determine if such approaches may alleviate the symptoms and pathology of GWI.

Evidence for CNS involvement in GWI also comes from animal modeling studies that used chemicals that were widely used by military personnel during the GW conflict³. These studies showed neurobehavioral deficits such as depression and cognitive problems corresponding with chronic brain astroglia and microglia activation, appearing at chronic post-exposure periods^{3–9}. Extensive characterization of these GWI mouse models using proteomic, lipidomics, and metabolomics technologies suggests the presence of inflammation and impaired lipid metabolism^{6,8}. Further analyses of these GWI mouse models at early post-exposure time-points (ranging from 1- to 5-months) showed changes in omega-3 docosahexaenoic acid (DHA) and ether-containing phospholipids

¹Roskamp Institute, 2040 Whitfield Ave, Sarasota, FL, 34243, USA. ²Open University, Milton Keynes, United Kingdom. ³James A. Haley Veterans' Hospital, Tampa, Florida, USA. ⁴NOVA Southeastern University, Ft. Lauderdale, FL, USA. ⁵Miami VAMC, Miami, FL, USA. ⁶Boston University School of Public Health, Boston, MA, USA. Correspondence and requests for materials should be addressed to U.J. (email: ujoshi@roskampinstitute.net)

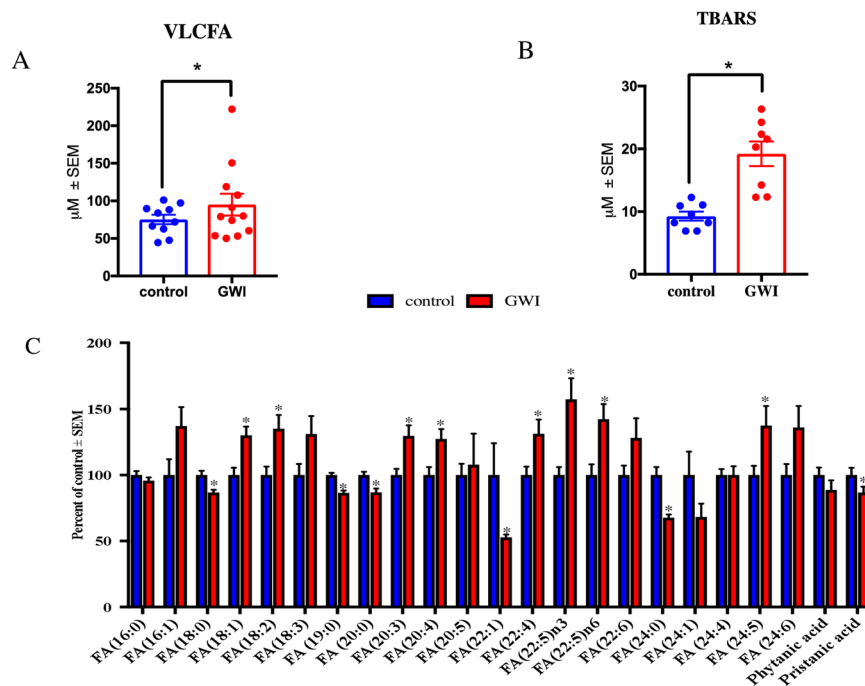


Figure 1. Plasma FFA profiles in veterans with GWI compared to GW control veterans. Mean $\pm$ SEM ($n = 10$ for controls and $n = 12$ for GWI). (A) When all VLCFA (C 22), were combined as one category, there was an overall increase in GWI compared to control GW veterans. (B) Quantification of lipid peroxidation product TBARS ($n = 8$ per group for these analyses only) in plasma showed that levels were significantly elevated in GWI compared to control veterans. (C) Profiling of FFA showed that several saturated (no double-bonds) and monounsaturated fatty acids (one double-bond) were decreased in the blood of veterans with GWI compared to controls. Pristanic acid was decreased in GWI compared to controls. The remaining long-chain fatty acids (14–21 carbons) and VLCFA species particularly omega-3 and omega-6 species, were significantly elevated in veterans with GWI compared to controls. * $p \leq 0.05$.

(PL), as well as very long chain-fatty acid (VLCFA) containing sphingomyelin (SM) species in the brains of GW agent-exposed mice compared to control mice^{5,10}. Many of these lipids decline with aging and chronicity of the post-exposure time-points⁸. These lipid changes are also detected in the blood of veterans with GWI¹¹. Since metabolism of VLCFA and synthesis of DHA and ether-containing PL is dependent upon normal peroxisomal function¹², these studies suggest a role of altered peroxisomal function in the chronic pathology of GWI.

Peroxisomes are the site of α -oxidation of 3-methyl branched-chain fatty acids (BCFA) and β -oxidation of VLCFA^{13–15}. Incomplete β -oxidation of VLCFA may lead to an overproduction of reactive oxygen species (ROS) or inadequate processing of ROS by the resident catalase in the peroxisomes, which may trigger oxidative stress and upregulate inflammatory responses^{16–18}. Recent studies have also shown an increased expression of genes related to oxidative stress and inflammation in a rat model of GWI¹⁹. Support for a role of inflammation in GWI also comes from clinical studies showing increases in pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF)- α , Interferon-gamma (IFN- γ), Interleukin 2 (IL-2) and IL-1 β , on immune cells and/or in plasma from veterans with GWI compared to healthy veterans^{20–23}. Collectively, these studies suggest that disturbances in peroxisomal β -oxidation may be associated with the oxidative stress and inflammation observed in GWI. Thus, targeting peroxisomes may be useful for treating GWI.

We, therefore, hypothesize that GW chemicals may have disturbed peroxisome function, which could impair β -oxidation or aberrantly increase VLCFA synthesis. As such, targeting peroxisome function may be beneficial for treating GWI. Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear hormone receptor which stimulates peroxisome proliferation, thereby increasing peroxisomal function and regulating lipid homeostasis^{24–28}. Furthermore, PPAR α agonists are also shown to reduce inflammation through genetic regulation of nuclear factor kappa B (NF κ B) in various CNS disorders^{29–32}. We, therefore, expect that Oleoylethanolamide (OEA), being a PPAR α agonist, may be of use in treating the underlying lipid dysfunction associated with GWI in a mouse model. Thus, the main goal of this study is to examine the fatty acid (FA) profiles associated with peroxisome function in blood from veterans with GWI and to test whether OEA treatment alters peroxisomal lipid metabolism and improves the chronic neurobehavioral deficits and glial activation in a mouse model of GWI.

Results

Peroxisome-associated VLCFA and BCFA are altered in plasma from veterans with GWI. In a pilot cross-sectional study of age- and gender-matched healthy GW veterans ($n = 10$) and those with GWI ($n = 12$), we examined pristanic acid (a BCFA), VLCFAs, and several free fatty acids (FFA). Figure 1A shows that levels of VLCFA were significantly increased ($p < 0.05$) in plasma from veterans with GWI compared to control

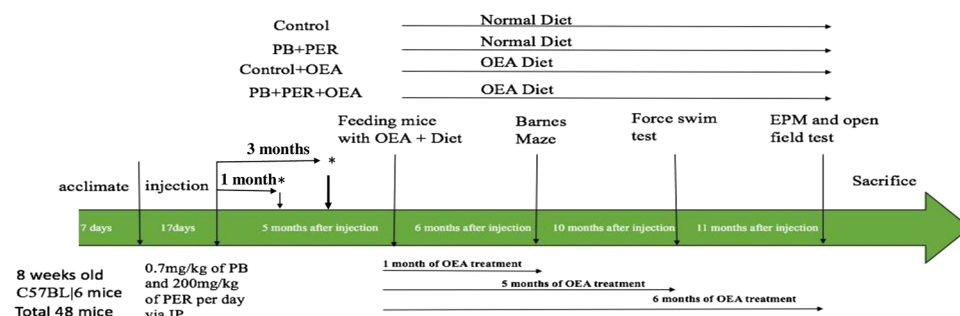


Figure 2. The experimental paradigm. The experimental timeline details the timing of GW chemical administration, treatment with OEA, behavioral testing, and tissue collection.

GW veterans. Plasma levels of malondialdehyde (MDA) were measured using a Thiobarbituric Acid Reactive Substance (TBARS) formed during the reaction of MDA with thiobarbituric acid. Levels of MDA were two-fold higher in GWI compared to control GW veterans ($p < 0.05$, Fig. 1B). Pristanic acid levels were decreased in GWI compared to controls ($p = 0.05$, Fig. 1C). An increase in several omega-3 (FA18:3, FA20:5n3, FA 22:5n3) and omega-6 (FA18:2, FA20:4, FA22:4 and FA 22:5) FFA was observed in plasma from GWI compared to control subjects (Fig. 1C). An effect size (d) ≥ 1.0 was detected for the differences between control and GWI subjects for VLCFA, pristanic acid and TBARS.

OEA treatment improves neurobehavioral deficits in a GWI mouse model. Based on the suggestion of possible peroxisomal β -oxidation dysfunction observed in blood samples from GWI subjects described above, we tested whether targeting peroxisomal β -oxidation with OEA would improve the chronic learning and memory deficits observed in a well-characterized mouse model of GWI^{6–8,33}. A timeline of the study procedures is provided in Fig. 2. As previously described, this mouse model is exposed to two GW chemicals: an anti-nerve agent pyridostigmine bromide (PB) and a pesticide permethrin (PER)^{8,33}. After GW chemical exposure, mice underwent testing with the Forced Swim Test (FST) at 1- and 3-months post-exposure. Control and GWI mice were divided into groups that either received oral OEA treatment in diet (administered orally in chow at 10 mg/kg daily) or the normal diet for a total of six months. The Barnes Maze test was used to assess learning in control and GWI mice after 1-month of oral OEA treatment (6-months post-exposure to GW chemicals) and long-term memory after 2-months of treatment (7-months post-exposure). Learning was assessed during the acquisition trials in which mice were allowed to freely explore the maze for 3 min and escape into the target box through one of the holes in the platform. On days 1 and 2, GWI mice traveled a longer distance to reach the target hole (TH) than control mice. However, GWI mice treated with OEA had the best performance, as demonstrated by having the shortest travel time when compared to control mice treated with OEA as well as compared to control and GWI mice on the normal chow diet ($p < 0.05$, Fig. 3A). During the probe trial at 2-months post-treatment, OEA treated GWI mice had a higher frequency of visiting the TH compared to GWI mice on a normal diet ($p < 0.05$, Fig. 3B). An effect size (f) = 0.4 was noted for these comparisons.

To test whether OEA corrected fatigue-like behavior in GWI mice, we performed the FST at 5-months post-treatment. While this test can be used for assessing depression, it is also helpful in quantifying fatigue by examining immobility, particularly when mice are repeatedly introduced to the FST apparatus^{34,35}, as is the case here, where all mice were subjected to the FST at 1- and 3-months post-exposure (Supplementary Figure 1A). We also observed that at 3-months post-exposure, immobility was similar between control and GWI mice for the first 2 min (Supplementary Figure 1B), but immobility increased in GWI mice with time, further suggesting that fatigue emerges with some time delay. At 5-months post-treatment (10-months post-exposure), GWI mice treated with OEA were less immobile than GWI mice on the control diet but mean velocity was not affected between the groups ($p < 0.05$, Fig. 3C,D, $f > 1.0$). However, OEA treated control mice were more immobile than control mice on normal diet (Fig. 3C). Anxiety/disinhibition was tested at 6-months post-treatment using the elevated plus maze (EPM) test, in which time spent in the closed arm indicates increased anxiety, whereas time spent in the open arms is indicative of disinhibition. Relative to controls, GWI mice spent more time in the open arms and an influence of OEA treatment was observed whereby time in open arms was reduced in OEA treated GWI mice ($p = 0.001$, Fig. 3E, $f = 0.59$). Time in the closed arms was reduced in GWI mice and was restored by OEA to similar levels as those observed in control mice on the normal diet (Fig. 3F) with no differences in the velocity between any of the treatment groups (Supplemental Fig. 2). Control mice on the OEA containing diet exhibited disinhibition when compared to control mice on the normal diet, but no anxiety-like behavior was observed. We used the open field test (OFT) to assess locomotor function, and found no differences between any of the groups for spending time in the center zone or at the perimeter of the circular platform of the OFT, suggesting no influence of OEA on locomotor function ($p > 0.05$, Supplemental Fig. 3).

Elevated levels of VLCFA in the brains of GWI mice were reduced by OEA treatment. Levels of VLCFA were significantly elevated in the brains of GWI compared to control mice. Compared to GWI mice on the normal diet, those treated with OEA had lower levels of VLCFA and were similar to control mice on the normal diet. Post-hoc analysis showed a statistically significant increase in the levels of VLCFA that were measured in the brains of GWI mice compared to controls, which were lowered in GWI mice with OEA treatment (Fig. 4A,

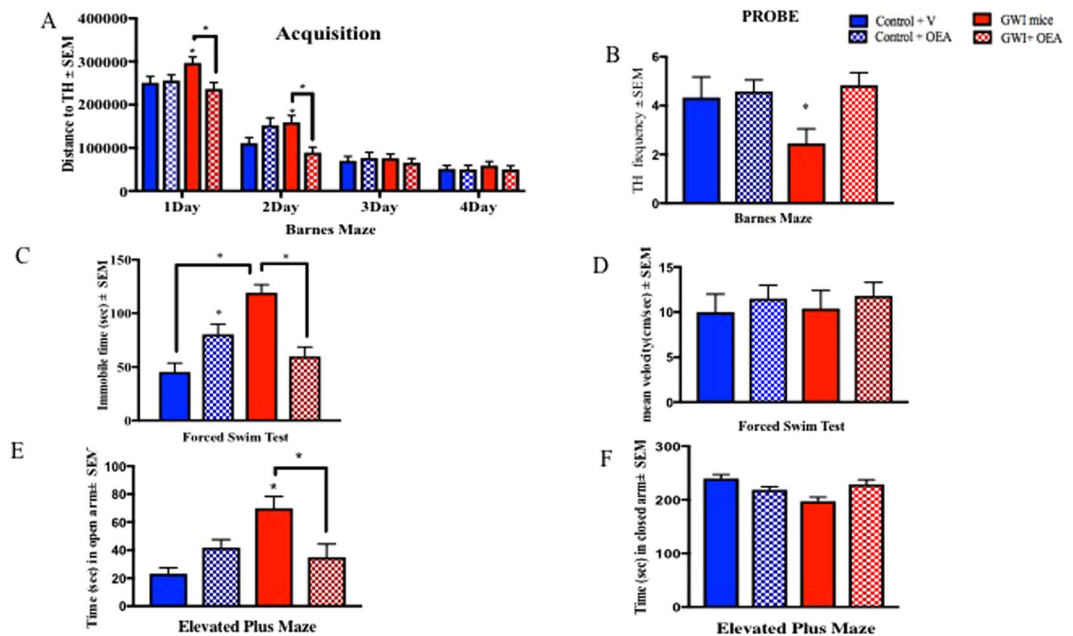


Figure 3. OEA treatment improves cognitive function and reduces fatigue and disinhibition type behavior in GWI mice. Mean $\pm$ SEM of control (n = 12), GWI mice (n = 11), control + OEA (n = 12) and GWI + OEA (n = 12). (A) One month after OEA treatment (6-months post-exposure to GW chemicals), acquisition trials were conducted to train mice to escape into the TH. For GWI mice treated with OEA, total distance to the TH was smallest, particularly on days 1 and 2, indicative of learning. As expected, GWI mice on normal chow had the worst performance with the largest total distance to the TH. Control mice treated with OEA had a higher latency to find the TH compared to control mice on the normal diet on day 2 only. (B) For the probe trials, conducted at 2-months post-treatment (7-months post-exposure), OEA treated GWI mice had a similar frequency of visits to the TH as control mice on normal chow and on OEA but a higher frequency of visits than GWI mice. (C) The immobile time was significantly increased in GWI mice compared to controls and was reduced in OEA treated GWI mice. (D) There was no change in the average speed among the different groups. (E) An examination of the time in the open arms of the EPM showed an increase of disinhibition in GWI mice on normal chow compared to both control groups and OEA treated GWI mice. (F) Total time in the closed arms was decreased in GWI mice compared with the control groups and OEA treated GWI mice. * $p \leq 0.05$.

$f = 1.0$). Similarly, levels of MDA in GWI mice were elevated compared to control mice but these levels were lower in OEA treated GWI mice (Fig. 4B, $f = 0.96$). Several omega-3 and omega-6 FFA were altered in GWI compared to control mice on normal diet (Fig. 4C and Supplementary Figure 4). In addition, GWI mice treated with OEA had similar FFA levels compared to those observed in control mice on a normal diet, whereas levels of these FFA were elevated in control mice treated with OEA (Fig. 4A,B). Supplementary Figures 5 and 6 show that OEA treatment increased PPAR α and PPAR γ co-activator 1 α (PGC-1 α) expression, irrespective of GW chemical exposure.

OEA reduces astroglia and microglia activation in GWI mice. Astroglia and microglia, which participate in protecting the brain and modulating inflammation^{36–39}, are upregulated in the brains of GWI mice at long-term post-exposure time-points⁸. Using immunohistochemistry followed by confocal microscopy, an increase in the glial fibrillary acidic protein (GFAP) staining of astroglia was observed in the cerebral cortex and the dentate gyrus (DG) of GWI compared to control mice, and was significantly decreased in OEA treated GWI mice compared to GWI mice on a normal diet ($p < 0.05$, Fig. 5A, $f = 0.89$ for the hippocampus and $f = 0.97$ for the cortex). Immunohistochemistry staining with ionized calcium binding adaptor molecule 1 (Iba1) and CD45 staining followed by light microscopy was used to examine microglia proliferation and activation, respectively. There was an elevation of microglia staining with Iba1 in the DG of GWI compared to control mice on normal chow, whereas OEA treated GWI mice had much lower staining in the DG with Iba1 compared to GWI mice on control diet (Fig. 6A, $f = 0.87$). There was no detectable staining of CD45 in either control or GWI mice with or without OEA treatment, indicating a lack of microglia activation as detected by CD45 (data not shown). There was no neuronal cell death, as detected by Nissl staining (data not shown).

OEA treatment reduces chronic brain inflammation in GWI mice. In order to determine if NF κ B-mediated pro-inflammatory pathways could be inhibited by OEA in the brains of GWI mice, we examined the ratios of the phosphorylated p65 (p-p65) to total p65 protein, which is a subunit of NF κ B, phosphorylated STAT3 (p-STAT3) and several pro-inflammatory cytokines. At 11-months post-exposure to GW chemicals, corresponding to 6-months of OEA treatment, there was a significant increase in the ratios of p-p65/p65 in the brains of GWI compared to control mice, whereas OEA treated GWI mice had lower ratios compared to GWI mice

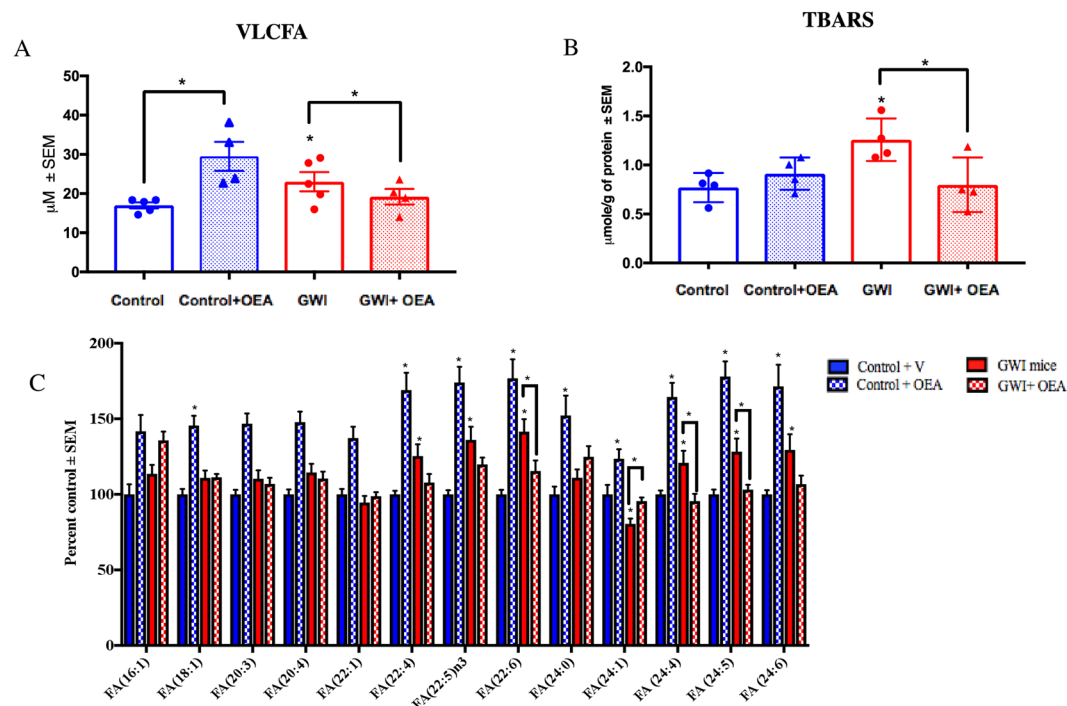


Figure 4. Brain lipid profiles show that OEA significantly decreases the VLCFA in the brains of GWI mice. Mean $\pm$ SEM expressed as percent control of mice on normal chow ($n = 4/5$ per group). (A) Levels of VLCFA were increased in the brains of GWI mice compared to control mice. OEA treated GWI mice had similar levels as in control mice. However, control mice treated with OEA had significantly higher levels of VLCFA compared to control mice that received normal diet. (B) Quantification of lipid peroxidation product TBARS showed that levels were significantly elevated in GWI compared to control mice on normal chow but were lower in OEA treated GWI mice compared to all other groups. (C) Compared to controls, GWI mice had higher levels of several major omega-6 and omega-3 FA (FA20:4, FA22:4, FA22:5, FA24:5 and FA24:6). Treatment with OEA decreased the levels of these FA in GWI mice. * $p \leq 0.05$.

on normal diet ($p < 0.001$, Fig. 7A,B, $f = 2.79$). Since p-STAT3 is thought to enhance the activation of NF κ B, we examined this protein and found it to be increased in the brains of GWI mice compared to controls, and reduced in OEA treated GWI mice compared to GWI mice on normal chow ($p < 0.05$, Fig. 7C,D, $f = 0.93$). Furthermore, several pro-inflammatory cytokines, including IL-1 β , IL-6, and IFN- γ were elevated in GWI mice but were significantly lower in GWI mice treated with OEA ($p < 0.05$, Fig. 7E, $f > 3.1$ for IL-1 β , $f = 1.6$ for IL-6 and $f = 0.75$ for IFN- γ). These cytokines were also altered in the plasma of GWI mice and normalized in GWI mice treated with OEA (Supplementary Figure 7). Supporting cell culture studies are provided in Supplementary Figure 8.

Chronically activated chemokine CCL2 and its receptor, CCR2, levels are reduced in OEA treated GWI mice. Chemokine receptor type 2 (CCR2) is involved in chemotaxis of myeloid cells in response to monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (MCP-1/CCL2)^{40–42}. A chronic increase in the CCR2 and CCL2 levels was observed in GWI mice ranging from 3- to 16-months post-exposure to GW chemicals (Supplementary Figure 11). We also examined their levels in the brain at 11-months post-exposure, which corresponded with 6-months of OEA treatment. Treatment with OEA lowered both CCR2 and CCL2 levels in GWI mice compared to GWI and control mice on normal diet (Fig 7F, $f = 1.49$ for CCL2 and $f = 0.66$ for CCR2).

Discussion

Our exploratory studies showed that lipids which are specially metabolized in peroxisomes, including VLCFA, Pristanic acid, and DHA, were altered in plasma from veterans with GWI. These findings were supported by our previous studies showing that omega-3 DHA and ether-containing PL were disturbed in veterans with GWI¹¹. Our preclinical mouse model studies also showed that VLCFA containing PL species were elevated in the brains of GWI compared to control mice^{8,10}. This further supports the relevance of impaired peroxisomal β -oxidation or aberrantly active biosynthesis of these FFA to the pathogenesis of GWI (Figure 8). Currently, there are no approved therapies that can treat the underlying pathology of GWI. The present treatment strategies mostly focus on pain management with non-steroidal anti-inflammatory drugs or opioids, reducing fatigue with vitamins/antioxidants and targeting mood/cognitive issues with antidepressants. These symptom management approaches do not modify the underlying disease process associated with GWI. Hence, there remains a need for developing treatments which target the underlying pathobiology of GWI. We used OEA as a stimulator of peroxisomal β -oxidation and showed that abnormal increases in brain VLCFA, tetracosahexaenoic, and DHA levels in GWI

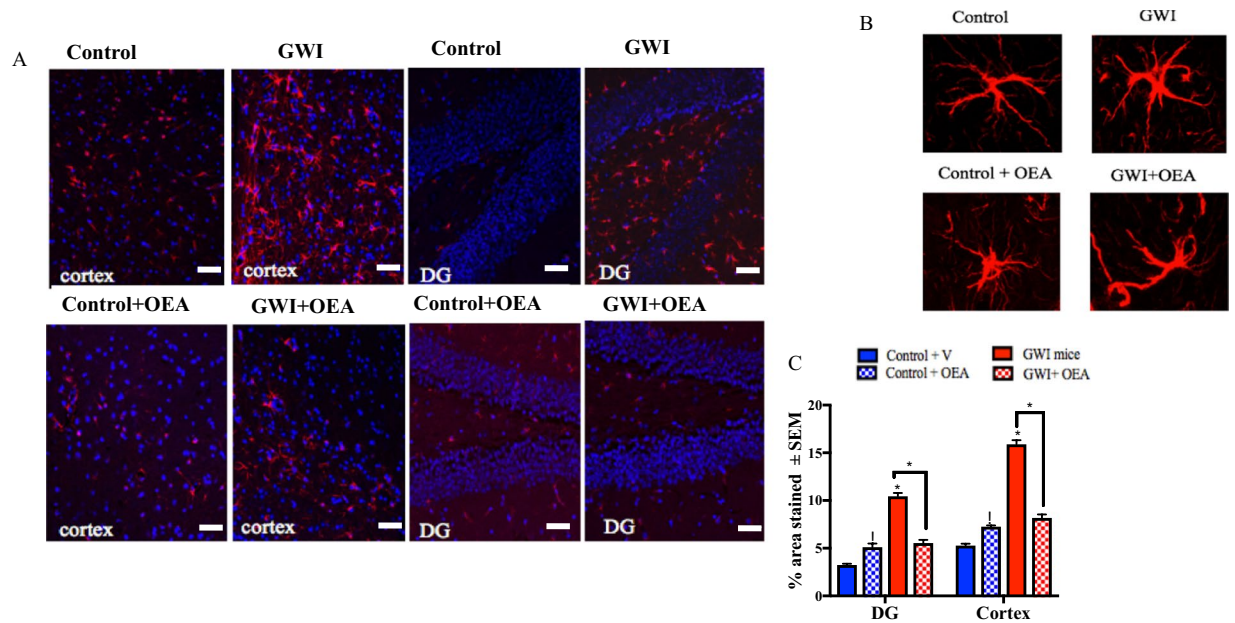


Figure 5. OEA treatment reduced elevated astroglia activation in GWI mice. Confocal microscopy images showing staining of astroglia with GFAP ($n = 4$ per group, 4 serial sections for each animal). Scale bar $50\mu\text{m}$. (A) Images show 20x GFAP staining (red) and DAPI (blue) in the cortex and the DG of mice from all 4 treatment groups. (B) Images show 100x magnification of GFAP stained astroglia in the cortex of each study treatment group. (C) Quantification of GFAP staining from 20x images. There are significant increases in GFAP staining for both the cortex and the DG within the hippocampus of GWI compared to control mice. GWI mice treated with OEA had similar levels as control mice on normal chow. Control mice treated with OEA had higher levels than control mice on the normal diet. * $p \leq 0.05$.

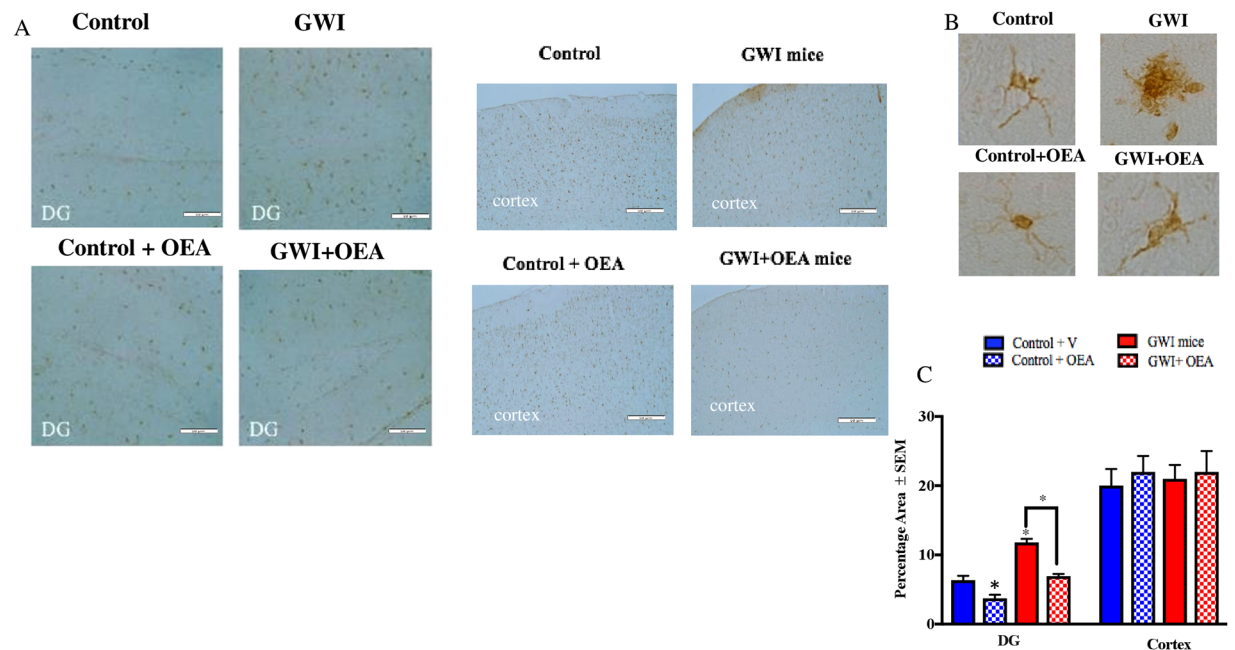


Figure 6. OEA treatment reduced microglia proliferation in GWI mice. Light microscopy images showing staining of microglia with IBA-1 ($n = 4$ per group, 4 serial section for each brain). Scale bar $50\mu\text{m}$. (A) Images show 20x Iba1 staining in the cortex and DG of mice from all 4 treatment groups. (B) Images show 100x magnification of Iba1 stained astroglia in the cortex of each study treatment group. (C) Quantification of Iba1 staining from 20x images. Levels of Iba1 staining within the DG was lower in OEA treated GWI mice compared to GWI mice on normal chow. Levels of Iba1 in GWI mice were higher in the DG compared to control mice. There were no differences in the cortices of GWI mice compared to control or OEA treated GWI mice * $p \leq 0.05$.

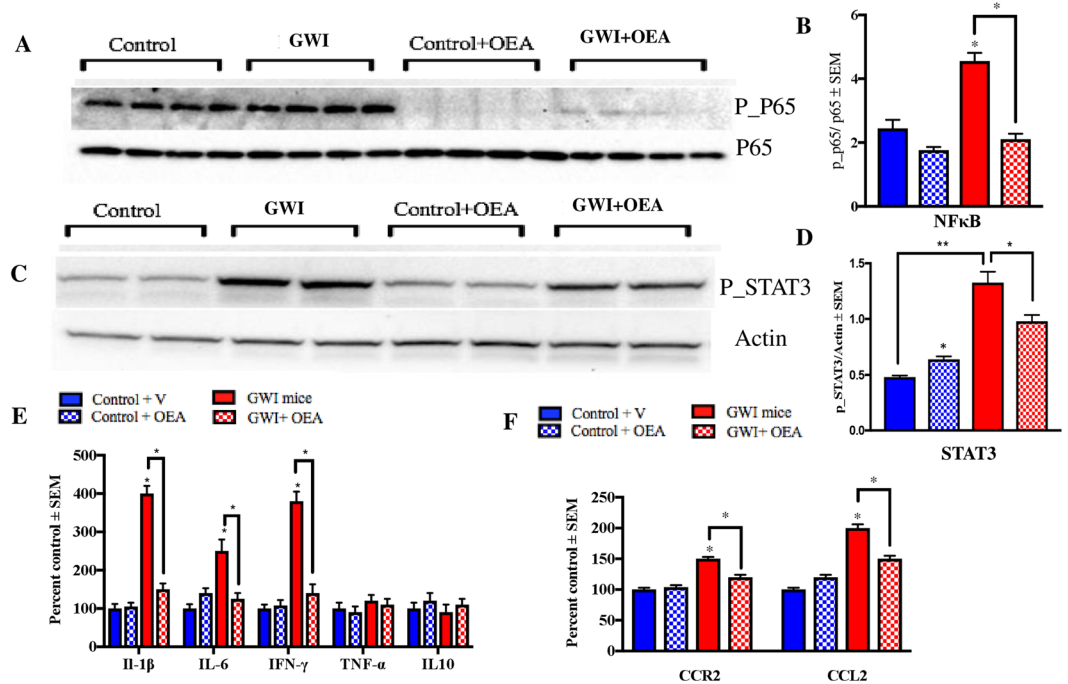


Figure 7. Levels of phosphorylated NFκB and STAT3 were reduced by OEA treatment in GWI mice. Mean ± SEM (shown as arbitrary units, $n = 4$ per group). (A,B) The ratio of p-P65/P65 was elevated in GWI mice at 11-months post-exposure. At this time-point, which corresponded with 6-months of OEA treatment, GWI mice having OEA treatment had a significantly lower ratio compared to GWI mice on normal chow. Control mice treated with OEA had non-significantly lower ratios compared to control mice on the regular diet. (C,D) Similarly, p-STAT3/Actin levels were significantly elevated in GWI compared to control mice and were also lower in OEA treated GWI mice compared to those on normal chow. Levels of STAT3 were also elevated in control mice treated with OEA compared to control mice on the normal diet. (E) Among the cytokines examined in the brain, IFN-γ, IL-6, and IL-1β were lower in OEA treated GWI mice compared to GWI mice on normal chow. Mice with GWI had elevated levels of these pro-inflammatory cytokines compared to control mice. These cytokines did not differ between control mice treated with OEA compared to control mice on the normal diet. (F) Treatment with OEA decreased the levels of CCR2 and CCL2 in the brain of GWI mice compared to GWI mice on normal chow. * $p \leq 0.05$.

mice were normalized after OEA treatment. These post-treatment changes also corresponded with reduced brain glia activation and inflammation and improved neurobehavioral deficits relevant to the clinical symptom presentation of GWI.

Memory impairment is one of the major complaints among veterans with GWI, and a recent meta-analysis comparing results of neuropsychological functioning in veterans with GWI across 14 studies has shown that veterans with GWI perform poorly on tasks relating to memory compared to controls^{43–50}, possibly due to damage or inflammation in the hippocampal region^{51–53}. Another recent study of neuropsychological functioning in military pesticide applicators from the Gulf War reported worse memory functioning in veterans exposed to pesticides and personal repellents during the war⁵⁴. GWI animal studies have consistently shown chronic impairment in learning and memory in several rodent models of GWI^{5,6,8,9,33,55}. Our current studies show that even after a couple of months of OEA treatment, GWI mice performed far better on both learning and memory-related tasks compared to all other groups, which is consistent with the currently known effects of OEA on cognition^{56–58} and may be of therapeutic value in GWI.

Other non-cognitive behavioral features were examined in this study with a particular emphasis on fatigue since clinical studies show that fatigue is one of the major symptoms of GWI, reported by nearly 79% of veterans with GWI^{1,3,30,46,47}. This is supported by an imaging study showing altered axial diffusion patterns within brain areas that link cortical regions involved in pain, fatigue, and cognition in a subset of veterans with GWI². At 1-month post-exposure, there was no difference between GWI and control mice on immobility during the FST, indicating no change in fatigue parameters. Interestingly, we detected increased immobility in GWI mice with repeated exposure to the FST apparatus at 3-months and a further increase at 10-months post-exposure. Interestingly, such effects were alleviated in GWI mice after 5-months of OEA supplementation. At this 10-month post-exposure timepoint, there were no indications of anxiety on the EPM and no locomotor problems or increased perimeter activity on the OFT in GWI mice. Instead, our findings on the EPM indicate disinhibition in GWI mice, evident by the fact that GWI mice were spending more time in the open arms of the EPM, which is consistent with a previous study reporting disinhibited behavior in the same model at 13-months post-exposure to GW chemicals³³. Disinhibition is suggested to be a consequence of lesions in the hippocampus and inhibition of dorsal hippocampal functioning^{59,60}. Collectively,

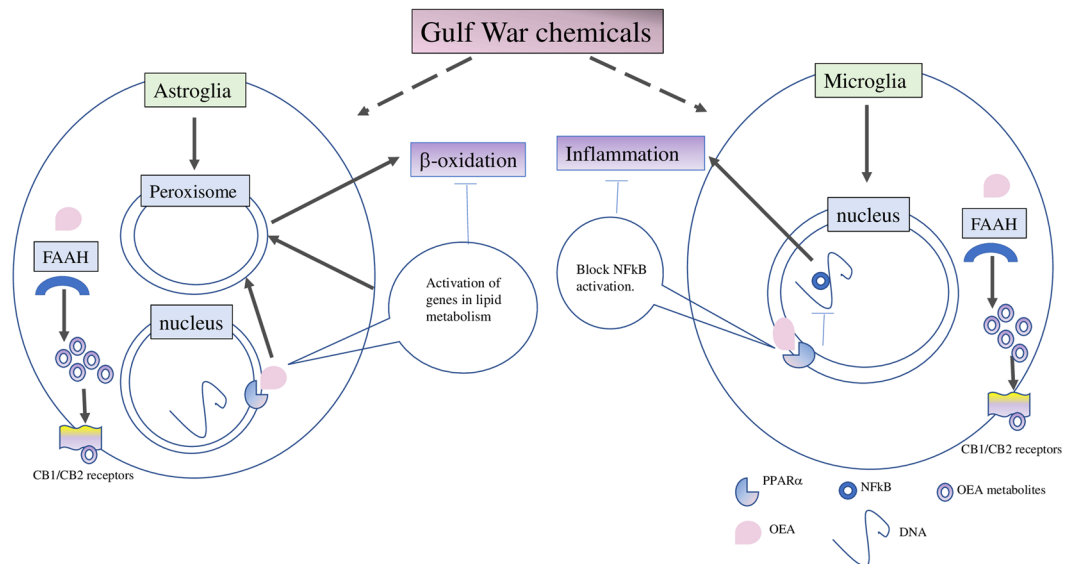


Figure 8. Gulf War chemicals dysregulate peroxisomal lipid metabolism in astroglia, contributing to microglia activation and inflammation which corresponds with neurobehavioral deficits. Our working hypothesis is that GW chemicals affect peroxisomal lipid metabolism in the astrocytes since astroglia activation occurs earlier than microglia activation in this GWI mouse model. We also propose that the failure of astroglia to perform its neuroprotective function contributes to the subsequent activation of microglia and neuroinflammation. Targeting peroxisomes with OEA is expected to reduce astroglia activation which corresponds with normalization of peroxisomal lipid metabolism. We also propose that OEA may also reduce NF κ B activation in microglia, thereby reducing neuroinflammation. In another scenario, OEA may undergo degradation by FAAH and oxygenation by other enzymes, generating additional bioactive metabolites which may target the cannabinoid receptors and also promote anti-inflammatory responses via synergistic activation of NF κ B.

these behavior studies suggest damage to the brain hippocampal region after GW chemical exposure and that OEA treatment could potentially alleviate these symptoms.

Consistent with previous studies^{6,9,33}, we observed astroglia activation in both the cortex and hippocampus of GWI mice that was reduced by OEA treatment. As before, Iba1 staining was increased in the hippocampus of GWI mice when it was examined at 16-months post-exposure⁸. However, there was no CD45 staining in either control or GWI mice, suggesting only increases in microglia number without an overt activation. This increase was also normalized after OEA treatment in GWI mice. As can be seen, both astroglia and microglia increases appear to be key pathologies in GWI, replicated in several rodent models of GWI^{6–9,33}. In fact, a role of astroglia involvement in GWI was suggested by an imaging study showing abnormal lactate utilization in subsets of GWI veterans and a blood biomarker study showing increased autoantibodies to GFAP in veterans with GWI^{61,62}. While previously thought to serve in a supportive capacity within the CNS, recent studies have shown that astroglia also plays a prominent role in regulating the innate immune responses in the brain by releasing chemokines which facilitate the activation, migration, and proliferation of microglia^{63,64}. Given that astroglia activation in this GWI model precedes changes in microglia, it is possible that the observed increase of microglia cells in the hippocampus may be a result of increased migration to control ongoing immune and inflammatory responses, which warrants further investigation.

While both astroglia and microglia can release CCL2, in conditions with an active immune component and an absence of obvious neuronal damage, astroglia appears to be the major producers of CCL2⁶⁵. Our current study shows that CCL2 levels are chronically elevated in GWI compared to the control which is consistent with findings of increased mRNA of this protein in another mouse model of GWI that was exposed to an organophosphate pesticide⁵⁴. The release of CCL2 helps recruit macrophage/microglia to the damaged area and attracts CCR2 expressing monocytes from the periphery in response to injury or chemical exposure^{66,67}, contributing to inflammation via CCR2 activation through NF κ B and STAT3 pathways^{40,41}. Given that we do not observe an overt neuronal death in our GWI mice at any of the chronic post-exposure timepoints^{6–8}, the increase in CCL2 is likely due to inflammation rather than cell death. Consistent with these studies, we also observed increases in phosphorylation of NF κ B and STAT3 in the brains of GWI mice, which corresponded with an increase in several pro-inflammatory cytokines, such as IL-1 β , IL-6, and IFN- γ . Increases in serum levels of these cytokines are also observed in veterans with GWI compared to control veterans^{20,68,69}.

Treatment with OEA can potentially alleviate the inflammatory aspects of GWI described above. This can be supported by our studies showing that OEA exerts anti-inflammatory effects in GWI by inhibiting NF κ B activity and downregulating many pro-inflammatory cytokines. However, metabolism of OEA by fatty acid amide hydrolase (FAAH) may target the brain cannabinoid receptors and some of the observed effects could be mediated by these alternative pathways and warrant an evaluation⁷⁰. Furthermore, given this impact of OEA on the

endocannabinoid system, translation of OEA treatment into humans in the context of cannabinoid use may produce some adverse effects and will require close monitoring.

One major limitation of this study is that OEA affected lipid homeostasis in control mice treated with OEA, which corresponded with lowered anxiolytic response in the EPM test and higher immobility in the FST. Despite that, we did not observe any adverse effect on memory or an increase in lipid peroxidation and inflammation in these mice. However, given the role of OEA in lipid metabolism and reducing inflammation, increasing its level in the absence of underlying inflammation or metabolic dysfunction warrants further examination. Our exploratory work into the mechanism of these effects suggests that PPAR α and PGC-1 α elevation could have contributed to possible increases in peroxisome proliferation and mitochondria biogenesis and that increases in lipids could reflect these changes^{71–73}. We do expect that additional pharmacokinetics and pharmacodynamics studies will help identify a safe and effective dose for chronic administration in veterans with GWI.

Conclusion

This study provides support that OEA treatment can restore normal peroxisomal lipid profiles in a well-characterized GWI mouse model, corresponding with improvement in neurobehavioral symptoms relevant to GWI. These studies also show that OEA treatment leads to a reduction of brain inflammation in this GWI mouse model, a pathology that is highly relevant to the etiology of GWI. These data provide critical support for future translational studies to find an optimal and safe dose of OEA for treating subjects with GWI.

Future Directions

Both clinical and animal studies now point to an association between peroxisomal dysfunction and GWI and, therefore, further evaluation of the enzymatic processes and proteins involved in the biosynthesis and metabolism of VLCFA and pristanic acid is required. Studies are also required to examine the influence of genes involved in transport and metabolism of these lipids. One of these genes include the apolipoprotein E (APOE), genetic variations of which are implicated in the differential modulation of CNS vulnerability to pesticide exposure^{74,75}. We and others have shown that microglia activation is associated with the chronic pathology of GWI in animal models^{8,9}. As such, translational studies using microglia imaging approaches in both veterans and animal models of GWI will significantly advance our understanding of the role of microglia in GWI⁷⁶. It would also be interesting to apply other technical approaches, such as live imaging of microglia using Translocator protein (TSPO)^{77–79} ligands or examining the presence of IL-1 β on microglia, to determine the activation state of microglia^{80,81}. Our studies also suggest a possible involvement of other myeloid cells in the brain that also warrants further investigation.

There are several different preclinical treatment approaches which are currently being explored that target different biological systems. Given the complexity of this illness, preclinical testing of drugs with different mechanisms of action are needed to maximize the translational potential of treating veterans with GWI. Future investigation is needed to examine a potential impact of OEA metabolism by FAAH on the observed treatment effects and OEA on neurogenesis since several studies have now shown reduced neurogenesis in GWI animal models^{9,33}, though the relevance of neurogenesis in human adults remains to fully understood^{82,83}.

Methods

Human Subjects. This study was approved by the Institutional Review Board (IRB) with all protocols conducted in accordance with relevant guidelines and regulations. Plasma samples from age- and gender-matched control veterans ($n = 10$) and veterans with GWI ($n = 12$) were compared in this study. A biorepository of plasma samples from GW veterans who previously consented to share their blood samples for future studies from the Boston Gulf War Illness Consortium (GWIC) and the Dynamic Modeling of GWI study was provided by our collaborators at the Boston University and NOVA Southeastern University sites. The GWI biorepository is approved by the institutional review boards (IRBs) at Boston University, NOVA Southeastern University and Miami Veterans Administration Medical Center (VAMC). All experiments were performed in accordance with the guidance from these oversight committees. Informed consent was obtained from all participating subjects. The GWI biorepository samples were all collected using the same written standard operating procedures for performing phlebotomy, plasma separation and aliquoting. All samples were stored at -80°C and were not previously thawed and refrozen. The Kansas GWI criteria⁷⁵ were used to determine cases of GWI and controls. The Kansas GWI criteria require that GW veterans show symptoms in at least 3 of 6 symptom domains (fatigue/sleep problems, pain, cognitive impairment, mood symptoms, gastrointestinal symptoms, respiratory symptoms, and skin abnormalities). Study participants were excluded if they reported being diagnosed with another medical condition that could explain their chronic health symptoms. The Kansas GWI case definition exclusions include veterans with a history of prior CNS or major psychiatric disorders that could affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson's Disease, Alzheimer's disease, schizophrenia). Controls were deployed veterans from the 1991 GW who did not meet the Kansas GWI criteria or exclusionary criteria listed above. Subjects also completed demographic and health symptom questionnaires. The recruitment strategy for the biorepository also excludes subjects currently participating in clinical trials, to minimize interferences from the effects of these medications at the time of the blood draw. Subjects are also asked if they are on any steroid treatments and, to the best of our knowledge, subjects have reported no. However, no other medical information is currently obtained. A detailed demographic table is provided in Table 1.

Animal Handling. All procedures on mice were approved by Roskamp Institute's Institutional Animal Care and Use Committee and were in compliance with the Office of Laboratory Animal Welfare and Laboratory animal care guidelines as described previously⁸. Eight-week-old male C57BL6 mice were purchased from Jackson Laboratory and allowed to acclimate to the new environment. All mice were placed on Standard rodent diet

	Control (GW veteran)	GW1
N total	10	12
Age (Mean $\pm$ SEM)	49.3 $\pm$ 2.6	46.1 $\pm$ 1.6 SE
Male (%)	100	100
Ethnicity		
Caucasian	5(50%)	4(28.5%)
African American	1(10%)	5(35.7%)
Hispanic	2(20%)	0
Asian	2(20%)	3(23%)

Table 1. Demographics of the Gulf War veteran cohort.

(Envigo, IN, USA) routine diet upon arrival. At 9 weeks of age ($n = 48$), 0.7 mg/kg of pyridostigmine bromide (PB) (Fisher Scientific) and 200 mg/kg of PER (Sigma Aldrich) in 100% Dimethyl sulfoxide (DMSO) was administered via intraperitoneal (i.p.) injection daily for 10 days, while the control group received only 100% DMSO^{6,8,33}. Subsequently, mice were allowed to age for 5-months post-exposure, a time point when we have observed cognitive impairment^{10,33}. Mice were again randomly assigned to either the OEA treatment or the control group. Mice in the OEA group were fed standard rodent chow (Envigo, MD, USA) containing a daily dose of 10 mg/kg OEA (based on the consumption of 5 g daily food intake) for up to 6 months, while the control group mice received standard chow only (11-months post-exposure, see Figure 2 for a timeline of the study). After 6 months of treatment, animals were euthanized for lipidomic, neuropathological, and biochemical studies.

OEA Synthesis method. A 1-L round-bottom flask was charged with a mixture of benzene and hexanes (360 mL, 1:1, v/v) and oleic acid (25 g, 88 mmol). The above solution was cooled on an ice-bath to which oxalyl chloride (20 mL, 232 mmol) was slowly added over a period of 30 minutes via an addition funnel. This was followed by an addition of a catalytic amount of Dimethylformamide (DMF) (1 mL, 13 mmol). The above reaction mixture was removed from ice and then stirred at room temperature for 5 hrs. The solvents were then removed under reduced pressure and replaced with anhydrous Dichloromethane (DCM) (360 mL). To this solution, triethylamine (50 mL, 359 mmol) and 2-aminoethanol (22 g, 360 mmol) was added. The reaction solution was stirred overnight at room temperature and was quenched with H₂O (360 mL). Layers were separated and then the organic layer was washed with H₂O (360 mL $\times$ 3) and brine (360 mL) and dried with anhydrous MgSO₄. After drying, the resulting residue was dissolved in n-hexane (500 mL) and was left in the refrigerator under -10°C overnight. The resulting product was filtered, washed, and dried in vacuo to a constant weight as a white solid (24.5 g, 86%). ¹H and ¹³C NMR of this product conform to literature reports.

The Barnes Maze test. At 1 month post-treatment with OEA, the Barnes maze trial was performed to assess learning. Acquisition trials were conducted over 4 days where each trial was conducted for 3 min, and 4 trials were conducted for each mouse per day. Briefly, each mouse was placed in the middle of the maze and the trial ended when the mouse entered the escape box or after 3 min. Bright flood lamps were used as motivators for mice to enter the escape box, where they stayed for 1 min. If a mouse did not reach the target hole within 3 min, the experimenter guided it to the escape box. A probe trial was conducted 24 hrs after the last training session. The escape box was removed for the probe trials. Each mouse was placed in the middle of the maze and allowed to explore for a fixed interval of 1.5 min. Another probe trial was administered at 2 months post-treatment to assess long-term memory. The Ethovision software was used to track mouse movement, and total distance traveled by each mouse on the platform was used to calculate the path length. Animals were subsequently euthanized for neuropathological and biochemical studies.

Forced Swim Test. Forced swim test was performed to measure fatigue-like behavior, according to the protocol published by Can and colleagues⁸⁴ and was administered at 1- and 3-months post-exposure to GW chemicals and then subsequently at 10-months post-exposure, corresponding with 5-months after OEA treatment. Furthermore, repeated exposure to FST is used to induce fatigue in mice⁸⁵. Mice were brought to the behavior testing room at least 30 minutes prior to the initiation of the testing in order to acclimate them to the testing conditions. The forced swim test (FST) apparatus was comprised of a cylindrical tank (30 cm height $\times$ 20 cm diameters) which was filled with warm tap water (approximately 22 $^{\circ}\text{C}$) to a depth of 25 cm, preventing the tail and feet from reaching the bottom of the apparatus. Each animal was placed in the tank for 6 minutes, after which the mouse was removed to a warm and dry environment. Data were recorded and captured using the Ethovision XT software version 7, and latency to stop swimming and time spent being immobile was recorded as the outcome measures.

The Elevated Plus Maze test. The EPM, used to determine anxiety, consisted of two open arms and two closed arms surrounded by high walls (40 cm) across from each other. The middle section that allows the animal to transit from arm to arm consisted of a square with dimensions of 12 $\times$ 12 cm. Mice could move freely within the maze for 5 min. Entries to each arm were recorded using the Ethovision system. Only one trial per mouse was administered. The distance traveled in the maze, the number of entries into each arm and the percentage of entries to open arms were calculated. The time spent in open and closed arms were analyzed as the outcome measures.

The Open Field test. The Open Field Test was used to determine locomotor activity and exploration activity of the mice. Mice were placed in the middle of the 100 cm diameter arena delimited by opaque walls and allowed to explore for 10 min. Distance traveled and time spent in the center zone (60 cm diameter) versus surrounding periphery were recorded by automated video tracking (Ethovision system). After each test, mice returned to their home cages and the arena was thoroughly cleaned with 70% ethanol.

Sample preparation. Mice were euthanized via cardiac puncture under anesthesia and all animals were transcardially perfused with PBS. Half brains (right hemisphere) and plasma were immediately frozen in liquid nitrogen and transferred to a -80°C freezer until further use. Using a Dounce homogenizer, each brain hemisphere was homogenized in chilled lysis buffer containing protease (Roche, Indianapolis IN) and phosphatase inhibitor (Pierce, Grand Island, NY) cocktails.

Immunohistochemistry and confocal microscopy. The left-brain hemispheres were fixed in 4% paraformaldehyde and embedded in paraffin. Sagittal sections (8 μm) were prepared and rehydrated in an ethanol gradient before the staining procedure. Glial fibrillary acid protein (GFAP; 1: 1000, Wako, Carpinteria, CA, USA) and ionized calcium binding adaptor molecule 1 (Iba1) antibodies (1: 5,000; Abcam, Cambridge, MA, USA) were used to stain astroglia and microglia, respectively. Primary antibodies were localized using respective fluorescent-labeled secondary antibodies. Slides were mounted in mounting media with DAPI (Abcam). ImageJ software was used to analyze the stained sagittal sections. The stained areas were calculated and expressed as a percentage of the field of view.

Enzyme-Linked Immunosorbent Assay. Brain samples were homogenized using a Dounce homogenizer, in chilled lysis buffer containing protease (Roche, Indianapolis IN) and phosphatase inhibitor (Pierce, Grand Island, NY) cocktails. Enzyme-Linked Immunosorbent Assay (ELISA) kits for mouse CCR2 and CCL2 protein (LSBio, Seattle, USA) were used to study levels of these proteins in the brain. Brain homogenates were diluted 1:4 with the sample diluent provided with the kits, and all procedures were performed as per the manufacturer's instructions. The total protein content of each sample was determined by the bicinchoninic acid (BCA) assay (ThermoFisher, Waltham, MA USA). Results were expressed in ng/mg of protein. The detection limit is typically $<0.156\text{ ng/ml}$ and $<10000\text{ pg/ml}$ for these ELISA kits, respectively. Intra-Assay: CV $<4.6\%$ Inter-Assay: CV $<7.6\%$ for CCR2 and Intra-Assay: CV $<5.6\%$ Inter-Assay: CV $<6\%$ for CCL2. There was no reported cross-reactivity with other proteins for the primary antibody used in these kits.

Brain cytokines, especially IFN- γ , IL-1 β , IL-10, IL-6, and TNF- α , were quantified using commercially available ELISA kits. LSBio ELISA kits were used to quantify IFN- γ (LS-F5065), IL-10 (LS-F9770) and IL-6 (LS-F2478) (LSBio Seattle, USA). IL-1 β and TNF- α (KMC0011 and BMS607-3) were quantified by using commercial ELISA kits (ThermoFisher, Waltham, MA USA). All procedures were performed as per the manufacturer's instructions. Cytokine concentrations were normalized against the total protein content determined by BCA. Results were then expressed as a percentage to control. There was no reported cross-reactivity with other proteins for the primary antibody used in these kits.

Western Blot. Following the BCA, equal amounts of protein (20 μg) from each sample were heated with Laemmli buffer containing beta-mercaptoethanol (BioRad, Hercules, CA, USA) and separated by 4–20% PAGE using a Tris-HCl buffer system and 18-well Criterion gels (Biorad) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Biorad) overnight at 90 mA. The membrane was blocked for 1 hour in 5% blocking milk (BioRad, Hercules, CA, USA). Then, each membrane was individually immunoprobed with a primary antibody against p-p65 phosphorylated at Ser536 (1:1000, Cell Signaling), total p65 NF κ B (1:1000, cell signaling), PPAR alpha (1:1000, cell signaling), p-STAT3 (1:1000, Cell Signaling) phosphorylated at Tyr 705, or actin (1:1000, Abcam) in blocking buffer overnight. After each primary antibody incubation, each membrane was incubated with the recommended dilution (1:5000) of corresponding horseradish peroxidase-conjugated secondary antibody (cell signaling) in blocking buffer at room temperature for 1 h. Protein bands were visualized using enhanced chemiluminescence detection reagents (Thermo Scientific, MA USA). Band intensities were analyzed using the ChemiDoc imaging system (Bio-Rad). Results were calculated using Image Lab software and normalized to the expression of actin protein in the samples.

Multiplex Cytokine Assay. Selected cytokine levels in the plasma were analyzed using Meso Scale Discovery (MSD) 96-Well MULTI-SPOT[®] Ultra-Sensitive V-PLEX Proinflammatory Panel 1 mouse Kit, using electrochemiluminescence detection on an MSD Sector Imager[™] 6000 with Discovery Workbench software (version 3.0.18) (MSD[®], Gaithersburg, MD, USA). Cytokines were measured using the TH1/TH2 8-plex kit, which included 8 markers: IFN- γ , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-13, and TNF- α . All assays were performed according to manufacturer's instructions, in duplicates. Plasma samples were diluted 1:2 and added to the plate which contained the capture antibody immobilized on a working electrode. Following incubation for 1 hour, SULFO_TAG labeled detection antibodies were added to the wells. Finally, MSD buffer was added which developed electrochemiluminescence and the plate was loaded into an MSD instrument for reading. Data were acquired using a SECTOR S 6000 plate reader (MSD). Results were then expressed as the percentage to control.

TBARS assay. Levels of MDA in brain homogenates were measured using a TBARS Assay Kit (Cayman Chemical, 10009055) as per the manufacturer's protocol. At boiling temperature, oxidized lipids produce MDA which can be measured calorimetrically at 530–540 nm.

Fatty acid analysis Assay. Total lipid extracts were prepared from brain homogenate and plasma samples by the Folch method⁸⁰ and modified to minimize the sample volume used. Briefly, a 5 μL aliquot of internal

Time	% Solvent A	% Solvent B	Flow Rate (nl/min)
00:00	70	30	250
01:00	50	50	250
40:00	2	98	250
50:00	2	98	250
50:01	70	30	250
65:01	70	30	250

Table 2. Gradient Program for Positive and negative Total Lipid Runs.

standard containing C17 fatty acid was spiked into 10 μ L of brain or plasma. Methanol (70 μ L) and chloroform (120 μ L) were then added. This mixture was centrifuged at $20,000 \times g$ for 10 minutes to pellet proteins and other cell debris, and the supernatant was transferred to a clean tube. Next, 0.88% KCl was added (40 μ L) at a volume of approximately 25% of the total reaction volume. This mixture was then vortexed for 1 minute and centrifuged as above to separate the phases. The upper phase was discarded and the lower phase was dried into a clean tube under vacuum. Samples were cleaned using 750 μ L, PVDF, 0.2 μ m centrifuge filters (Thermo Scientific) prior to analysis. Filters were conditioned prior to use by centrifuging with 1:1 chloroform: methanol (v/v) (200 μ L) at $10,000 \times g$ for 5 minutes and discarding the flow-through. Dried lipid extracts were resuspended in 1:1 chloroform: methanol (v/v) loaded onto the conditioned centrifuge filters, and centrifuged as above. Flow-through was then dried directly into an autosampler vial under vacuum and stored at -80°C until further processing. Cleaned lipid extracts were resuspended in LC solvents (50 μ L) at a ratio of 70% solvent A (27% IPA, 42% water, 21% ACN, 0.1% formic acid, 10 mM ammonium formate), and 30% solvent B (90% IPA, 10% ACN, 0.1% formic acid, 10 mM ammonium formate) for LCMS analysis of total lipids. A Thermo EASY nLC 1000 liquid chromatograph coupled with a Thermo LTQ/Orbitrap mass spectrometer with nanoflex ESI source was used for nano-HPLC-MS sample analysis. Samples were injected onto an Acclaim PepMapTM 100, 75 μ m $\times$ 2 cm, nanoViper, C18, 3 μ m, 100A trapping column using mobile phase A as the loading solvent with the outlet flow directed to waste. Following sample loading, the outlet flow was directed to the analytical column (Acclaim PepMapTM RSLC, 75 μ m $\times$ 15 cm, nanoViper, C18, 2 μ m, 100A) for chromatographic separation of lipid species. Gradient elution was carried out by the gradient programs shown below in Table 2. All samples were kept at 7°C in a cooled autosampler tray for the duration of the analysis. Data was acquired by full scan MS in both positive and negative modes with a mass range of 130–2000 m/z (13 μ scans/sec, spray voltage: 1500 V, resolution: 30,000, max inject time: 200msec). TracefinderTM software (Thermo Scientific) was used for peak identification and integration for lipid species in each run. Target compound lists of expected analytes for each chosen lipid class were used to find peaks of interest with ion windows of 5 ppm mass accuracy for the expected ions.

Statistical analyses. Post-hoc power calculations of only the primary outcomes for human studies are performed given the exploratory nature of this work. For human studies, the primary outcome of interest was peroxisomal lipids and therefore VLCFA, pristanic acid and TBARS levels were used. For the sample size used in the study, a power of $>95\%$ with the effect size $d = 2.86$ at $\alpha = 0.05$ was detected for TBARS using the means and the standard deviations (SD) for control and GWI subjects. A power of 84% was detected for pristanic acid with a Cohen $d = 1.17$ at $\alpha = 0.05$ whereas a power of 74% was detected with $d = 1.0$ at $\alpha = 0.05$ for VLCFA. For behavior studies, we considered cognitive deficits as the primary outcome measure and the probe data were used for these calculations and observed a power of 65% with $f = 0.43$ at $\alpha = 0.05$. For the FST, a power of $>95\%$ was observed with the $f = 1.1$ at $\alpha = 0.05$. For EPM, we detected a power of $>90\%$ with the $f = 0.59$ at $\alpha = 0.05$. Power calculations of biochemistry were restricted to VLCFA and TBARS measurements as primary outcomes of interest. For VLCFA, a power of 88% was detected with $f = 1.0$ at $\alpha = 0.05$. For the TBARS assay, we detected a power of 79% with the $f = 0.96$ at $\alpha = 0.05$. Both astroglia and microglia activation in the hippocampus were considered primary outcome measures for immunohistochemistry analyses. For astroglia activation, we obtained a power of 72% with $f = 0.89$ at $\alpha = 0.05$ for the hippocampus and a power of 79% with $f = 0.97$ at $\alpha = 0.05$ for the cortex staining. For *iba1*, we observed a power of 71% with $f = 0.87$ at $\alpha = 0.05$. Data are expressed as mean $\pm$ SEM. Differences between means were assessed using one-way analysis of variance (ANOVA) or t-tests as appropriate. Neuropathological, western blot and ELISA data for protein markers described above were analyzed using ANOVA to determine statistical significance. For the lipidomics studies, a mixed linear model regression (MLM), Fisher's least significant difference (LSD) correction, and the Benjamini–Hochberg procedure (B-H) were used for multiple-test corrections and to control the false discovery rate (FDR) for hypothesis testing of primary outcomes and as applicable. Barnes Maze data were analyzed using MLM for the acquisition trials and ANOVA for the probe trials. All other behavior tests were analyzed using ANOVA. All data were analyzed using SPSS version 22.0.0 (IBM Corporation, Armonk, NY). B-H ($\alpha = 0.05$) was calculated using Excel. $P < 0.05$ was considered significant.

References

- White, R. F. *et al.* Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: Effects of toxicant exposures during deployment. *Cortex* **74**, 449–475 (2016).
- Rayhan, R. U. *et al.* Increased Brain White Matter Axial Diffusivity Associated with Fatigue, Pain and Hyperalgesia in Gulf War Illness. *PLoS One* **8** (2013).
- Binns, J. *et al.* Gulf War Illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations. 1–465 (2008).

4. Terry, A. V. *et al.* Chronic, intermittent exposure to chlorpyrifos in rats: protracted effects on axonal transport, neurotrophin receptors, cholinergic markers, and information processing. *J. Pharmacol. Exp. Ther.* **322**, 1117–1128 (2007).
5. Abdullah, L. *et al.* Lipidomic profiling of phosphocholine containing brain lipids in mice with sensorimotor deficits and anxiety-like features after exposure to gulf war agents. *NeuroMolecular Med.* **14**, 349–361 (2012).
6. Abdullah, L. *et al.* Proteomic CNS profile of delayed cognitive impairment in mice exposed to Gulf War agents. *Neuro Molecular Med.* **13**, 275–288 (2011).
7. Zakirova, Z. *et al.* A chronic longitudinal characterization of neurobehavioral and neuropathological cognitive impairment in a mouse model of Gulf War agent exposure. *Front. Integr. Neurosci.* **9**, 71 (2015).
8. Abdullah, L. *et al.* Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness. *Toxicology* **372**, 22–33 (2016).
9. Parihar, V. K., Hattiangady, B., Shuai, B. & Shetty, A. K. Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. *Neuropsychopharmacology* **38**, 2348–62 (2013).
10. Abdullah, L. *et al.* Chronic elevation of phosphocholine containing lipids in mice exposed to Gulf War agents pyridostigmine bromide and permethrin. *Neurotoxicol. Teratol.* **40**, 74–84 (2013).
11. Emmerich, T. *et al.* Phospholipid profiling of plasma from GW veterans and rodent models to identify potential biomarkers of Gulf War Illness. *PLoS One* **12** (2017).
12. Ferdinandusse, S. *et al.* Identification of the peroxisomal beta-oxidation enzymes involved in the biosynthesis of docosahexaenoic acid. *J. Lipid Res.* **42**, 1987–95 (2001).
13. Casteels, M., Foulon, V., Mannaerts, G. P. & Van Veldhoven, P. P. Alpha-oxidation of 3-methyl-substituted fatty acids and its thiamine dependence. *European Journal of Biochemistry* **270**, 1619–1627 (2003).
14. Watkins, P. A. Very-long-chain acyl-CoA synthetases. *Journal of Biological Chemistry* **283**, 1773–1777 (2008).
15. Mashek, D. G. & Greenberg, A. S. Serum TAG analysis differentiates between genetic and obesity-associated NAFLD. *Diabetes* **63**, 42–44 (2014).
16. Terlecky, S. R., Terlecky, L. J. & Giordano, C. R. Peroxisomes, oxidative stress, and inflammation. *World J. Biol. Chem.* **3**, 93–7 (2012).
17. Ivashchenko, O. *et al.* Intraperoxisomal redox balance in mammalian cells: oxidative stress and interorganellar cross-talk. *Mol. Biol. Cell* **22**, 1440–1451 (2011).
18. Koepke, J. I., Wood, C. S., Terlecky, L. J., Walton, P. A. & Terlecky, S. R. Progeric effects of catalase inactivation in human cells. *Toxicol. Appl. Pharmacol.* **232**, 99–108 (2008).
19. Shetty, G. A. *et al.* Chronic Oxidative Stress, Mitochondrial Dysfunction, Nrf2 Activation and Inflammation in the Hippocampus Accompany Heightened Systemic Inflammation and Oxidative Stress in an Animal Model of Gulf War Illness. *Front. Mol. Neurosci.* **10**, 1–20 (2017).
20. Khaiboullina, S. F. *et al.* Cytokine expression provides clues to the pathophysiology of Gulf War illness and myalgic encephalomyelitis. *Cytokine* **72**, 1–8 (2015).
21. Broderick, G. *et al.* Exploring the diagnostic potential of immune biomarker coexpression in gulf war illness. *Methods Mol. Biol.* **934**, 145–164 (2012).
22. Vojdani, A. & Thrasher, J. D. Cellular and humoral immune abnormalities in Gulf War veterans. *Environ. Health Perspect.* **112**, 840–846 (2004).
23. Zhang, Q. *et al.* Changes in immune parameters seen in Gulf War veterans but not in civilians with chronic fatigue syndrome. *Clin. Diagn. Lab. Immunol.* **6**, 6–13 (1999).
24. Bordet, R. *et al.* PPAR: a new pharmacological target for neuroprotection in stroke and neurodegenerative diseases. *Biochem Soc Trans* **34**, 1341–1346 (2006).
25. Jung, T. W. *et al.* Rosiglitazone protects human neuroblastoma SH-SY5Y cells against MPP+ induced cytotoxicity via inhibition of mitochondrial dysfunction and ROS production. *J. Neurol. Sci.* **253**, 53–60 (2007).
26. Gray, E., Ginty, M., Kemp, K., Scolding, N. & Wilkins, A. Peroxisome proliferator-activated receptor- α agonists protect cortical neurons from inflammatory mediators and improve peroxisomal function. *Eur. J. Neurosci.* **33**, 1421–1432 (2011).
27. Forman, B. M., Chen, J. & Evans, R. M. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors and. *Proc. Natl. Acad. Sci.* **94**, 4312–4317 (1997).
28. Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M. & Kliewer, S. A. Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **272**, 3406–10 (1997).
29. Cunard, R. *et al.* WY14,643, a PPAR alpha ligand, has profound effects on immune responses *in vivo*. *J. Immunol.* **169**, 6806–6812 (2002).
30. Jones, D. C., Ding, X. & Daynes, R. a. Nuclear receptor peroxisome proliferator-activated receptor alpha (PPARalpha) is expressed in resting murine lymphocytes. The PPARalpha in T and B lymphocytes is both transactivation and transrepression competent. *J. Biol. Chem.* **277**, 6838–45 (2002).
31. Dasgupta, S., Roy, A., Jana, M., Hartley, D. M. & Pahan, K. Gemfibrozil ameliorates relapsing-remitting experimental autoimmune encephalomyelitis independent of peroxisome proliferator-activated receptor-alpha. *Mol. Pharmacol.* **72**, 934–946 (2007).
32. Gocke, A. R. *et al.* Transcriptional modulation of the immune response by peroxisome proliferator-activated receptor-{alpha} agonists in autoimmune disease. *J. Immunol.* **182**, 4479–87 (2009).
33. Zakirova, Z. *et al.* Gulf War agent exposure causes impairment of long-term memory formation and neuropathological changes in a mouse model of Gulf War Illness. *PLoS One* **10** (2015).
34. Duan, F. F., Guo, Y., Li, J. W. & Yuan, K. Antifatigue Effect of Luteolin-6-C-Neohesperidoside on Oxidative Stress Injury Induced by Forced Swimming of Rats through Modulation of Nrf2/ARE Signaling Pathways. *Oxid. Med. Cell. Longev.* **2017** (2017).
35. Xu, M., Liang, R., Li, Y. & Wang, J. Anti-fatigue effects of dietary nucleotides in mice. *Food Nutr. Res.* **61**, 1334485 (2017).
36. Gadea, A. & López-Colomé, A. M. Glial transporters for glutamate, glycine, and GABA: II. GABA transporters. *Journal of Neuroscience Research* **63**, 461–468 (2001).
37. Kacem, K., Lacombe, P., Seylaz, J. & Bonvento, G. Structural organization of the perivascular astrocyte endfeet and their relationship with the endothelial glucose transporter: A confocal microscopy study. *Glia* **23**, 1–10 (1998).
38. Gehrmann, J., Matsumoto, Y. & Kreutzberg, G. W. Microglia: Intrinsic immunoregulator cell of the brain. *Brain Research Reviews* **20**, 269–287 (1995).
39. Kreutzberg, G. W. Microglia: A sensor for pathological events in the CNS. *Trends in Neurosciences* **19**, 312–318 (1996).
40. Jimenez, F. *et al.* CCR2 plays a critical role in dendritic cell maturation: possible role of CCL2 and NF-kappa B. *J. Immunol.* **184**, 5571–81 (2010).
41. Chen, K. *et al.* Signal relay by CC chemokine receptor 2 (CCR2) and formylpeptide receptor 2 (Fpr2) in the recruitment of monocyte-derived dendritic cells in allergic airway inflammation. *J. Biol. Chem.* **288**, 16262–16273 (2013).
42. Macanas-Pirard, P. *et al.* The CCL2/CCR2 axis affects transmigration and proliferation but not resistance to chemotherapy of acute myeloid leukemia cells. *PLoS One* **12** (2017).
43. Maule, A. L. *et al.* Meta-analysis of self-reported health symptoms in 1990–1991 Gulf War and Gulf War-era veterans. *BMJ Open* **8**, e016086 (2018).
44. Sullivan, K. *et al.* Cognitive functioning in treatment-seeking Gulf War veterans: Pyridostigmine bromide use and PTSD. *J. Psychopathol. Behav. Assess.* **25**, 95–103 (2003).

45. Anger, W. K. *et al.* Neurobehavioral deficits in Persian Gulf veterans: Evidence from a population-based study. *J. Int. Neuropsychol. Soc.* **5**, 203–212 (1999).
46. Sillanpaa, M. C. *et al.* Gulf War veterans: A neuropsychological examination. *J. Clin. Exp. Neuropsychol.* **19**, 211–219 (1997).
47. Binder, L. M. *et al.* Subjective cognitive complaints, affective distress, and objective cognitive performance in Persian Gulf War veterans. *Arch. Clin. Neuropsychol.* **14**, 531–6 (1999).
48. Bunegin, L., Mitzel, H. C., Miller, C. S., Gelineau, J. F. & Tolstyk, G. P. Cognitive performance and cerebrohemodynamics associated with the Persian Gulf Syndrome. *Toxicol. Ind. Health* **17**, 128–137 (2001).
49. Lange, G., Van Nierkerk, A. & Meyer, B. J. Detection of an artifact on lumbar SPECT. *Clin. Nucl. Med.* **26**, 446–448 (2001).
50. Storzbach, D. *et al.* Psychological differences between veterans with and without Gulf War unexplained symptoms. *Psychosom. Med.* **62**, 726–735 (2000).
51. Menon, S. *et al.* A longitudinal study of anticardiolipin antibody levels and cognitive functioning in systemic lupus erythematosus. *Arthritis Rheum.* **42**, 735–741 (1999).
52. Vythilingam, M. *et al.* Smaller head of the hippocampus in Gulf War-related posttraumatic stress disorder. *Psychiatry Res. - Neuroimaging* **139**, 89–99 (2005).
53. Weiner, M. W. Magnetic Resonance and Spectroscopy of the Human Brain in Gulf War Illness. 1–11 (2005).
54. Koo, B. B. *et al.* Corticosterone potentiates DFP-induced neuroinflammation and affects high-order diffusion imaging in a rat model of Gulf War Illness. *Brain. Behav. Immun.* **67**, 42–46 (2018).
55. Terry, A. V. Functional Consequences of Repeated Organophosphate Exposure: Potential Non-Cholinergic Mechanisms. *Pharmacol. Ther.* **v**, 265–275 (2012).
56. Campolongo, P. *et al.* Fat-induced satiety factor oleoylethanolamide enhances memory consolidation. *Proc. Natl. Acad. Sci. USA* **106**, 8027–8031 (2009).
57. Yang, L. C. *et al.* Chronic oleoylethanolamide treatment improves spatial cognitive deficits through enhancing hippocampal neurogenesis after transient focal cerebral ischemia. *Biochem. Pharmacol.* **94**, 257–269 (2015).
58. Plaza-Zabala, A. *et al.* Effects of the endogenous PPAR- α agonist, oleoylethanolamide on MDMA-induced cognitive deficits in mice. *Synapse* **64**, 379–389 (2010).
59. Bannerman, D. M. *et al.* Regional dissociations within the hippocampus - Memory and anxiety. *Neuroscience and Biobehavioral Reviews* **28**, 273–283 (2004).
60. Da Cruz e Alves-de-Moraes, L. B., Ribeiro-Paes, J. T., Longo, B. M., Ferrazoli, E. G. & De Andrade, T. G. C. S. Effect of the bone marrow cell transplantation on elevated plus-maze performance in hippocampal-injured mice. *Behav. Brain Res.* **248**, 32–40 (2013).
61. Rayhan, R. U. *et al.* Exercise Challenge in Gulf War Illness Reveals Two Subgroups with Altered Brain Structure and Function. *PLoS One* **8** (2013).
62. Abou-Donia, M. B. *et al.* Screening for novel central nervous system biomarkers in veterans with Gulf War Illness. *Neurotoxicol. Teratol.* **61**, 36–46 (2017).
63. McKimmie, C. S. & Graham, G. J. Astrocytes modulate the chemokine network in a pathogen-specific manner. *Biochem. Biophys. Res. Commun.* **394**, 1006–1011 (2010).
64. Ramesh, G., Maclean, A. G. & Philipp, M. T. Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. *Mediators Inflamm.* **2013** (2013).
65. Conductier, G., Blondeau, N., Guyon, A., Nahon, J. L. & Rovère, C. The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. *J. Neuroimmunol.* **224**, 93–100 (2010).
66. Selenica, M. L. B. *et al.* Diverse activation of microglia by chemokine (C-C motif) ligand 2 overexpression in brain. *J. Neuroinflammation* **10** (2013).
67. Parillaud, V. R. *et al.* Analysis of monocyte infiltration in MPTP mice reveals that microglial CX3CR1 protects against neurotoxic over-induction of monocyte-attracting CCL2 by astrocytes. *J. Neuroinflammation* **14** (2017).
68. Parkitny, L., Middleton, S., Baker, K. & Younger, J. Evidence for abnormal cytokine expression in Gulf War Illness: A preliminary analysis of daily immune monitoring data. *BMC Immunol.* **16**, 57 (2015).
69. Craddock, T. J. A. *et al.* Achieving remission in Gulf War Illness: A simulation-based approach to treatment design. *PLoS One* **10** (2015).
70. Sagar, D. R. *et al.* Dynamic regulation of the endocannabinoid system: Implications for analgesia. *Molecular Pain* **5** (2009).
71. Watt, M. J., Southgate, R. J., Holmes, A. G. & Febbraio, M. A. Suppression of plasma free fatty acids upregulates peroxisome proliferator-activated receptor (PPAR) α and δ and PPAR coactivator 1 α in human skeletal muscle, but not lipid regulatory genes. *J. Mol. Endocrinol.* **33**, 533–44 (2004).
72. Edvardsson, U. *et al.* PPAR α activation increases triglyceride mass and adipose differentiation-related protein in hepatocytes. *J. Lipid Res.* **47**, 329–40 (2006).
73. Garcia-Roves, P. *et al.* Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. *Proc. Natl. Acad. Sci.* **104**, 10709–10713 (2007).
74. Basaure, P. *et al.* Postnatal chlorpyrifos exposure and apolipoprotein E (APOE) genotype differentially affect cholinergic expression and developmental parameters in transgenic mice. *Food Chem. Toxicol.* **118**, 42–52 (2018).
75. Peris-Sampedro, F. *et al.* Apolipoprotein E (APOE) genotype and the pesticide chlorpyrifos modulate attention, motivation and impulsivity in female mice in the 5-choice serial reaction time task. *Food Chem. Toxicol.* **92**, 224–235 (2016).
76. Torres-Platas, S. G. *et al.* Morphometric characterization of microglial phenotypes in human cerebral cortex. *J. Neuroinflammation* **11** (2014).
77. Arlicot, N. *et al.* Evaluation of CLINDE as potent translocator protein (18 kDa) SPECT radiotracer reflecting the degree of neuroinflammation in a rat model of microglial activation. *Eur. J. Nucl. Med. Mol. Imaging* **35**, 2203–2211 (2008).
78. Winkler, A. *et al.* The translocator protein ligand [18F]DPA-714 images glioma and activated microglia *in vivo*. *Eur. J. Nucl. Med. Mol. Imaging* **39**, 811–823 (2012).
79. Arlicot, N. *et al.* Detection and quantification of remote microglial activation in rodent models of focal ischaemia using the TSPO radioligand CLINDE. *Eur. J. Nucl. Med. Mol. Imaging* **37**, 2371–2380 (2010).
80. Folch, J., Lees, M. & Sloane Stanley, G. H. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497–509 (1957).
81. Flygt, J. *et al.* Neutralization of Interleukin-1 β following Diffuse Traumatic Brain Injury in the Mouse attenuates the loss of Mature Oligodendrocytes. *J. Neurotrauma* **1–40** <https://doi.org/10.1089/neu.2018.5660> (2018).
82. Boldrini, M. *et al.* Human Hippocampal Neurogenesis Persists throughout Aging. *Cell Stem Cell* **22**, 589–599.e5 (2018).
83. Sorrells, S. F. *et al.* Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* **555**, 377–381 (2018).
84. Can, A. *et al.* The mouse forced swim test. *J. Vis. Exp.* e3638 <https://doi.org/10.3791/3638> (2012).
85. Zhang, Z. *et al.* NLRP3 inflammasome activation mediates fatigue-like behaviors in mice via neuroinflammation. *Neuroscience* **358**, 115–123 (2017).

Acknowledgements

This work is supported by two CDMRP awards (GW1300045 and GW150056) to Dr. Laila Abdullah, a VA Merit award (1I01CX000469) and a CDMRP award (GW080094) to Dr. Fiona Crawford, and by the Roskamp Foundation. Plasma samples from GW veterans were made possible through a CDMRP GWI consortium award (GW120037) to Dr. Kimberly Sullivan, and a VA merit and a CDMRP award to Dr. Nancy Klimas. We would like to thank Anastasia Edsell and Maxwell Eisenbaum for their assistance with reviewing & editing the manuscript. Disclaimer: The contents do not represent the views of the Department of Veterans Affairs or the United States Government.

Author Contributions

Conceptualization: Laila Abdullah, James Evans, Fiona Crawford, Utsav Joshi. Data Curation: Utsav Joshi., Tanja Emmerich, Ross Joseph, Sarah Oberlin, Nicole Saltiel, Joseph Ojo, James Evans. Funding acquisition, Laila Abdullah, James Evans, Michael Mullan, Fiona Crawford. Methodology, Utsav Joshi, Ross Joseph, Nicole Saltiel, Heather Langlois, Sarah Oberlin, Benoit Mouzon, James Evans, Carlyn Lungmus, Laila Abdullah. Project administration and Supervision: Laila Abdullah, Fiona Crawford. Resources, Utsav Joshi, Ross Joseph, Nicole Saltiel, Sarah Oberlin, Heather Langlois, James Evans, Carlyn Lungmus, Laila Abdullah, Daniel Paris, Chao. Writing – original draft: Utsav Joshi and Laila Abdullah. Writing – review & editing: Utsav Joshi, Nicole Saltiel and Laila Abdullah. Collaborator- Nancy Klimas and Kimberly Sullivan.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-018-31242-7>.

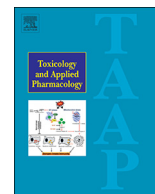
Competing Interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2018



Increased butyrate priming in the gut stalls microbiome associated-gastrointestinal inflammation and hepatic metabolic reprogramming in a mouse model of Gulf War Illness

Ratanesh Kumar Seth^{a,1}, Diana Kimono^{a,1}, Firas Alhasson^a, Sutapa Sarkar^a, Muayad Albadrani^a, Stephen K. Lasley^b, Ronnie Horner^c, Patricia Janulewicz^d, Mitzi Nagarkatti^e, Prakash Nagarkatti^e, Kimberly Sullivan^d, Saurabh Chatterjee^{a,*}

^a Environmental Health and Disease Laboratory, Department of Environmental Health Sciences, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA

^b Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine, Peoria, IL, USA

^c Department of Health Services Policy and Management, Arnold School of Public Health, University of South Carolina, Columbia, SC, USA

^d Department of Environmental Health, Boston University School of Public Health, Boston University, Boston, MA, USA

^e Department of Pathology Microbiology and Immunology, USC School of Medicine, University of South Carolina, Columbia, SC, USA

ARTICLE INFO

Keywords:

Permethrin
Pyridostigmine bromide
Gut dysbiosis
TLR4
Claudin-2
Cytokines

ABSTRACT

Most of the associated pathologies in Gulf War Illness (GWI) have been ascribed to chemical and pharmaceutical exposures during the war. Since an increased number of veterans complain of gastrointestinal (GI), neuroinflammatory and metabolic complications as they age and there are limited options for a cure, the present study was focused to assess the role of butyrate, a short chain fatty acid for attenuating GWI-associated GI and metabolic complications. Results in a GWI-mouse model of permethrin and pyridostigmine bromide (PB) exposure showed that oral butyrate restored gut homeostasis and increased GPR109A receptor copies in the small intestine (SI). Claudin-2, a protein shown to be upregulated in conditions of leaky gut was significantly decreased following butyrate administration. Butyrate decreased TLR4 and TLR5 expressions in the liver concomitant to a decrease in TLR4 activation. GW-chemical exposure showed no clinical signs of liver disease but a significant alteration of metabolic markers such as SREBP1c, PPAR- α , and PFK was evident. Liver markers for lipogenesis and carbohydrate metabolism that were significantly upregulated following GW chemical exposure were attenuated by butyrate priming in vivo and in human primary hepatocytes. Further, Glucose transporter Glut-4 that was shown to be elevated following liver complications were significantly decreased in these mice after butyrate administration. Finally, use of TLR4 KO mice completely attenuated the liver metabolic changes suggesting the central role of these receptors in the GWI pathology. In conclusion, we report a butyrate specific mechanistic approach to identify and treat increased metabolic abnormalities in GWI veterans with systemic inflammation, chronic fatigue, GI disturbances, metabolic complications and weight gain.

1. Introduction

Gulf War Illness (GWI) has been characterized as a chronic multi-symptom illness with pathology that includes neuronal inflammation leading to cognitive deficiencies, chronic fatigue, joint and muscle pain and gastrointestinal complications (White et al., 2016; Kerr, 2015; Gulf War and Health, 2016). GWI research has identified toxicant chemical exposures in the war theater including sarin nerve gas, pyridostigmine bromide (PB) anti-nerve gas pills, insecticides and insect repellents, to

be prime reasons for most symptoms reported by the veterans (White et al., 2016). A study of military pesticide applicators from the GW recently reported increased cognitive decrements in attention, memory and information processing speed in veterans with combined exposures to PB, pesticides and insect repellents (Sullivan et al., 2018). Animal models of GWI have also shown chronic impairment in learning and memory, fatigue and gastrointestinal dysfunction when exposed to GW-relevant chemicals including PB, pesticides and the insect repellent permethrin (Abdullah et al., 2016; Parihar et al., 2013). GWI has

* Corresponding author.

E-mail address: Schatt@mailbox.sc.edu (S. Chatterjee).

¹ Ratanesh Kumar Seth and Diana Kimono contributed equally.

emerged as a primarily neuroimmune disorder with greater inflammatory effects noted when GW-relevant toxicants were combined with corticosterone in animal models to mimic the physical and mental stressors of war deployment (Koo et al., 2018). However the basis of gastrointestinal complications and parallel networks for neuronal complications have remained largely elusive (Georgopoulos et al., 2017; O'Callaghan et al., 2015; O'Callaghan et al., 2016). Though the larger epidemiological studies list GI complications as a major health problem in only a subset of GW veterans, there are hardly any reports that show hepatic metabolic disturbances as a widely reported symptom (White et al., 2016; Kerr, 2015; Haley et al., 2013). This may be due to the silent nature of the presentation of these symptoms and unknown links to a wider systemic complication in GW veterans following sedentary lifestyles/physical disabilities/diet/aging over a longer period of time (Coughlin et al., 2011). However, with increased incidences of obesity and persistence of symptoms in GW-veterans and the US population in general, the causes, outcomes, and extent of metabolic disturbances can no longer be ignored. The long-ranging implications of such silent changes in the GI tract and the liver following chemical exposure form the basis of the present study.

We have shown recently the unambiguous role of the gut microbiome in causing neuronal inflammation largely due to gut leaching and systemic endotoxemia (Alhasson et al., 2017). The altered gut bacterial signature obtained following Gulf War chemical exposure caused a TLR4-linked inflammatory surge in the GI tract and could be traced in the frontal cortex (Alhasson et al., 2017). The observations reported paved the way for newer investigations into wider systemic inflammatory complications in extraneuronal organs that might not have a clear phenotype yet may be a basis for multisymptom illness as described in GWI.

GWI is also characterized by the presence of chronic fatigue. Most classifications in the past have listed chronic fatigue as one of the most widely reported symptom burdens in GW veterans (Kerr, 2015; Gulf War and Health, 2016; Coker, 1996). Interestingly, changes in the microbiome of affected patients with chronic fatigue syndrome/fibromyalgia have been strongly associated with the causes of this illness (Nagy-Szakal et al., 2017; Morris et al., 2016; Giloteaux et al., 2016). Chronic fatigue is also strongly linked to widescale changes both in the gut bacteria and the systemic metabolism with the latter believed to have roots in the liver though skeletal muscles also play a major role (Naviaux et al., 2016). Persistent alteration of liver metabolism following changes in gut microbiome and its subsequent effects on systemic metabolism may affect the development of chronic fatigue via the altered availability of NADPH, ATP, and cofactors for various biochemical pathways (Naviaux et al., 2016).

Most chronic liver diseases like fatty liver disease and biliary fibrosis are silent in a presentation at the clinic and remain asymptomatic until it reaches an irreversible stage (Milic & Stimac, 2012). Nonalcoholic fatty liver disease or cholestatic liver disease have chronic fatigue as one of the symptoms (Schreuder et al., 2008; Zakharia et al., 2018). The fatigue associated with these silent liver complications have been assigned to lipotoxicity, insulin and leptin resistance, endocrinopathies and metabolic syndrome (Kaltsas et al., 2010). Interestingly, we have shown that environmental chemicals alter liver metabolism by increasing glucose transporters in the liver fibroblasts, elevating expressions of PPAR- α , PFK and decreasing PPAR- γ levels with a concomitant rise in leptin (Seth et al., 2013a). These changes in the liver have been associated with an altered microbiome following consumption of a diet rich in high fat and low fiber for a long period of time at least in the murine models (Chiu et al., 2017; Marra & Svegliati-Baroni, 2018).

With strong emphasis on the altered microbiome being associated with pathologies of inflammatory bowel disease, chronic liver disease, chronic fatigue syndrome and neuronal complications (all have an inflammatory component) via the gut-brain axis, it is important that studies be focused on bacterial metabolites within the gut that might be linked to some or all the pathways that link these systemic

complications. Interestingly, GWI patients present significant symptoms that resemble some or all these in the clinics. Thus bacterial metabolites like butyric acid, propionic acid and acetate need to be considered as molecules of interest in treating multiple symptoms of GWI since these molecules have shown promising results in the clinic to cure IBD and dysbiosis related complications (Sun et al., 2017; Zhang et al., 2016; Imhann et al., 2018). Further, Butyrate-producing bacteria such as Roseburia species supplementation rescued patients from IBD (Imhann et al., 2018). Butyrate, in particular, is a short chain fatty acid (SCFA) that primarily interacts with the GPR109A and is an immunosuppressant widely known to increase T-regulatory cells in the intestine and a prominent HDAC1 inhibitor (Sekhavat et al., 2007; Singh et al., 2014).

The present study tests the hypothesis that GW chemical exposure causes a decrease in butyrate-producing bacteria and concomitant butyrate priming in the gut through oral supplementation attenuates GI inflammation, gut leaching and metabolic abnormalities in the liver and higher systemic leptin levels. The study uses state of the art genomic approaches and an oral priming by butyrate for elucidating genus-specific changes in gut bacteria, and human hepatocytes treated with an insect repellent permethrin, (used in Gulf War theater) for mechanistic investigations.

2. Materials and methods

2.1. Materials

Pyridostigmine bromide (PB), Permethrin (Per), Lipopolysaccharides (LPS), Corticosterone and Sodium butyrate (NaBT) were purchased from Sigma- Aldrich (St. Louis, MO). Anti- claudin-2, anti-occludin, anti-TLR4, anti-flotillin, anti-HMGB1, anti-Leptin and anti-IL1 β primary antibodies were purchased from Abcam (Cambridge, MA). Anti-TLR5 primary antibody was purchased from Santacruz Biotechnology (Dallas, TX). Species-specific biotinylated conjugated secondary antibodies and Streptavidin-HRP (Vectastain Elite ABC kit) were purchased from Vector Laboratories (Burlingame, CA). Fluorescence-conjugated (Alexa Fluor) secondary antibodies, ProLong Gold antifade mounting media with DAPI were purchased from Thermofisher Scientific (Grand Island, NY) and all other chemicals which were used in this project purchased from sigma only if otherwise specified. Paraffin-embedding of tissue sections on slides were done by AML laboratories (Baltimore, MD). Microbiome analysis was done by Second Genome, the microbiome company (San Francisco, CA).

2.2. Animals

Adult wild-type male (C57BL/6J mice) and adult mice that contained the disrupted TLR4 gene (TLR4 KO) (B6-B10ScN-Tlr4^{lps-del}/JthJ) were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were implemented in accordance with NIH guideline for human care and use of laboratory animals and local IACUC standards. All procedures were approved by The University of South Carolina at Columbia, SC. Mice were housed individually and fed with chow diet at 22–24 °C with a 12-h light/ 12-h dark cycle. All mice were sacrificed after animal experiments had been completed. Right after anesthesia, blood from the mice was drawn using cardiac puncture, in order to preserve serum for the experiments. The mice liver was collected for further experiments immediately after terminal euthanasia. Fecal pellets and luminal contents were collected from the animals, followed by dissection of the small intestine. The tissues were fixed using 10% neutral buffered formalin. Distal segments of small intestines were used for the staining and visualizations.

2.3. Rodent model of Gulf War Illness (GWI)

Mice were exposed to Gulf War chemicals based on established

rodent models of Gulf War Illness with some modifications (O'Callaghan et al., 2015; Zakirova et al., 2015). The treated wild-type mice group (GWI) and treated TLR4 KO mice group (TLR4 KO) were dosed tri-weekly for one week with PB (2 mg/kg) and Permethrin (200 mg/kg) via the oral route. After completion of PB/Permethrin dosages, mice were administered corticosterone intraperitoneally (i.p.) with a dose of 100 µg/mice/day for 5 days of the week for one week. The dose of corticosterone was selected from the study which exposed mice to 200 mg/L of corticosterone through drinking water. The i.p. dose of corticosterone had similar immunosuppression as examined by low splenic T cell proliferation (data not shown). The vehicle control group (Veh) of mice received saline injections and vehicle for oral gavage in the same paradigm. Another group of wild-type mice was exposed with PB, Permethrin and corticosterone similar to GWI group of mice and co-exposed with sodium butyrate (GWI + NaBT) 10 mg/kg via the oral route.

2.4. Microbiome analysis

Fecal pellets and luminal contents were collected from the animals of each group after sacrifice and then sent to Second Genome and School of Medicine, the University of South Carolina for microbiome analysis. The second Genome performed nucleic acid isolation with the MoBio PowerMag Microbiome kit (Carlsbad, CA) according to manufacturer's guidelines and optimized for high-throughput processing V3-V4 sequencing and bioinformatic analysis.

2.5. Cell culture

Freshly isolated primary human hepatocytes were obtained from Liver Tissue Cell Distribution System, University of Minnesota, Minneapolis, MN. Plated hepatocytes were maintained in DMEM media supplemented with 10% FBS until treated. Cells were then serum starved in DMEM supplemented with 1.5% FBS for 8 h and exposed to vehicle control and chemicals. Cells were then treated with vehicle (Veh Cont), LPS (1 µM), LPS + NaBT (LPS 1 µM and Sodium Butyrate 0.2 mg/mL) for 24 h. After experiment cells were harvested for mRNA extraction and gene expression analysis.

2.6. Laboratory methods

2.6.1. Immunohistochemistry

The distal part of small intestine was collected from mice and fixed in 10% neutral buffered formalin. The fixed tissues were rolled, paraffin embedded and cut in 5 µm thick section. These sections were subjected to deparaffinization using a standard protocol. Epitope retrieval solution and steamer (IHC-Word, Woodstock, MD) were used for epitope retrieval for deparaffinized sections. 3% H₂O₂ was used for the recommended time to block the endogenous peroxidase. After serum blocking, the tissue was incubated overnight at 4.0 °C with primary antibody IL1β. Species-specific biotinylated conjugated secondary antibodies and streptavidin conjugated with HRP were used to implement antigen-specific immunohistochemistry. 3,3'-Diaminobenzidine (DAB) (Sigma Aldrich, St Louis, MD) was used as a chromogenic substrate. Mayer's Hematoxylin solution (Sigma Aldrich) was used as a counter-stain. Sections were washed between the steps using phosphate buffered saline 1 ×. Finally, stained sections were mounted with Simpo-mount (GBI laboratories, Mukilteo, WA). Tissue sections were observed using Olympus BX51 microscope (Olympus, America). Cellsens software from Olympus America (Center Valley, PA) was used for morphometric analysis of images.

2.6.2. Immunofluorescence staining

Paraffin-embedded distal part of the small intestine or liver sections were deparaffinized using a standard protocol. Epitope retrieval solution and steamer were used for epitope retrieval of sections. Primary

antibodies such as anti-Claudin-2, anti-Occludin, anti-GPR109A, anti-TLR4, anti-Flotillin, and anti-TLR5 were used at the recommended dilution. Species-specific secondary antibodies conjugated with Alexa Fluor (633-red and 488-green) were used at advised dilution. In the end, the stained sections were mounted using Prolong gold antifade reagent with DAPI. Sections were observed under-Olympus fluorescence microscope using 20 ×, 40 × or 60 × objective lenses.

2.6.3. Real-time quantitative PCR

mRNA expression in small intestine, liver, and human primary hepatocytes was examined by quantitative real-time PCR analysis. Total RNA was isolated from each 25 mg liver tissue or 15 mg small intestine tissue or 1 × 10⁶ primary human hepatocytes cell by homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and purified with the use of RNeasy mini kit columns (Qiagen, Valencia, CA). cDNA was synthesized from purified RNA (1 µg) using iScript cDNA synthesis kit (Bio-rad, Hercules, CA) following the manufacturer's standard protocol. Real-time qPCR (qRTPCR) was performed with the gene-specific primers using SsoAdvanced SYBR Green Supermix and CFX96 thermal cycler (Bio-rad, Hercules, CA). Threshold Cycle (Ct) values for the selected genes were normalized against respective samples internal control (18S). Each reaction was carried out in triplicates for each gene and for each sample. The relative fold change was calculated by the 2^{-ΔΔCt} method. The sequences for the primers used for Real-time PCR are provided in Table 1.

2.6.4. Elisa

Serum Leptin and serum HMGB1 was estimated using ELISA kits from Abclonal Biotechnologies (Woburn, MA) following manufacturer protocol. Serum IL1β was estimated using an ELISA kit from ProteinTech (Rosemont, IL) following manufacturer protocol.

2.6.5. Serum biochemistry tests

Biochemical analysis of mouse serum was done for ALT, urea Nitrogen, creatinine, cholesterol, triglycerides and glucose from the University of Georgia college of veterinary medicine.

2.7. Statistical analysis

Prior to initiation of the study, we conducted calculations for each

Table 1
Real-time PCR primer sequences.

Gene ^a	Primer sequence (5' to 3' orientation)
MM_IL-1β	Sense: CCTCGGCCAAGACAGGTGCGC Antisense: TGCCCATCAGAGGCAAGGAGGA
MM_MCP-1	Sense: CACAGTTGCCGGCTGGAGCAT Antisense: GTAGCAGCAGGTGAGTGGGGC
MM_TNF-α	Sense: CAACGCCCTCCTGGCCAACG Antisense: TCGGGGACGCCTTGCCCTT
MM_SREBP1c	Sense: GGAACAGACACTGGCCGA Antisense: AAGTCACTGTCTTGGTTGTTGAT
MM_PPAR-α	Sense: AGACCTTCGGCAGCTGGTCAC Antisense: GTGGCAACGGCCTGCCATCT
MM_PPAR-γ	Sense: TTCGCTGATGCACTGCCTAT Antisense: GGAATGCGAGTGGTCTTCCA
MM GLUT-1	Sense: CCTGTCTCTTCTACCCAACC Antisense: GCAGGAGTGTCCGTGTCTTC
MM GLUT-4	Sense: CACCGGCAGCCTCTTATCAT Antisense: CACCGAGACCAACGTGAAGA
MM_PFK	Sense: GCGGTGAAACTCCGAGGAA Antisense: GTTGCTCTTGACAATCTCTCATCAG
Hs_SREBP1c	Sense: CATGGATTGCACCTTCGAA Antisense: GGCGAGGGAAGTCACTGTCTT
Hs_PPAR-γ	Sense: GGCTTCATGACAAGGGAGTTTC Antisense: AACTCAAACCTGGGCTCCATAAAG

^a MM: Mouse specific primers, Hs: Human specific primers.

experimental condition with appropriate preliminary data to confirm that the sample number is sufficient to achieve a minimum statistical power of 0.80 at an alpha of 0.05. All in vivo and in vitro experiments were repeated three times with 3 mice per group ($N = 3$; data from each group of three mice were pooled). Student's *t*-test was used to compare means between two groups at the termination of treatment. A one-way ANOVA was applied as needed, to evaluate differences among treatment groups followed by the Bonferroni post-hoc correction for intergroup comparisons.

3. Results

3.1. Butyrate production is key to gut health in GW-chemical exposure and microbial dysbiosis

We have shown previously that GW chemical exposure caused a significant alteration in microbial population when compared to untreated controls with significant increases in Firmicutes-Bacteroidetes ratio, a trend that is uniformly observed in IBD, neuroinflammation and metabolic syndrome. The changes were consistent with the neuroinflammatory phenotype in the mouse model of GWI. On in-depth analysis of the microbial data, we found that GW-chemical exposed group showed a marked decrease in *Lactobacillus*, and *Bifidobacterium* sp., the genus being responsible for producing the short chain fatty acid butyrate. Interestingly butyrate has been shown to attenuate IBD and resists proinflammatory changes in the small intestine (Fig. 1A) ($p < 0.05$). The two genus showed a > 5 fold (log scale) decrease in abundance (Fig. 1A). Butyrate priming through oral gavage and its presence during exposure significantly elevated the levels of *Bifidobacterium*, butyrogenic bacteria *Roseburia* sp. and *Lactobacillus* (i, ii and iii) (Fig. 1B) ($p < 0.05$) when compared to GWI alone with the first two genera showing an increase up to $> 60\%$ when compared to GWI. The percentages noted in the figure are compared to the overall abundance of all genus detected in the metagenomic analysis. The comparisons between GWI and GWI + NaBT groups were done using GWI as the base line. Such a comparison showed a $> 60\%$ increase of these genus in GWI + NaBT group when compared to GWI alone (Fig. 1B). The observations in Fig. 1A led to the rationale for using Butyrate as a viable molecule for attenuating microbiome-associated inflammatory phenotype and the subsequent changes observed in the GWI model. Butyrate exerts its actions via binding to the niacin receptor. GPR109A has been recently discovered to bind butyrate and stimulate the activation of Treg cells thus suppressing TH17 mediated proinflammatory events (Singh et al., 2014). Our results showed that there is a significant decrease in the protein levels of GPR109A in GW-chemical exposed group when compared to untreated controls (Fig. 2A and D) ($p < 0.05$). Butyrate presence in the intestine via feeding GW-exposed mice through an oral gavage significantly increased GPR109A protein levels in the villi regions when compared to GWI-group (Fig. 2A and D) ($p < 0.05$) suggesting that butyrate presence resisted the decrease in GPR109A protein levels thus helping butyrate to exert its actions in the dysbiosis-affected small intestine and restore gut-epithelial cell integrity and metabolic homeostasis.

3.2. Butyrate priming through oral route restores tight junction protein levels

The epithelial tight junction determines the paracellular water and ion movement in the intestine and also prevents uptake of larger molecules, including antigens, in an uncontrolled manner where Claudin-2 and Occludin play a major role and are perceived as a marker for leaky gut (Luettig et al., 2015). Our results from immunofluorescence microscopy for the immunoreactivity of Claudin-2 showed a significant increase in GW-chemical exposed group when compared to untreated controls (Fig. 2B and E) ($p < 0.05$), thus confirming our previously reported data. Butyrate presence in GW-chemical exposed group showed a significant decrease in that group when compared to GW-

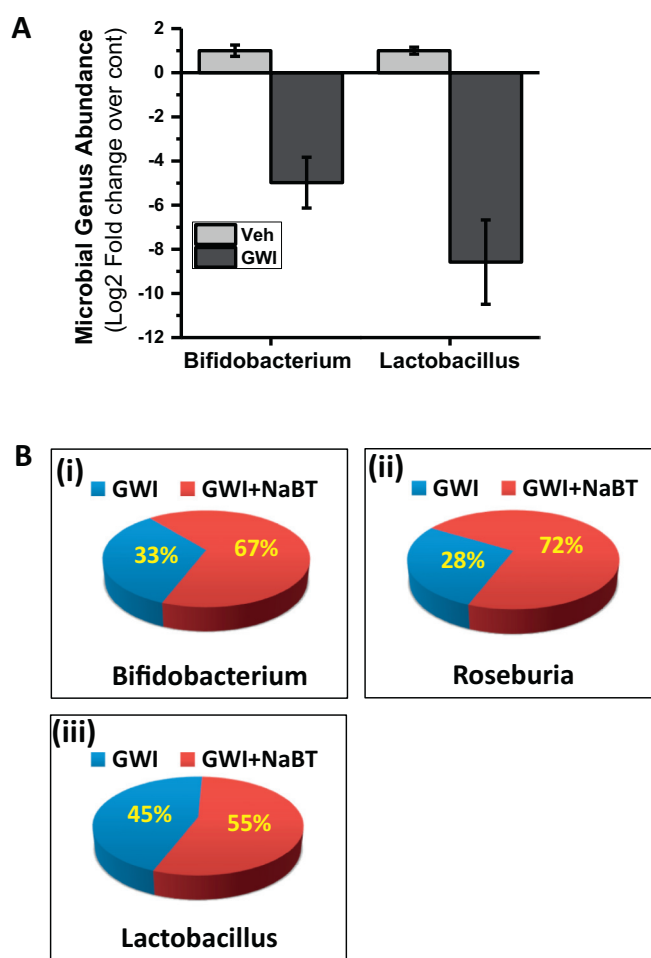


Fig. 1. Gut microbiome alteration in mice model of Gulf War Illness (GWI). **A.** The proportional abundance of microbial genera: Graphical representation of the most abundant taxa of bacteria at the genus level. Groups compared are gulf war illness group (wild-type mice exposed to gulf war chemicals) (GWI, $n = 3$) and control group fed with vehicle (Veh, $n = 3$) (p -value: < 0.05). **B.** Percentage abundance of gut bacteria *Bifidobacterium* (i), *Roseburia* (ii), and *Lactobacillus* (iii) in a group of mice co-exposed with Gulf war chemicals and Sodium butyrate (GWI + NaBT, $n = 3$) as compared with GWI mice ($n = 3$) (p -value: < 0.05).

chemical exposed group alone suggesting a parallel role of butyrate in Claudin-2 protein levels in the small intestine (Fig. 2B and E) ($p < 0.05$). The results also showed that butyrate priming nearly restored the Claudin-2 levels to untreated controls (Fig. 2B). Similarly, the protein level of another tight junction protein Occludin was significantly decreased in GW-chemical exposed groups (Fig. 2C and F) and sodium butyrate treatment significantly restored the levels of Occludin in the intestine. The results suggested that Butyrate may have a previously unconfirmed role in modulating Claudin-2 and Occludin proteins in the small intestine though IL10A-dependent repression of Claudin-2 has been shown (Zheng et al., 2017).

3.3. Butyrate priming in the intestine attenuates proinflammatory phenotype in the intestine via a decrease in TLR4 activation

Since gut leaching was predominant in GWI mouse model and resulted in endotoxemia, we studied whether butyrate priming helped in attenuating the proinflammatory microenvironment in the small intestine (Alhasson et al., 2017). Results showed that butyrate administration through an oral route decreased TLR4 colocalization (as shown by white circles), a hallmark of its activation in GW-chemical exposed

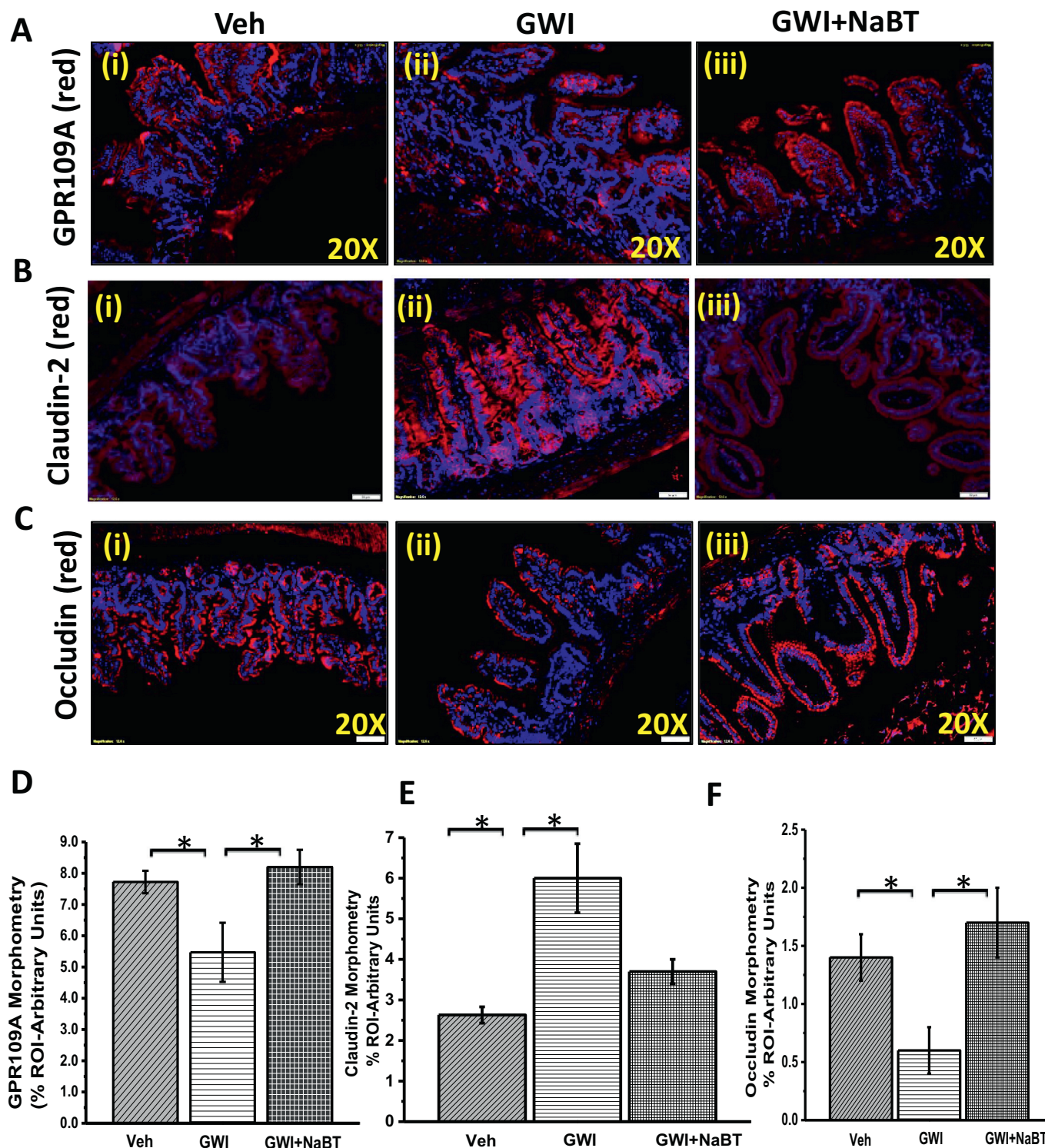


Fig. 2. Change in gut microbiome in GWI alter niacin receptor (GPR109A) and tight junction proteins in the intestine. **A.** The expression pattern of butyrate and niacin receptor GPR109A was assessed by immunofluorescence microscopy. The representative images showed immunoreactivity of GPR109A in the distal part of the small intestine of veh control group of mice (veh, $n = 3$), gulf war illness group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$). **B and C.** The expression pattern of Claudin-2 and Occludin (tight junction proteins) was assessed by immunofluorescence microscopy. Tissue levels of Claudin-2 (B) and Occludin (C) in Vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$) was assessed by immunofluorescent microscopy after labeling the protein with the red fluorescent secondary antibody and counterstained with DAPI (blue). **D–F.** The bar diagram shows the quantitative morphometric analysis of fluorescence intensities of GPR109A (D), Claudin-2 (E), and Occludin (F) immunoreactivity in the region of interest (ROI) in the small intestine. (* $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

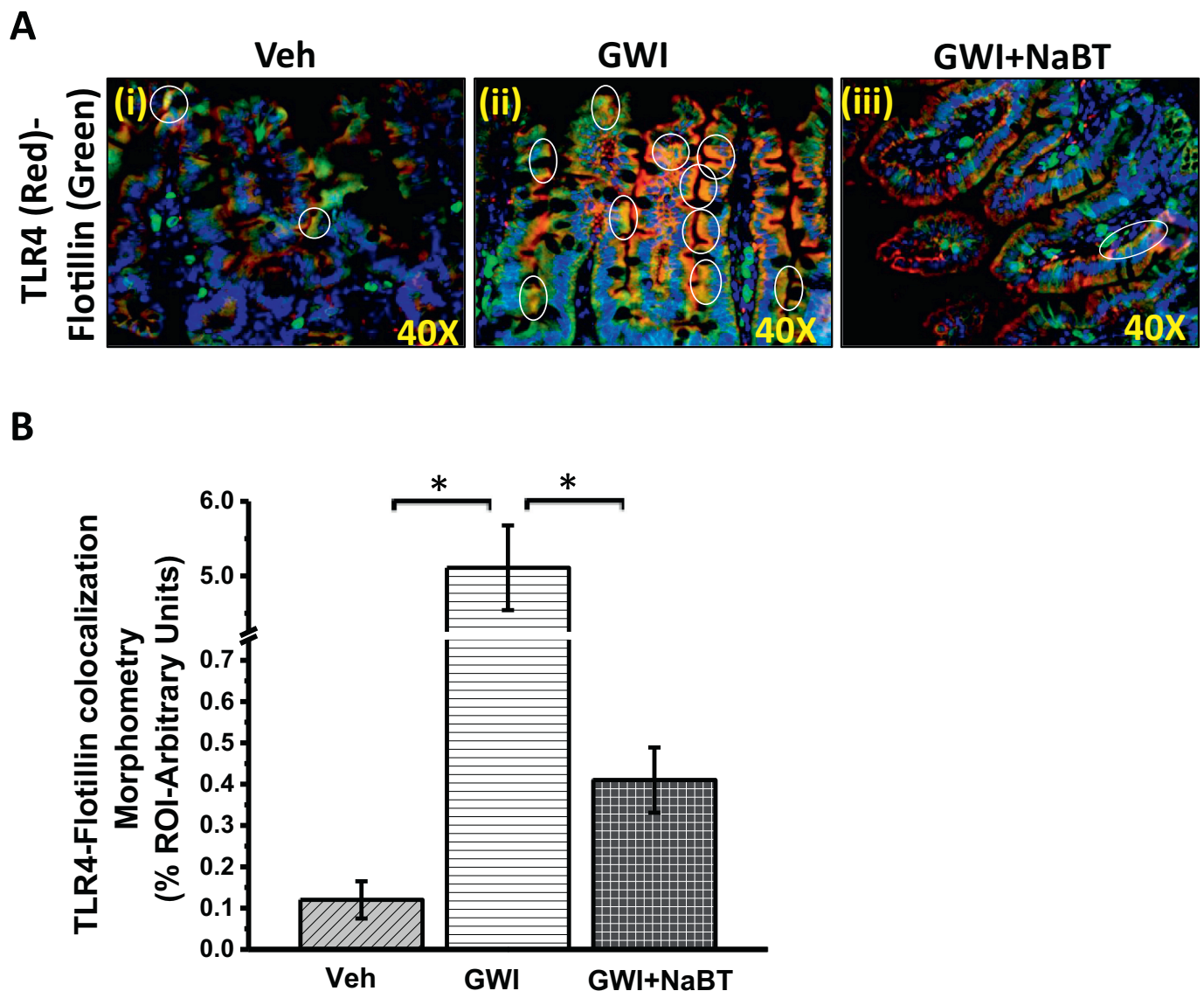


Fig. 3. Sodium butyrate priming in a rodent model of GWI attenuates TLR4 activation in the small intestine. **A.** Immunofluorescence microscopy of small intestine showing TLR4 (red) trafficking to the lipid rafts (green) of the small intestine tissue, an essential process for TLR4 activation causing a co-localization of TLR4 in flotillin-rich rafts (yellow). Representative images of TLR4-flotillin co-localization in the small intestine of vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$) shown by white circles covering the yellow spots created by an overlay of red (TLR4) and green (Flotillin). Images were taken at higher magnification ($40\times$ oil). **B.** Graphical representation of the quantitative morphometric analysis of colocalization events in the region of interest (ROI) in the small intestine. Images for analysis were randomly chosen in different microscopic fields. Data is represented as Mean $\pm$ SEM and $^*(p < 0.05)$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

group when compared to GW-group alone (Fig. 3A and B) ($p < 0.05$). Notably, the results also confirmed our earlier observations of an increased TLR4 trafficking to lipid rafts in GW chemical exposed group when compared to untreated controls (Fig. 3A and B) ($p < 0.05$). TLR4 activation was followed by increased IL-1 β protein levels in the villi regions but not in crypts of GW chemical exposed group when compared to untreated controls (Fig. 4A and B) ($p < 0.05$). Also, butyrate priming significantly decreased the IL-1 β levels in the same regions when compared to GW-Chemical exposed group (Fig. 4A and B). Gene expressions of IL-1 β , monocyte chemoattractant MCP-1 and TNF- α were significantly decreased in Butyrate administered group when compared to GW-chemical exposed group (Fig. 4C) ($p < 0.05$). Interestingly, serum IL-1 β significantly increased in GW- chemical exposed groups (66.71 ± 1.98 pg/mL) as compared to vehicle control group (39.95 ± 1.8 pg/mL) (Fig. 4D). However, Butyrate exposure to the

GW-Chemical exposed mice showed a significant decrease in the serum IL-1 β (34.56 ± 1.26 pg/mL) (Fig. 4D). The results suggested that butyrate presence helped attenuate intestinal inflammation primarily from a TLR4 pathway however it could not rule out other parallel inflammatory pathways in the gut such as histone deacetylases.

Similar to pathogen-associated molecular patterns (PAMPs), that can trigger a proinflammatory response, sterile inflammation can be triggered by endogenous molecules from a necrotic or damaged cell that can activate several proinflammatory pathways including TLR4 (Garcia-Martinez et al., 2015). Such endogenous molecules are collectively called Damage-associated molecular patterns or DAMPs. We have shown previously that HMGB1 and leptin can be released from several organ systems and can trigger a proinflammatory cascade (Chatterjee et al., 2013; Chandrashekar et al., 2016; Chandrashekar et al., 2017). We quantified the released HMGB1 and leptin in mouse serum

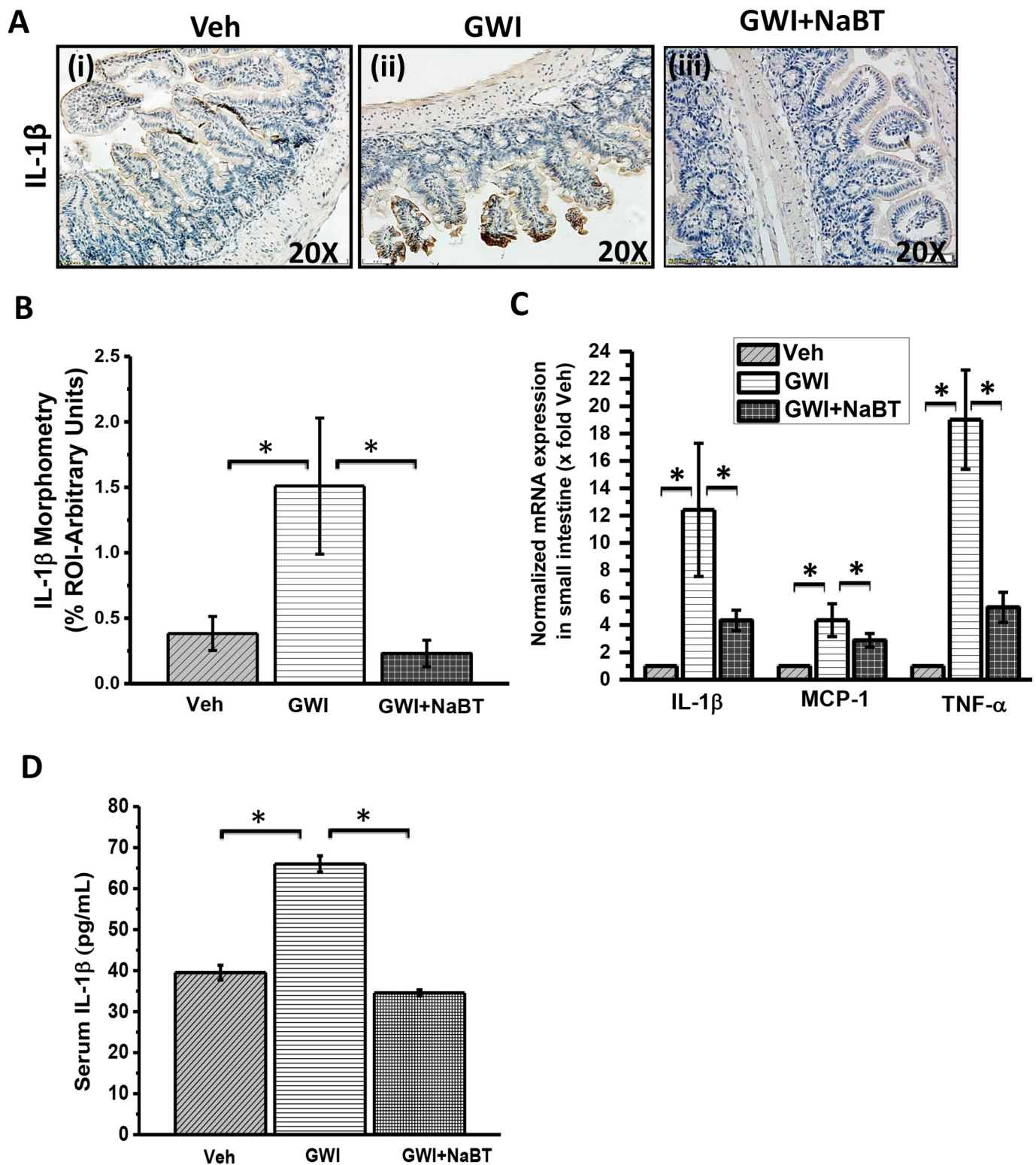
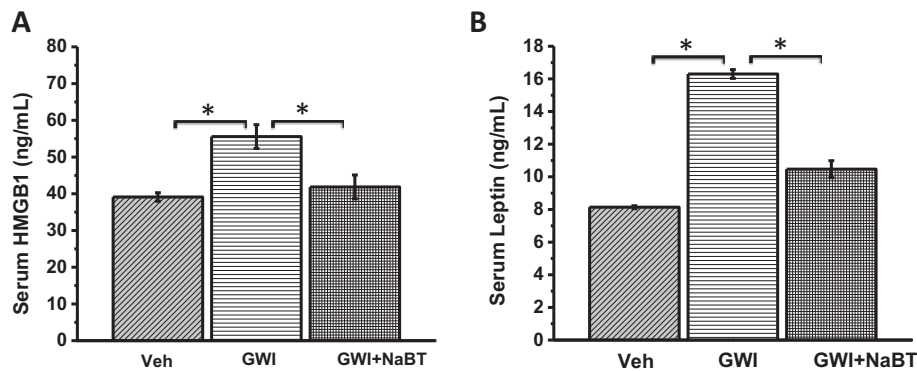


Fig. 4. Sodium butyrate priming in a rodent model of GWI improves proinflammatory phenotype in small intestine mediated by the TLR4 pathway. **A.** Small intestine tissue slices were probed for IL-1 β immunoreactivity in vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$) using immunohistochemistry. Specific immunoreactivity to IL-1 β is evident by dark brown spots. **B.** Graphical representation of morphometric analysis of the IL-1 β immunoreactivity in tissue slices. Data normalized against vehicle control (veh) $^*(p < 0.05)$. **C.** Quantitative real-time PCR (qRT-PCR) analysis of inflammatory markers in the small intestine. mRNA expression of IL-1 β , MCP-1, and TNF- α was analyzed in the samples of vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$). Normalized mRNA expression is represented as a fold change of vehicle control (veh) on Y-axis. Data points represented with Mean $\pm$ SEM $^*(p < 0.05)$. **D.** Graphical representation of serum IL-1 β in pg/mL of the samples of vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



and leptin western blot bands. The data was normalized to a total serum protein (Ponceau S). Y-axis depicts the HMGB1/Ponceau S ratio (B) and leptin/Ponceau S ratio (C) from Veh, GWI and GWI + NaBT groups. * $p < 0.05$ is considered statistically significant. D-E. Graphical representation of serum HMGB1 (D) and serum leptin (E) in ng/mL of the samples of vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$).

using competitive ELISA techniques. Result showed that GW-chemical exposed groups had significantly higher levels of HMGB1 and leptin in the serum when compared to untreated controls (Fig. 5A and B) ($p < 0.05$). Butyrate priming significantly decreased the level of HMGB1 while a decrease in leptin levels was not significant when compared to GW-chemical exposed group (Fig. 5A and B) ($p < 0.05$). The results suggested that circulatory DAMPs can be soluble mediators of ectopic inflammatory events distant to the small intestine while butyrate priming may attenuate such effects and help identify therapeutic targets in the systemic inflammatory phenotype seen in GWI.

3.4. Increased TLR activation in the liver is attenuated by butyrate priming in the intestine

TLR activation is observed in organ systems following gut dysbiosis (Reichardt et al., 2017; Henao-Mejia et al., 2012). TLRs especially TLR4, TLR2, and TLR5 have been shown to increase tissue inflammation (Henao-Mejia et al., 2012). Interestingly, TLR-induced metabolic deregulation is increasingly seen as an important event in metabolic syndrome (Boutagy et al., 2016). Our results showed that GW chemical exposed group showed a significantly increased TLR4 activation (trafficking to lipid rafts) in the liver especially in the sinusoidal cells (white circles) (Fig. 6A and B). TLR4 trafficking significantly decreased in butyrate administered group when compared to GW-chemical exposed group (Fig. 6A and B). Interestingly, butyrate administration markedly increased TLR4 protein (red) levels in the liver but could not be observed in the rafts of the membrane (yellow), a sign that TLR4 protein was increased but the activation was attenuated by butyrate administration (Fig. 6A). TLR5 a protein that is activated the following binding with flagellin also increased in the liver of GW exposed mice but was significantly decreased in the butyrate administered group (Fig. 6C and D). The results suggested that increased circulatory levels of DAMPs or a leaky gut-associated flagellin might have resulted in activation of TLR4 and TLR5 in the liver of GW chemical exposed group but was blocked by the presence of butyrate.

3.5. TLR4 activation is associated with metabolic changes and inflammatory response in the liver but the phenotypic liver injury is predominantly absent

The liver is the principal organ for gluconeogenesis, lipogenesis and cholesterol metabolism (Bechmann et al., 2012). Recent studies have put a great deal of emphasis on liver metabolic reprogramming in conditions of metabolic syndrome that include hepatic expression of lipid and glucose metabolism markers, hepatic insulin and leptin resistance (Seth et al., 2013a; Chen et al., 2012). Interestingly, the fatty

liver disease is associated with a long-term metabolic alteration (often years to manifest) and inflammatory response in the liver till the disease phenotype surfaces and is rightly called a silent disease (Milic & Stimac, 2012; Doycheva et al., 2017). We studied both the changes in hepatic metabolic markers and the inflammatory response arising from a TLR4 activation following GW-chemical exposure and microbial dysbiosis to ensure whether we can detect early responses in the liver that can manifest into liver disease years later. Results showed that hepatic SREBP1c, a molecule predominantly responsible for lipogenesis was upregulated following GW-chemical exposure when compared to untreated controls (Fig. 7A) ($p < 0.05$) (Geidl-Flueck & Gerber, 2017). Butyrate administration significantly decreased SREBP1c gene expression in the hepatic lobule when compared to GW-chemical exposed group (Fig. 7A) ($p < 0.05$). PPAR- α is a transcription factor and a major regulator of lipid metabolism in the liver (Badman et al., 2007). PPAR- α is activated under conditions of energy deprivation and is necessary for the process of ketogenesis (Badman et al., 2007). PPAR- α was significantly upregulated in the GW-chemical exposed group when compared to untreated controls while butyrate administration significantly decreased and restored the PPAR- α levels when compared to GW-chemical exposed group (Fig. 7A) ($p < 0.05$). PPAR- γ is an important player in liver fat metabolism and is known to be increased in benign steatosis but is significantly down-regulated in models of liver injury (Seth et al., 2013b). Our results showed that PPAR- γ was significantly decreased in GW chemical exposed group when compared to untreated controls and butyrate priming reversed this downregulation when compared to GW-chemical exposed group (Fig. 7A) ($p < 0.05$). The results were in agreement with our previous studies in a liver metabolic disease that had similar decreases in PPAR- γ (Seth et al., 2013b).

We have shown previously that liver metabolic disorders triggered by environmental contaminants can increase the expression of Phosphofructokinase (PFK) (Seth et al., 2013b). PFK, a rate-limiting enzyme in the glycolytic pathway was significantly upregulated in GW chemical exposed group when compared to untreated controls while butyrate administration significantly restored the PFK levels (Fig. 7A) ($p < 0.05$). Hepatic class I glucose transporters (GLUT) have limited role in the liver but recent studies show their importance in hepatic disease states (Karim et al., 2012). We and others have shown that GLUT-1 and GLUT-4 are regulated by leptin and purinergic signaling and they are upregulated in fatty liver disease primarily in hepatic stellate cells (Chandrasekaran et al., 2016; Tang & Chen, 2010). Our results show that both GLUT-1 and GLUT-4 were upregulated in the GW chemical exposed groups when compared to untreated controls however butyrate administration significantly decreased the GLUT-1 and GLUT-4 levels when compared to GW-chemical exposed groups

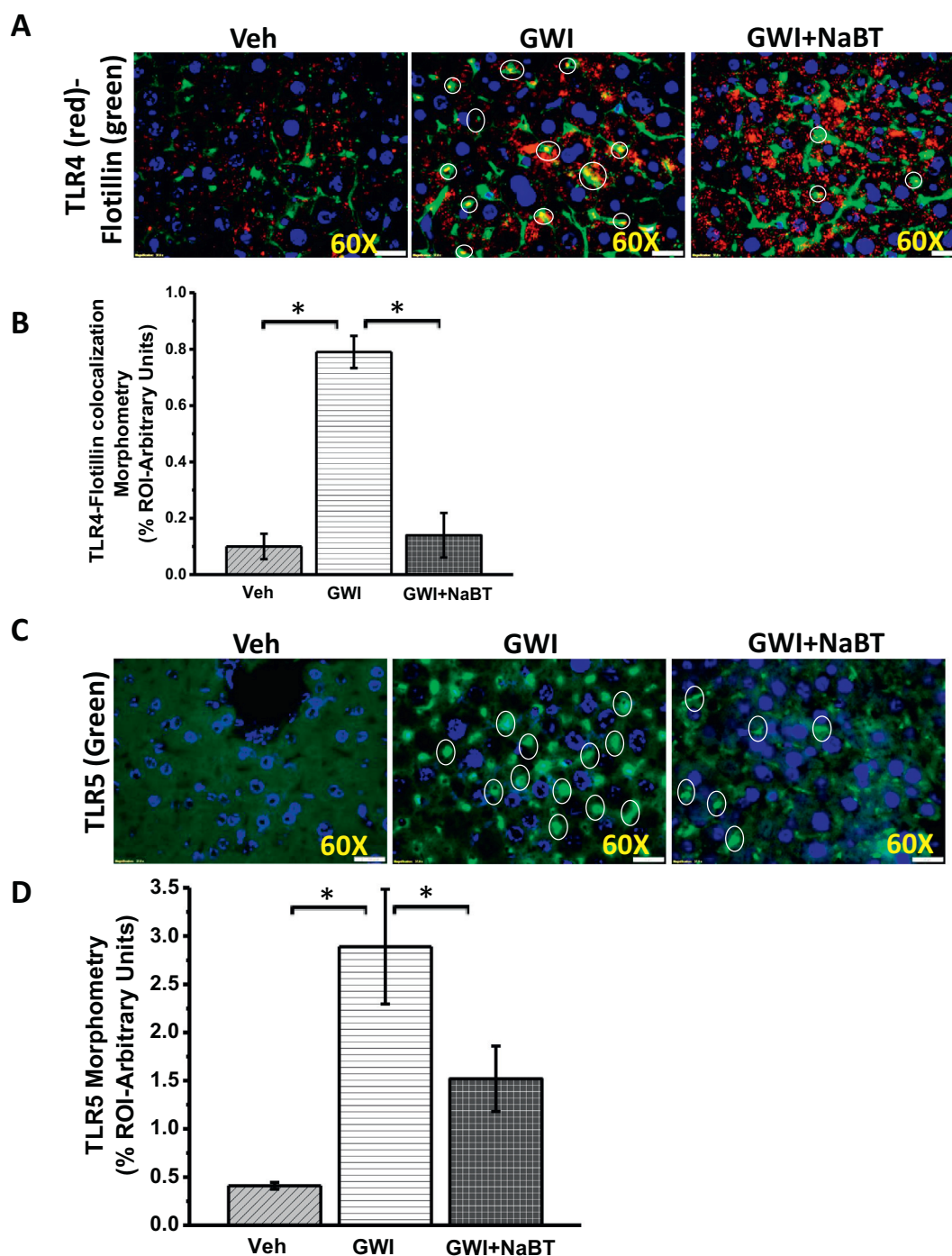


Fig. 6. Sodium butyrate treatment in a rodent model of GWI attenuates TLR4 activation in Liver. **A.** Immunofluorescence microscopy of liver slices showing TLR4 (red) trafficking to the lipid rafts (green), an essential process for TLR4 activation causing a co-localization of TLR4 in flotillin-rich rafts (yellow). Representative images of TLR4-flotillin co-localization in the liver of vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$) shown by white circles covering the yellow spots created by an overlay of red (TLR4) and green (Flotillin). Images were taken at higher magnification ($60\times$ oil). **B.** Graphical representation of the quantitative morphometric analysis of colocalization events in the liver. Images for morphometric analysis were randomly chosen in different microscopic fields. Data is represented as Mean $\pm$ SEM $^*(p < 0.05)$. **C.** Tissue levels of TLR5 immunoreactivity in vehicle control group of mice (Veh, $n = 3$), gulf war chemical treated group of mice (GWI, $n = 3$) and a group of mice co-exposed with GWI and sodium butyrate (GWI + NaBT, $n = 3$) mouse liver samples as observed by immunofluorescent microscopy after labeling the TLR5 protein with the green fluorescent secondary antibody and counterstained by DAPI (blue). **D.** The bar diagram shows the quantitative morphometric analysis of fluorescence intensities of TLR5 immunoreactivity in the liver tissue. Images for morphometric analysis of TLR5 were randomly chosen in different microscopic fields. Data is represented as Mean $\pm$ SEM $^*(p < 0.05)$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

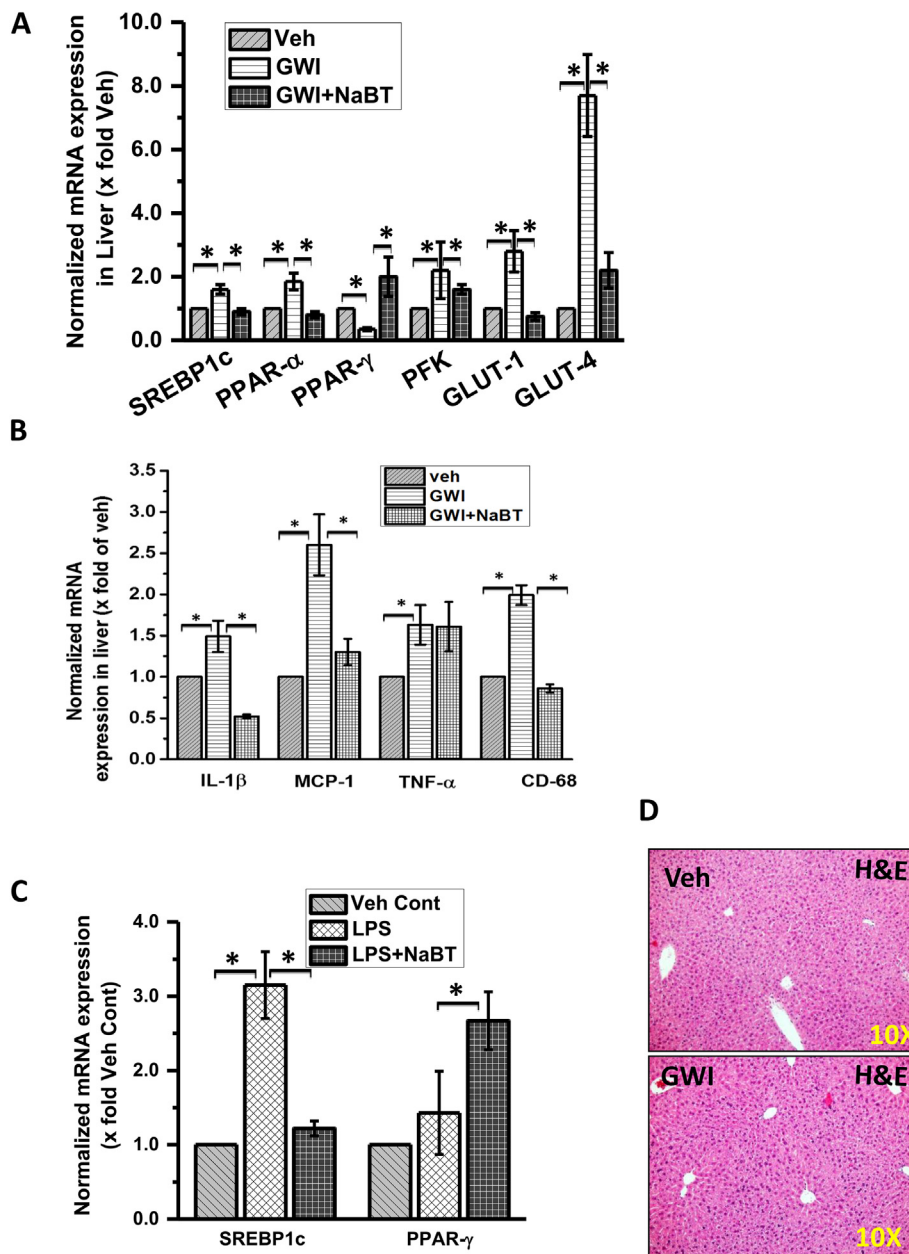


Fig. 7. TLR4 activation is associated with metabolic changes and inflammatory response in the liver but the phenotypic liver injury is predominantly absent. **A.** Quantitative real-time PCR (qRT-PCR) analysis principle carbohydrate metabolic markers (PFK, GLUT-1, and GLUT-4) and fat metabolic markers (SREBP1c, PPAR-α, and PPAR-γ) in the liver tissue. mRNA expression of SREBP1c, PPAR-α, PPAR-γ and PFK, GLUT-1, GLUT-4 and **B.** mRNA expression analysis of inflammatory marker IL-1β, MCP-1, TNF-α, and Kupffer cell activation marker CD68 were analyzed in the liver sample of vehicle control group of mice (Veh, n = 3), gulf war chemical treated group of mice (GW, n = 3) and a group of mice co-exposed with GW and sodium butyrate (GW + NaBT, n = 3). Normalized mRNA expression is represented as a fold change of Vehicle control (veh) on Y-axis. Data points represented with Mean ± SEM *(p < 0.05). **C.** mRNA expression of SREBP1c, PPAR-γ were analyzed in the primary human hepatocytes cells treated with lipopolysaccharide (LPS) and Co-treated with LPS and sodium butyrate (LPS + NaBT). Normalized mRNA expression is represented as a fold change of Vehicle control (Veh Cont) on Y-axis. Data points represented with Mean ± SEM *(p < 0.05). **D.** Representative Hematoxylin and Eosin stained (H&E) images of liver sections showed liver pathophysiology of vehicle control group of mice (Veh, n = 3), gulf war chemical treated a group of mice (GW, n = 3). Images were taken at 10× magnification.

(Fig. 7A) (p < 0.05). Further, to investigate the role of GW-Chemical exposure in exacerbating the inflammatory response in liver, hepatic mRNA expression profiles of interleukin (IL)-1β, monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor (TNF)-α and Kupffer cell activation marker CD68 were analyzed. Results indicated that there was a significant increase in the mRNA expression profiles of IL-1β, MCP-1, TNF-α and CD68 in GW Chemical exposed mice livers compared with vehicle treated mice livers (Fig. 7B) (p < 0.05). Interestingly, mice groups co-exposed with GW chemicals and sodium butyrate showed significantly decreased level of IL-1β, MCP-1, and CD68 but not TNF-α. The results suggested a similar role of higher leptin and/or heightened inflammation in causing the increase but remained to be seen whether it was cell or organ specific.

The liver has multiple cell types and includes cells of epithelial, endothelial, fibroblast and macrophage lineages. They perform multiple functions including metabolic, cellular defense and wound healing. The liver lobule comprises of 90% hepatocytes which are epithelial in origin and is a center for most of the metabolic functions. We used human primary hepatocytes, primed with lipopolysaccharide (LPS)

(concentrations found in our previous study (Alhasson et al., 2017)) to study the effects of metabolic dysregulation if any due to GW-chemical exposure. Results showed that LPS primed hepatocytes showed a significant increase in lipogenesis mediator SREBP1c while butyrate co-exposure decreased these levels (Fig. 7C) (p < 0.05). Similar to our in vivo data, LPS primed hepatocytes showed a significant increase in PPAR-γ gene expression when co-exposed to butyrate while LPS only or untreated controls showed no change in the PPAR-γ levels (Fig. 7C) (p < 0.05).

Hematoxylin and Eosin stains of liver tissue sections obtained from GW-chemical exposed group showed no signs of lipid accumulation or macrophage infiltration or Mallory body formation signifying the absence of advanced stage inflammatory foci or liver disease (Fig. 7D). Though histopathology of the liver section from each mice group clearly showed that there was no sign of liver damage or development of Nonalcoholic steatohepatitis, we resorted to clinical chemistry analysis for more detailed outcomes. To confirm such observations we performed clinical chemistry analysis of mouse serum samples for ALT, BUN, creatinine, total cholesterol, triglyceride and serum glucose. The

Table 2
Serum chemistry analysis.

Serum chemistry	Vehicle (n = 3)	GW1 (n = 3)	GW1 + NaBT (n = 3)
ALT (U/L)	6	10	6
BUN (mg/dl)	32	28	24
Creatinine (mg/dl)	ND	ND	ND
Cholesterol (mg/dl)	78	82	98
Triglycerides (mg/dl)	84	102	84

ND = Creatinine result is less than the linearity of the assay 0.2 mg/dL.

clinical chemistry data showed (Table 2) that there was no significant difference in serum ALT (showed a marked increase in GW1 group but was not significant between groups), BUN, creatinine and total cholesterol upon GW Chemical exposure as compared to vehicle control group. However, co-exposure with sodium butyrate decreased the levels of ALT, BUN but not the total cholesterol (Table 2). The triglyceride levels were significantly increased in GW1 chemical exposed mice groups as compared to untreated control groups, while co-exposure with sodium butyrate caused the triglyceride level to be decreased significantly when compared to GW1 group ($p < 0.05$) suggesting a slow but incremental risk of fatty liver in the future (Table 2). The glucose levels showed no significant difference between groups (data not shown).

3.6. TLR4 drives the metabolic alterations in GW-chemical exposed liver

TLR4 induced downstream proinflammatory signaling has been found to aid in insulin resistance (Razolli et al., 2015). Prolonged insulin resistance has been shown to cause metabolic disturbances in the liver, skeletal muscle and adipose tissue (Boutagy et al., 2016). Since microbiome associated gut leaching and systemic endotoxemia were reported in the mouse model of GW1 and the present study found metabolic changes in the liver, we studied the direct role of TLR4 in causing the metabolic changes. The results showed that TLR4 knockout (TLR4 KO) mice had decreased TLR5 expression in the liver when compared to GW-chemical exposed group (Fig. 8A and B) ($p < 0.05$). TLR4 KO mice had significantly decreased expression of Class I glucose transporter GLUT-4, PFK, PPAR- α , and lipogenesis mediator SREBP1c when compared to GW-chemical exposed group while GLUT-1 showed no change in the expression suggesting GLUT-1 might not be regulated by TLR4 (Fig. 8C) ($p < 0.05$). The results suggested that TLR4 activation following systemic endotoxemia might be responsible for the ectopic metabolic alterations in the liver but is unable to present any significant changes in liver disease phenotype. The results also are in agreement with epidemiological studies where veterans deployed in GW don't report liver abnormalities in the clinics based on the typical symptoms.

4. Discussion

Epidemiological studies have shown a strong correlation between GW toxicant exposures and cognitive/neurological complications but there are also reports of chronic fatigue, gastrointestinal disturbances and occasional cases of metabolic syndrome (White et al., 2016). Our study shows that microbial dysbiosis owing to GW-chemical exposure causes a significant decrease in healthy gut bacteria like *Bifidobacterium* and *Lactobacillus* (LeBlanc et al., 2017). Interestingly, they are a class of bacteria that generate butyrate in the gut (LeBlanc et al., 2017). Recent studies have shown a beneficial effect of butyrate in preclinical studies involving colitis and IBD (Sun et al., 2017). The above results prompted us to use sodium butyrate administration through an oral route as a priming agent throughout the chemical exposure time so that a restored butyrate in the gut could prevent and prime the gut against the dysbiosis, inflammatory leaching, and

generation of systemic mediators in the small intestine. Results also showed the role of butyrate in increasing the levels of the butyrogenic bacteria, increasing the expression of butyrate receptor GPR109A, decreasing Claudin-2 and decreasing TLR4 activation. We have shown recently that GW chemical exposure causes gut dysbiosis, the disintegration of gut membrane causing leaching and systemic endotoxemia (Alhasson et al., 2017) that eventually led to TLR4 activation. We also showed a causal role of dysbiosis to the neuroinflammation in frontal cortex thus raising a possibility of the existence of a “Gut-Brain-Axis” in GW1 similar to other pathological conditions (Alhasson et al., 2017). This axis may act in parallel to some of the direct toxic effects of GW chemical exposures on the brain tissue (O'Callaghan et al., 2015; Zakirova et al., 2015). Sodium Butyrate priming, as shown in our data might reverse the pathology associated with GW chemical exposure since it restored gut health, reversed gut barrier integrity and decreased SI inflammation (decreased IL-1 β) while increasing the possibility of increased butyrate binding to GPR109A due to higher availability of this receptor in SI. Notably, butyrate priming also decreased the release of HMGB1 and leptin though slightly in circulation albeit from the intestinal epithelial cells but other sources like liver cannot be ruled out. The source might be the damaged epithelial cells in the small intestine since the potential generation of free radical species has been shown before and oxidative stress in the intestinal epithelial cells and macrophages could release HMGB1 (Tang et al., 2012; Sappington et al., 2002). On the other hand, HMGB1 release due to gut integrity changes also causes oxidative stress and cell necrosis as have been reported in other studies (Sappington et al., 2002). Though leptin is primarily released from adipocytes and liver, chemical/food-induced leptin release have been shown in the gut and has been traced in duodenal juice (Sobhani et al., 2000; Guilmeau et al., 2003). Thus our finding of increased leptin in circulation following GW chemical exposure might be a result of the leaky gut or liver though the exact source remains to be determined at this time. The release of both leptin and HMGB1 and its modulation by butyrate priming in the gut points to the intestine as a source of these inflammatory mediators along with endotoxin and has tremendous implication determining ectopic/endocrine pathology of GW1.

In spite of well-coordinated symptom reporting in GW1 about chronic fatigue in most of the studies, the causes of such chronic fatigue have been limited to abnormalities in neurological pathways or mitochondrial dysfunction without an organ-specific definition (Koslik et al., 2014). Presence of symptoms related to metabolic syndrome or liver diseases is rare (Kelsall et al., 2009). Interestingly, fatigue is also associated with metabolic syndrome and various liver diseases (Carey et al., 2015; Sweet et al., 2017; Jensen et al., 2017). Though we hardly see literature reporting liver abnormalities in GW1, asymptomatic metabolic abnormalities in the liver (as evident in silent liver diseases like NAFLD), can contribute to chronic fatigue. These facts mentioned above led us to examine the liver pathology likely affected by higher circulatory mediators like endotoxin, leptin, and HMGB1. Owing to the tremendous role of the liver in carbohydrate metabolism, we focused on the role of circulatory HMGB1 and leptin on (a) hepatic TLR4 activation and (b) alterations in both lipid and carbohydrate metabolism. Our results showed a significant increase in TLR4 trafficking to the lipid rafts, a hallmark of activation of the TLR4 pathway in the liver following exposure to GW-chemicals. Also, there was a subsequent increase in TLR5 levels in the liver with both TLR4 activation and TLR5 levels showing decreases after butyrate priming. These results assume huge significance since TLR4 activation has been found to cause insulin resistance, uptake of free fatty acids for triglyceride production in macrophages and sterol biosynthesis (Boutagy et al., 2016; Feingold et al., 2012). On the other hand, stearic acid has been shown to promote TLR4 mediated inflammation (Song et al., 2006; Anderson et al., 2012). Our results of increased expression of genes such as SREBP1c and PPAR- α which play a major role in liver lipogenesis (cholesterol biosynthesis and import) might be the result of the increased TLR4 activation and

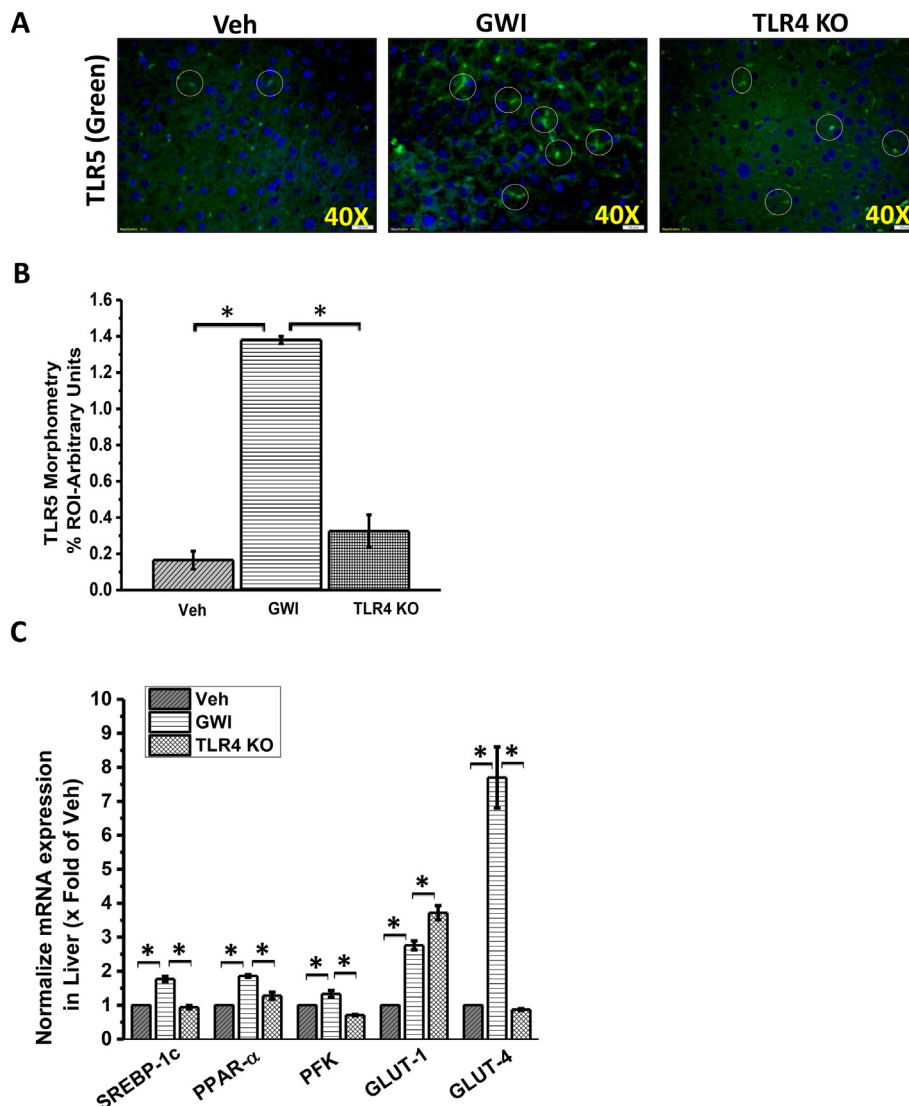


Fig. 8. TLR4 drives the metabolic alterations in GW-chemical exposed liver. **A.** Tissue levels of TLR5 in Gulf War chemical treated a group of wild-type mice (GWI, $n = 3$) and a group of TLR4 knockout mice (TLR4 KO, $n = 3$). Mouse liver samples as observed by immunofluorescence microscopy after labeling the TLR5 protein with green fluorescent secondary antibody and nuclear counterstaining by DAPI (blue). Images were taken at $60\times$ (oil) magnification. **B.** The bar diagram showed the quantitative morphometric analysis of fluorescence intensities of TLR5 immunoreactivity in the liver tissue. $^{*}(p < 0.05)$. **C.** mRNA expression analysis of principle carbohydrate metabolic markers (PFK, GLUT-1, and GLUT-4) and fat metabolic markers (SREBP1c, PPAR- α) in the liver tissue of vehicle, GWI and TLR4 KO mice. Normalized mRNA expression is represented as a fold change of vehicle control (veh) on Y-axis. Data points represented with Mean $\pm$ SEM $^{*}(p < 0.05)$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the subsequent cascade of events that alter liver metabolism following GW-chemical exposure. Interestingly, both SREBP1c and LXRs control lipid metabolism and it remains to be seen whether an increase in SREBP1c in the GW-chemical exposed liver was an adaptive way to suppress a chronic TLR4 activation thus mounting an anti-inflammatory response as is seen in some studies (Iizuka & Horikawa, 2008; Oishi et al., 2017). There are numerous reports which find increased glucose metabolism following NF- κ B activity which is downstream of TLR4 activation (Mauro et al., 2011). Our results of increased expression of phosphofructokinase, a rate-limiting enzyme for glycolysis show that a TLR4 mediated mechanism might play a role in driving a glycolytic pathway in the liver. Notably, isolated hepatocytes when stimulated with TLR4 ligand LPS or GW-chemical Permethrin did not show an increase in PFK but exhibited a 3-fold increase in SREBP1c over vehicle control suggesting that hepatocytes along with macrophages may be targets of TLR4 activation thus playing a vital role in the reprogramming of lipid metabolism. Further, Class I glucose transporters GLUT-1 and GLUT-4 was elevated in the GW-chemical exposed liver. The result assumes significance since inflammation in the liver has been shown to increase glucose uptake in hepatic stellate cells in a mouse model of fatty liver disease (Chandrasekaran et al., 2016). Our results of decreased inflammation and subsequent metabolic disturbances in the liver following butyrate priming may shed some light on the inhibitory role of butyrate on histone deacetylase activity (Khan & Jena, 2014).

Butyrate is a known HDAC inhibitor (Khan & Jena, 2015). Studies show that butyrate can act as an HDAC inhibitor and decrease NF κ B activity (Segain, 2000). We found a decrease in NF κ B activity following butyrate administration but was found to be insignificant (data not shown). Also, butyrate can act independently of TLR4 activation by inhibiting HDACs (Chang et al., 2014). Future studies should target the extensive role of butyrate in HDAC inhibition in Gulf War Illness that may be independent of TLR4 activation. Importantly, the results of altered expressions of the metabolic genes failed to induce any histological changes that support inflammatory or metabolic liver disease following GW-chemical exposure. This is of high significance since liver diseases take years to manifest and most remain asymptomatic (silent) thus evading most clinical observations. It remains to be seen whether the unavailability of reports related to liver complications in GWI is due to the silent nature of the manifestations that are only limited to changes in the expressions of metabolic genes and would perhaps take years to show any phenotypic disease. Finally, our studies with TLR4 gene-deficient mice exposed to GW chemicals reversed the levels of TLR5 and expressions for SREBP1c, PPAR- α , PFK and GLUT-4 emphasizing the fact that TLR4 activation was indeed responsible for the metabolic reprogramming in the liver. Further, the reversal of TLR4-inducible systemic release of DAMPS and metabolic changes in the liver bodes well for a potential use of this compound for a gut-targeted therapy in GWI veterans.

In summary, we show that GW-chemical exposure in mice and subsequent systemic inflammation following a dysbiosis in the gut could cause significant changes in the way the liver metabolizes lipid and carbohydrate with no detectable pathology while butyrate resists those changes. The study will help us advance our efforts to scrutinize clinical symptom reporting in the liver and re-evaluate the way we approach the therapeutic aspect of GWI by targeting multiple physiological pathways. Uses of short-chain fatty acids or probiotics can help in such pursuits.

Grant support

This work was supported by a pilot funding received from the Gulf War Illness Research Consortium to Saurabh Chatterjee (Parent DOD grant # W81XWH-13-2-0072, PI: Dr. Kimberly Sullivan) and P01AT003961 (Project 4) to Saurabh Chatterjee, P01AT003961, P20GM103641, R01AT006888, R01ES019313, R01MH094755 and VA Merit Award BX001357 to Mitzi Nagarkatti and Prakash S. Nagarkatti.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

The authors gratefully acknowledge the technical services of Benny Davidson at the IRF, University of South Carolina School of Medicine and AML Labs (Baltimore MD), and University of Georgia college of veterinary medicine for support in clinical blood chemistry test. We also thank the Instrumentation resource facility (IRF) at the University of South Carolina for equipment usage and consulting services.

References

- Abdullah, L., Evans, J.E., Joshi, U., Crynen, G., Reed, J., Mouzon, B., et al., 2016. Translational potential of long-term decreases in mitochondrial lipids in a mouse model of Gulf War Illness. *Toxicology* 372, 22–33.
- Alhasson, F., Das, S., Seth, R., Dattaroy, D., Chandrashekar, V., Ryan, C.N., et al., 2017. Altered gut microbiome in a mouse model of Gulf War illness causes neuroinflammation and intestinal injury via leaky gut and TLR4 activation. *PLoS One* 12, e0172914.
- Anderson, E.K., Hill, A.A., Hasty, A.H., 2012. Stearic acid accumulation in macrophages induces toll-like receptor 4/2-independent inflammation leading to endoplasmic reticulum stress-mediated apoptosis. *Arterioscler. Thromb. Vasc. Biol.* 32, 1687–1695.
- Badman, M.K., Pissios, P., Kennedy, A.R., Koukos, G., Flier, J.S., Maratos-Flier, E., 2007. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* 5, 426–437.
- Bechmann, L.P., Hannivoort, R.A., Gerken, G., Hotamisligil, G.S., Trauner, M., Canbay, A., 2012. The interaction of hepatic lipid and glucose metabolism in liver diseases. *J. Hepatol.* 56, 952–964.
- Boutagy, N.E., McMillan, R.P., Frisard, M.I., Hulver, M.W., 2016. Metabolic endotoxemia with obesity: is it real and is it relevant? *Biochimie* 124, 11–20.
- Carey, E.J., Ali, A.H., Lindor, K.D., 2015. Primary biliary cirrhosis. *Lancet* 386, 1565–1575.
- Chandrashekar, V., Das, S., Seth, R.K., Dattaroy, D., Alhasson, F., Michelotti, G., et al., 2016. Purinergic receptor X7 mediates leptin induced GLUT4 function in stellate cells in nonalcoholic steatohepatitis. *Biochim. Biophys. Acta* 1862, 32–45.
- Chandrashekar, V., Seth, R.K., Dattaroy, D., Alhasson, F., Ziolenka, J., Carson, J., et al., 2017. HMGB1-RAGE pathway drives peroxynitrite signaling-induced IBD-like inflammation in murine nonalcoholic fatty liver disease. *Redox Biol.* 13, 8–19.
- Chang, P.V., Hao, L., Offermanns, S., Medzhitov, R., 2014. The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proc. Natl. Acad. Sci. U. S. A.* 111, 2247–2252.
- Chatterjee, S., Ganini, D., Tokar, E.J., Kumar, A., Das, S., Corbett, J., et al., 2013. Leptin is key to peroxynitrite-mediated oxidative stress and Kupffer cell activation in experimental non-alcoholic steatohepatitis. *J. Hepatol.* 58, 778–784.
- Chen, Y., Choi, S.S., Michelotti, G.A., Chan, I.S., Swiderska-Syn, M., Karaca, G.F., et al., 2012. Hedgehog controls hepatic stellate cell fate by regulating metabolism. *Gastroenterology* 143 (1319–29 e111).
- Chiu, C.C., Ching, Y.H., Li, Y.P., Liu, J.Y., Huang, Y.T., Huang, Y.W., et al., 2017. Nonalcoholic fatty liver disease is exacerbated in high-fat diet-fed Gnotobiotic mice by colonization with the gut microbiota from patients with nonalcoholic Steatohepatitis. *Nutrients* 9.
- Coker, W.J., 1996. A review of Gulf War illness. *J. R. Nav. Med. Serv.* 82, 141–146.
- Gulf war and health. In: Cory-Slechta, D., Wedge, R. (Eds.), Update of Health Effects of Serving in the Gulf War, 2016. vol. 10 National Academy of Sciences, Washington DC.
- Coughlin, S.S., Kang, H.K., Mahan, C.M., 2011. Selected health conditions among overweight, obese, and non-obese veterans of the 1991 Gulf War: results from a survey conducted in 2003–2005. *Open Epidemiol. J.* 4, 140–146.
- Doycheva, I., Watt, K.D., Rifai, G., Abou Mrad, R., Lopez, R., Zein, N.N., et al., 2017. Increasing burden of chronic liver disease among adolescents and young adults in the USA: a silent epidemic. *Dig. Dis. Sci.* 62, 1373–1380.
- Feingold, K.R., Shigenaga, J.K., Kazemi, M.R., McDonald, C.M., Patzek, S.M., Cross, A.S., et al., 2012. Mechanisms of triglyceride accumulation in activated macrophages. *J. Leukoc. Biol.* 92, 829–839.
- Garcia-Martinez, I., Shaker, M.E., Mehal, W.Z., 2015. The therapeutic opportunities in damage-associated molecular pattern-driven metabolic diseases. *Antioxid. Redox Signal.* 23, 1305–1315.
- Geidl-Flueck, B., Gerber, P.A., 2017. Insights into the hexose liver metabolism-glucose versus fructose. *Nutrients* 9.
- Georgopoulos, A.P., James, L.M., Carpenter, A.F., Engdahl, B.E., Leuthold, A.C., Lewis, S.M., 2017. Gulf War illness (GWI) as a neuroimmune disease. *Exp. Brain Res.* 235, 3217–3225.
- Giloteaux, L., Goodrich, J.K., Walters, W.A., Levine, S.M., Ley, R.E., Hanson, M.R., 2016. Reduced diversity and altered composition of the gut microbiome in individuals with myalgic encephalomyelitis/chronic fatigue syndrome. *Microbiome* 4, 30.
- Guilmeau, S., Buyse, M., Tsocas, A., Laigneau, J.P., Bado, A., 2003. Duodenal leptin stimulates cholecystokinin secretion: evidence of a positive leptin-cholecystokinin feedback loop. *Diabetes* 52, 1664–1672.
- Haley, R.W., Charuvastra, E., Shell, W.E., Buhner, D.M., Marshall, W.W., Biggs, M.M., et al., 2013. Cholinergic autonomic dysfunction in veterans with Gulf War illness: confirmation in a population-based sample. *JAMA Neurol.* 70, 191–200.
- Henao-Mejia, J., Elinav, E., Jin, C., Hao, L., Mehal, W.Z., Stroum, T., et al., 2012. Inflammasome-mediated dysbiosis regulates progression of NAFLD and obesity. *Nature* 482, 179–185.
- Iizuka, K., Horikawa, Y., 2008. ChREBP: a glucose-activated transcription factor involved in the development of metabolic syndrome. *Endocr. J.* 55, 617–624.
- Imhann, F., Vich Vila, A., Bonder, M.J., Fu, J., Gevers, D., Visschedijk, M.C., et al., 2018. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. *Gut* 67, 108–119.
- Jensen, O., Bernklev, T., Jelsness-Jorgensen, L.P., 2017. Fatigue in type 1 diabetes: a systematic review of observational studies. *Diabetes Res. Clin. Pract.* 123, 63–74.
- Kaltsas, G., Vgontzas, A., Chrousos, G., 2010. Fatigue, endocrinopathies, and metabolic disorders. *PM & R* 2, 393–398.
- Karim, S., Adams, D.H., Lalor, P.F., 2012. Hepatic expression and cellular distribution of the glucose transporter family. *World J. Gastroenterol.* 18, 6771–6781.
- Kelsall, H.L., McKenzie, D.P., Sim, M.R., Leder, K., Forbes, A.B., Dwyer, T., 2009. Physical, psychological, and functional comorbidities of multisymptom illness in Australian male veterans of the 1991 Gulf War. *Am. J. Epidemiol.* 170, 1048–1056.
- Kerr, K.J., 2015. Gulf War illness: an overview of events, most prevalent health outcomes, exposures, and clues as to pathogenesis. *Rev. Environ. Health* 30, 273–286.
- Khan, S., Jena, G., 2014. Sodium butyrate, a HDAC inhibitor ameliorates eNOS, iNOS and TGF-beta1-induced fibrogenesis, apoptosis and DNA damage in the kidney of juvenile diabetic rats. *Food Chem. Toxicol.* 73, 127–139.
- Khan, S., Jena, G., 2015. The role of butyrate, a histone deacetylase inhibitor in diabetes mellitus: experimental evidence for therapeutic intervention. *Epigenomics* 7, 669–680.
- Koo, B.B., Michalovic, L.T., Calderazzo, S., Kelly, K.A., Sullivan, K., Killiany, R.J., et al., 2018. Corticosterone potentiates DFP-induced neuroinflammation and affects high-order diffusion imaging in a rat model of Gulf War Illness. *Brain Behav. Immun.* 67, 42–46.
- Koslik, H.J., Hamilton, G., Golomb, B.A., 2014. Mitochondrial dysfunction in Gulf War illness revealed by ³¹P phosphorus magnetic resonance spectroscopy: a case-control study. *PLoS One* 9, e92887.
- LeBlanc, J.G., Chain, F., Martin, R., Bermudez-Humaran, L.G., Courau, S., Langella, P., 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. *Microb. Cell Factories* 16, 79.
- Luetting, J., Rosenthal, R., Barmeyer, C., Schulzke, J.D., 2015. Claudin-2 as a mediator of leaky gut barrier during intestinal inflammation. *Tissue Barriers* 3, e977176.
- Marra, F., Svegliati-Baroni, G., 2018. Lipotoxicity and the gut-liver axis in NASH pathogenesis. *J. Hepatol.* 68 (2), 280. <http://dx.doi.org/10.1016/j.jhep.2017.11.014>. (Epub 2017 Nov 14. Review, PMID: 29154964).
- Mauro, C., Leow, S.C., Anso, E., Rocha, S., Thotakura, A.K., Tornatore, L., et al., 2011. NF-kappaB controls energy homeostasis and metabolic adaptation by upregulating mitochondrial respiration. *Nat. Cell Biol.* 13, 1272–1279.
- Milic, S., Stimac, D., 2012. Nonalcoholic fatty liver disease/steatohepatitis: epidemiology, pathogenesis, clinical presentation and treatment. *Dig. Dis.* 30, 158–162.
- Morris, G., Berk, M., Carvalho, A.F., Caso, J.R., Sanz, Y., Maes, M., 2016. The role of microbiota and intestinal permeability in the pathophysiology of autoimmune and Neuroimmune processes with an emphasis on inflammatory bowel disease type 1 diabetes and chronic fatigue syndrome. *Curr. Pharm. Des.* 22, 6058–6075.
- Nagy-Szakal, D., Williams, B.L., Mishra, N., Che, X., Lee, B., Bateman, L., et al., 2017. Fecal metagenomic profiles in subgroups of patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Microbiome* 5, 44.
- Naviaux, R.K., Naviaux, J.C., Li, K., Bright, A.T., Alaynick, W.A., Wang, L., et al., 2016. Metabolic features of chronic fatigue syndrome. *Proc. Natl. Acad. Sci. U. S. A.* 113, E5472–E5480.
- O'Callaghan, J.P., Kelly, K.A., Locker, A.R., Miller, D.B., Lasley, S.M., 2015. Corticosterone primes the neuroinflammatory response to DFP in mice: potential

- animal model of Gulf War Illness. *J. Neurochem.* 133, 708–721.
- O'Callaghan, J.P., Michalovicz, L.T., Kelly, K.A., 2016. Supporting a Neuroimmune basis of Gulf War Illness. *EBioMedicine* 13, 5–6.
- Oishi, Y., Spann, N.J., Link, V.M., Muse, E.D., Strid, T., Edillor, C., et al., 2017. SREBP1 contributes to resolution of pro-inflammatory TLR4 signaling by reprogramming fatty acid metabolism. *Cell Metab.* 25, 412–427.
- Parihar, V.K., Hattiangady, B., Shuai, B., Shetty, A.K., 2013. Mood and memory deficits in a model of Gulf War Illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. *Neuropsychopharmacology* 38, 2348–2362.
- Razolli, D.S., Moraes, J.C., Morari, J., Moura, R.F., Vinolo, M.A., Velloso, L.A., 2015. TLR4 expression in bone marrow-derived cells is both necessary and sufficient to produce the insulin resistance phenotype in diet-induced obesity. *Endocrinology* 156, 103–113.
- Reichardt, F., Chassaing, B., Nezami, B.G., Li, G., Tabatabavakili, S., Mwangi, S., et al., 2017. Western diet induces colonic nitroergic myenteric neuropathy and dysmotility in mice via saturated fatty acid- and lipopolysaccharide-induced TLR4 signalling. *J. Physiol.* 595, 1831–1846.
- Sappington, P.L., Yang, R., Yang, H., Tracey, K.J., Delude, R.L., Fink, M.P., 2002. HMGB1 B box increases the permeability of Caco-2 enterocytic monolayers and impairs intestinal barrier function in mice. *Gastroenterology* 123, 790–802.
- Schreuder, T.C., Verwer, B.J., van Nieuwkerk, C.M., Mulder, C.J., 2008. Nonalcoholic fatty liver disease: an overview of current insights in pathogenesis, diagnosis and treatment. *World J. Gastroenterol.* 14, 2474–2486.
- Segain, J.P., Raingeard de la Bletiere, D., Bourreille, A., Leray, V., Gervois, N., Rosales, C., et al., 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* 47, 397–403.
- Sekhavat, A., Sun, J.M., Davie, J.R., 2007. Competitive inhibition of histone deacetylase activity by trichostatin a and butyrate. *Biochem. Cell Biol.* 85, 751–758.
- Seth, R.K., Kumar, A., Das, S., Kadiiska, M.B., Michelotti, G., Diehl, A.M., et al., 2013a. Environmental toxin-linked nonalcoholic steatohepatitis and hepatic metabolic reprogramming in obese mice. *Toxicol. Sci.* 134, 291–303.
- Seth, R.K., Kumar, A., Das, S., Kadiiska, M.B., Michelotti, G., Diehl, A.M., et al., 2013b. Environmental toxin-linked nonalcoholic steatohepatitis and hepatic metabolic reprogramming in obese mice. *Toxicol. Sci.* 134 (2), 291–303. <http://dx.doi.org/10.1093/toxsci/kft104>. Epub 2013 May 2.
- Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., et al., 2014. Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis. *Immunity* 40, 128–139.
- Sobhani, I., Bado, A., Vissuzaine, C., Buyse, M., Kermorgant, S., Laigneau, J.P., et al., 2000. Leptin secretion and leptin receptor in the human stomach. *Gut* 47, 178–183.
- Song, M.J., Kim, K.H., Yoon, J.M., Kim, J.B., 2006. Activation of toll-like receptor 4 is associated with insulin resistance in adipocytes. *Biochem. Biophys. Res. Commun.* 346, 739–745.
- Sullivan, K., Kregel, M., Bradford, W., Stone, C., Thompson, T.A., Heeren, T., et al., 2018. Neuropsychological functioning in military pesticide applicators from the Gulf War: effects on information processing speed, attention and visual memory. *Neurotoxicol. Teratol.* 65, 1–13.
- Sun, M., Wu, W., Liu, Z., Cong, Y., 2017. Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *J. Gastroenterol.* 52, 1–8.
- Sweet, P.H., Khoo, T., Nguyen, S., 2017. Nonalcoholic fatty liver disease. *Prim. Care* 44, 599–607.
- Tang, Y., Chen, A., 2010. Curcumin prevents leptin raising glucose levels in hepatic stellate cells by blocking translocation of glucose transporter-4 and increasing glucokinase. *Br. J. Pharmacol.* 161, 1137–1149.
- Tang, D., Kang, R., Coyne, C.B., Zeh, H.J., Lotze, M.T., 2012. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol. Rev.* 249, 158–175.
- White, R.F., Steele, L., O'Callaghan, J.P., Sullivan, K., Binns, J.H., Golomb, B.A., et al., 2016. Recent research on gulf war illness and other health problems in veterans of the 1991 gulf war: effects of toxicant exposures during deployment. *Cortex* 74, 449–475.
- Zakharia, K., Tabibian, A., Lindor, K.D., Tabibian, J.H., 2018. Complications, symptoms, quality of life and pregnancy in cholestatic liver disease. *Liver Int.* 38 (3), 399–411. <http://dx.doi.org/10.1111/liv.13591>. Epub 2017 Oct 3.
- Zakirova, Z., Crynen, G., Hassan, S., Abdullah, L., Horne, L., Mathura, V., et al., 2015. A chronic longitudinal characterization of neurobehavioral and Neuropathological cognitive impairment in a mouse model of gulf war agent exposure. *Front. Integr. Neurosci.* 9, 71.
- Zhang, M., Zhou, Q., Dorfman, R.G., Huang, X., Fan, T., Zhang, H., et al., 2016. Butyrate inhibits interleukin-17 and generates Tregs to ameliorate colorectal colitis in rats. *BMC Gastroenterol.* 16, 84.
- Zheng, L., Kelly, C.J., Battista, K.D., Schaefer, R., Lanis, J.M., Alexeev, E.E., et al., 2017. Microbial-derived butyrate promotes epithelial barrier function through IL-10 receptor-dependent repression of Claudin-2. *J. Immunol.* 199, 2976–2984.