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AWARD NUMBER: W81XWH-13-2-0072

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

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RECIPIENT: CDMRP GWIRP Program Manager - Mr. Brett Chaney

REPORT DATE: Oct 2019

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Choose Distribution Statement A or B. (Reference https://mrmc.amedd.army.mil/index.cfm?pageid=researcher_resources.technical_reporting for additional information.)

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REPORT DOCUMENTATION PAGE			Form Approved 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 (0704-1188), Washington, DC 20503			
1. REPORT DATE: **OCTOBER 2019**	**2. REPORT TYPE** **ANNUAL**	**3. Report Type and Period Covered** 30 Sep 2018 – 29 Sep 2019	
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			**19a. NAME OF RESPONSIBLE PERSON: USAMRMC**
16. Security Classification of Report			**19b. TELEPHONE NUMBER**
a. U	b. ABSTRACT **c. THIS PAGE** U U	17. LIMITATION OF ABSTRACT U	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)

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1. INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

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: Provide a brief list of keywords (limit to 20 words).

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-nine years after the 1991 Gulf War, 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; Jeffrey et al., 2019; Belgrad et al., 2019; Joshi et al., 2019). 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 (Miller and O'Callaghan 2019; Belgrad et al., 2019; Joshi et al., 2019; Janulewicz et al., 2019; Januelwicz 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; Jeffrey et al., 2019). 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

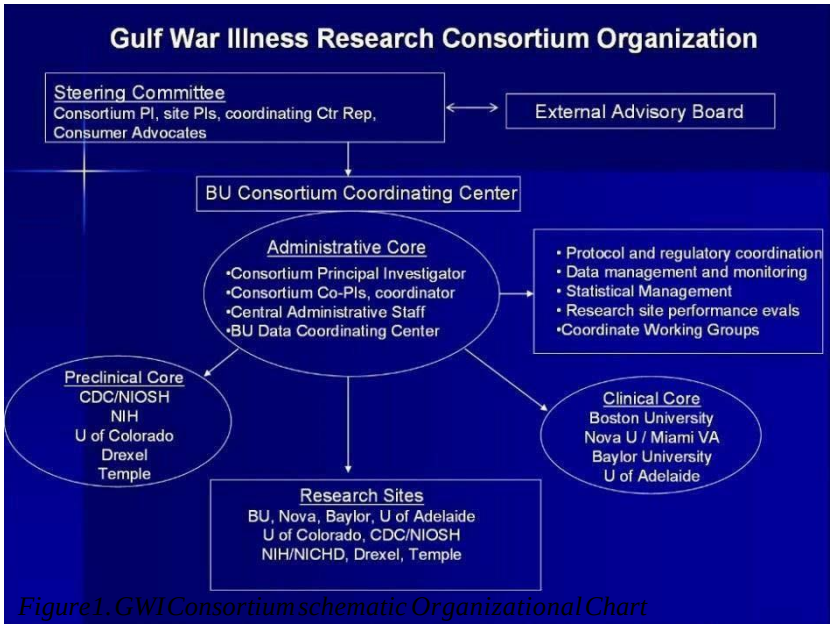


Figure 1. GWI Consortium schematic Organizational Chart

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.

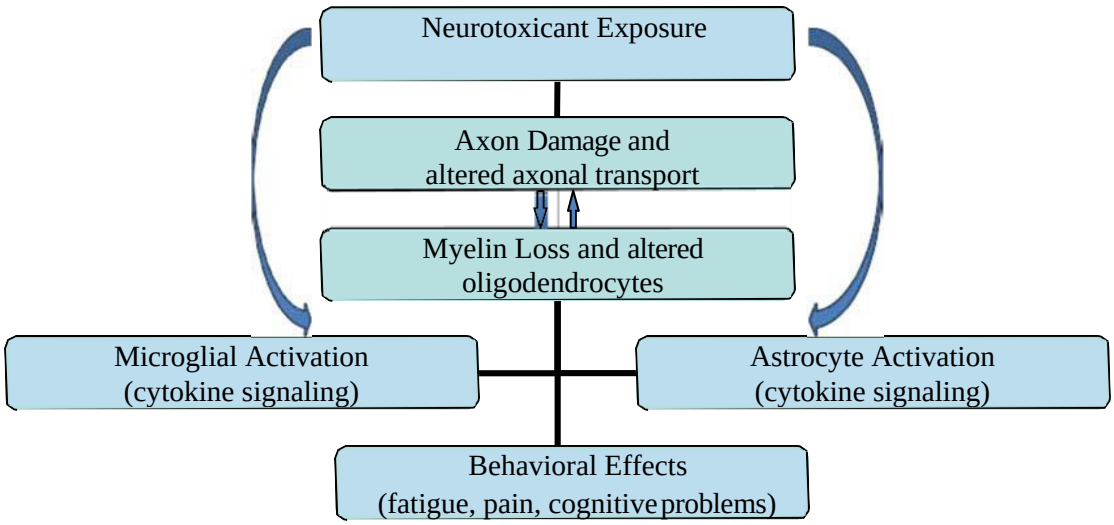
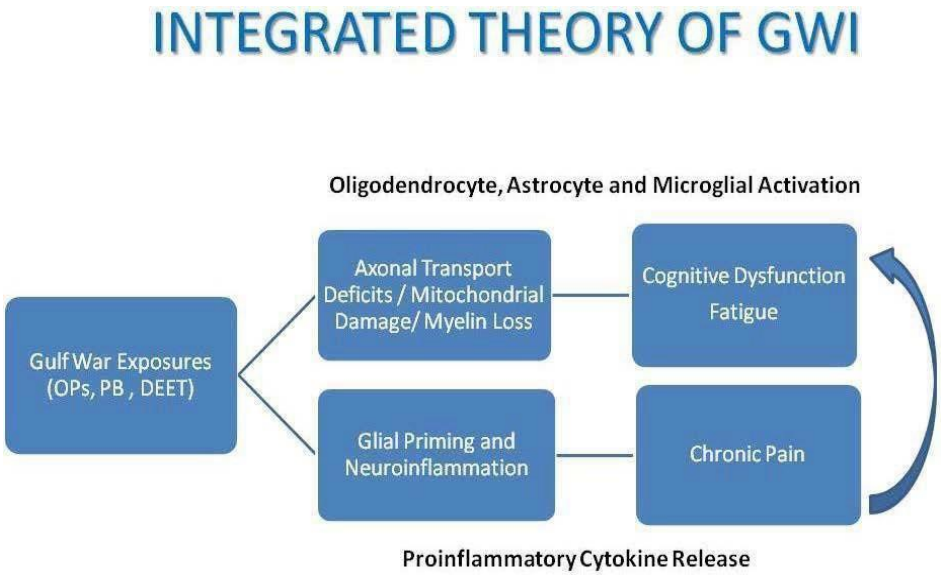


Figure 2. Schematic Representation of Hypothesized GWI Mechanisms

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.



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 f Disease Control/NIOSH, National Institutes of Health, Drexel University, Temp 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 6 is inclusive of Tasks 1-8 above. The statement of work for year 6 primarily describes the completion of the initial 10 sub-studies. In addition, in year 6, 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 seventh years 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; May 2018. 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 C o l l e g e 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 now that the

Data Use Agreements between Hines VA and BU and Miami VA are approved.

DUA document has been finalized between BU and Hines VA and signed by the BU attorney. Final revised and IRB approved versions of the DUAs from Boston and Miami were sent to VA Central Office and have now been approved and signed. We can begin sending blood samples to Dr. Klimas at the Miami VA for cytokine analysis. Dr. Klimas will perform the cytokine analyses and send the results to Hines VA. Hines VA will conduct the statistical analyses and complete this validation study.

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. Most animal studies are now completed with the exception of the CDC site where treatment studies remain ongoing.

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 to complete subject recruitment there. All current staff have completed IRB and IACUC trainings necessary for their work with animal and human studies. The Miami site hired and trained an additional research assistant to complete recruitment for the study at their site. Only BU site continues to recruit for control participants at this time.

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 been largely completed, 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 now completed recruitment and clinical appointments at their site. Boston, Miami and Texas sites added new clinical staff to their teams to complete subject recruitment. 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
01-09-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
02-06-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
03-022-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
04-03-2019	Monthly Web-meeting	Pre-clinical and clinical study updates

05-01-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
06-05-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
07-10-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
08-07-2019	GWIC in-person meeting	Pre-clinical and clinical study updates
09-04-2019	Monthly Web-meeting	Pre-clinical and clinical study updates
10-02-2019	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, Coller, 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, 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. Coller, 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 bi-monthly phone calls were conducted with testing staff as subject recruitment was 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 until completion at the Houston and Miami 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.

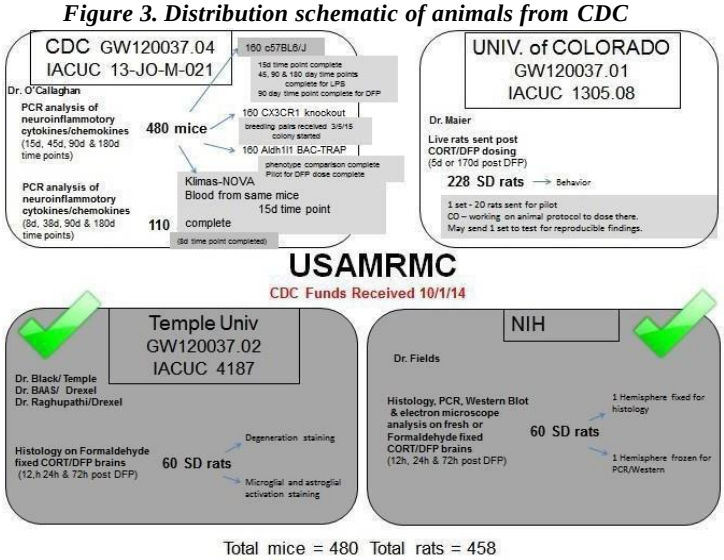
Task 2d. Obtain stored blood samples from Hines VA study and send to Miami VA for analysis.

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 was put on a newer DUA template by Hines VA staff and the new version was reviewed and signed. The Miami VA IRB has approved the DUA and sent it to the Hines VA. Hines VA reviewed and approved the DUA. Final revised and IRB approved versions of the DUAs from Boston and Miami were sent to VA Central Office for review in mid-June and have received final signature approval in December 2019. Blood samples will now be sent to Dr. Klimas at the Miami VA for analyses and processing. Dr. Klimas will perform the cytokine testing and send the results to the Hines VA. Hines VA will conduct the statistical analyses and report the results back to GWIC for comparison with GWIC results and write-up for publication.

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

Monthly web meeting and working group meetings were ongoing during the past year 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 (Belgrad et al., 2019; Michalowicz et al., 2019; Joshi et al., 2019; Janulewicz et al., 2019; Seth et al., 2019; Kimono et al., 2019; 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 where the pilot study has now been performed.

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. Additional perfused brains from the 5-week time point representing current GWI veteran time points were shipped to Dr. Koo at Boston University and were brain imaged. Results will follow shortly.

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. These results were published by NIH investigators in Belgrad et al., 2019 and 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 made cell cultures from embryonic mice and rats for studies of myelination and the co- cultures were 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 were combined with these neuronal cultures in multicompartment 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 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. These results have been published in Belgrad et al., 2019.

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 (Michlowicz et al., 2019; Koo et al., 2017; O'Callaghan et al., 2015).

Dr. Fields then 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 now been published in Glia as Belgrad et al., 2019 (see appendix).

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

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

4a. Assess for axonal transport integrity in rodent and cell models exposed to either GW-relevant neurotoxicants or cytokines (Drexel, Temple).

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 now 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 neurotoxicants 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 Disopropyl 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. In future studies, we would like to experiment with additional time points and treatment durations to further characterize pathology in these neurons.

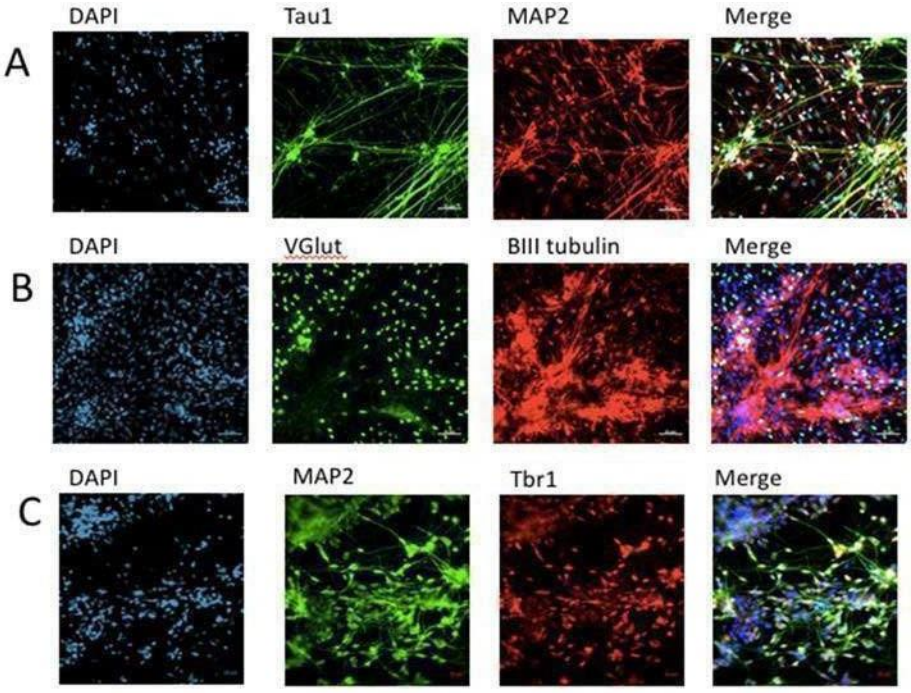


Figure 2. Neuronal validation of hiPSC-derived neurons. (A) Tau1 stains for total tau and is used to mark mature neurons. MAP2 is a microtubule-associated protein.

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

(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.

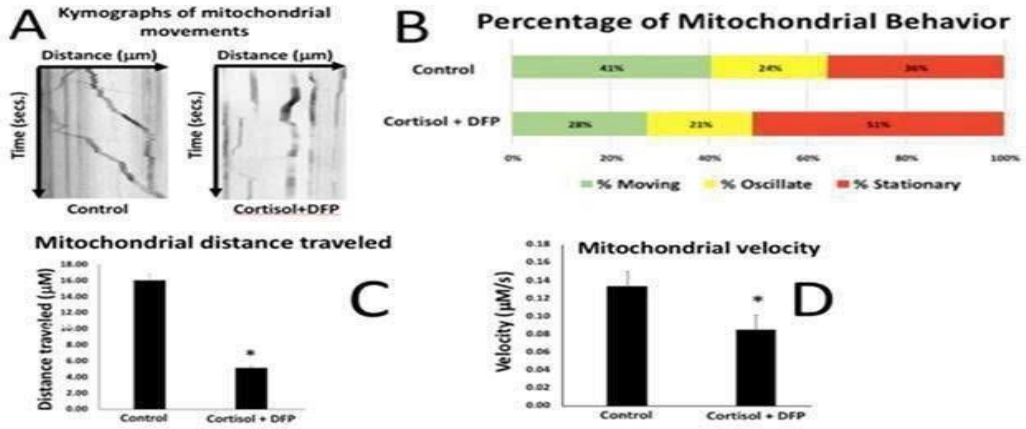


Figure 3. Neurotoxicant/stress- mediated i mpairment of mitochondrial transport.

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.

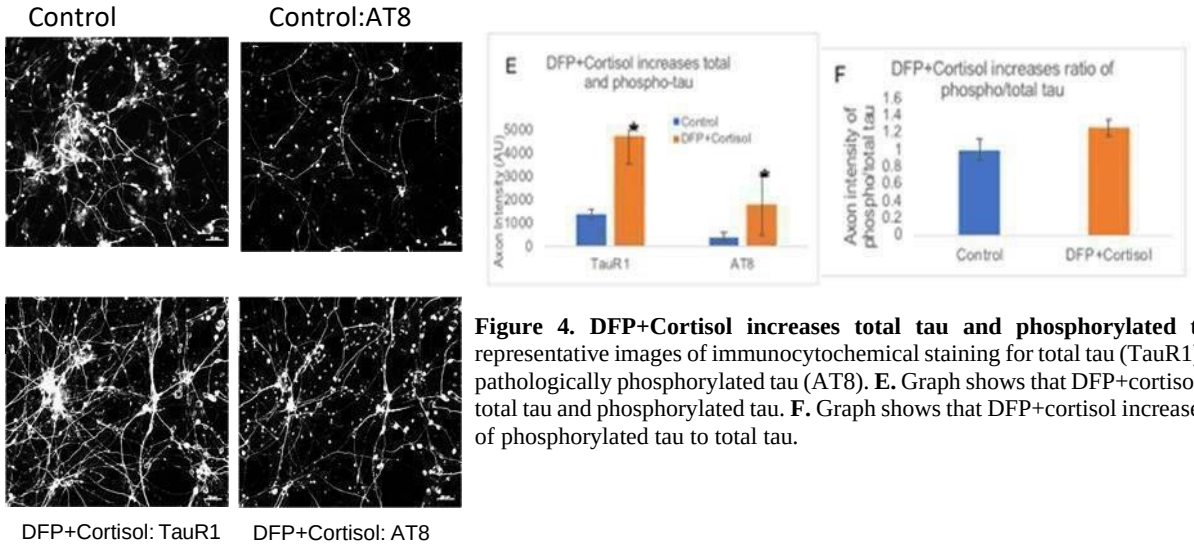


Figure 4. DFP+Cortisol increases total tau and phosphorylated tau. A-D. representative images of immunocytochemical staining for total tau (TauR1) and early pathologically phosphorylated tau (AT8). **E.** Graph shows that DFP+cortisol increases total tau and phosphorylated tau. **F.** Graph shows that 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. If confirmed, these results would correspond with recent findings from the CNS autoantibody study showing increased levels of tau autoantibodies in veterans with GWI (Abou-donia et al., 2017).

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 neurotoxicants 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.

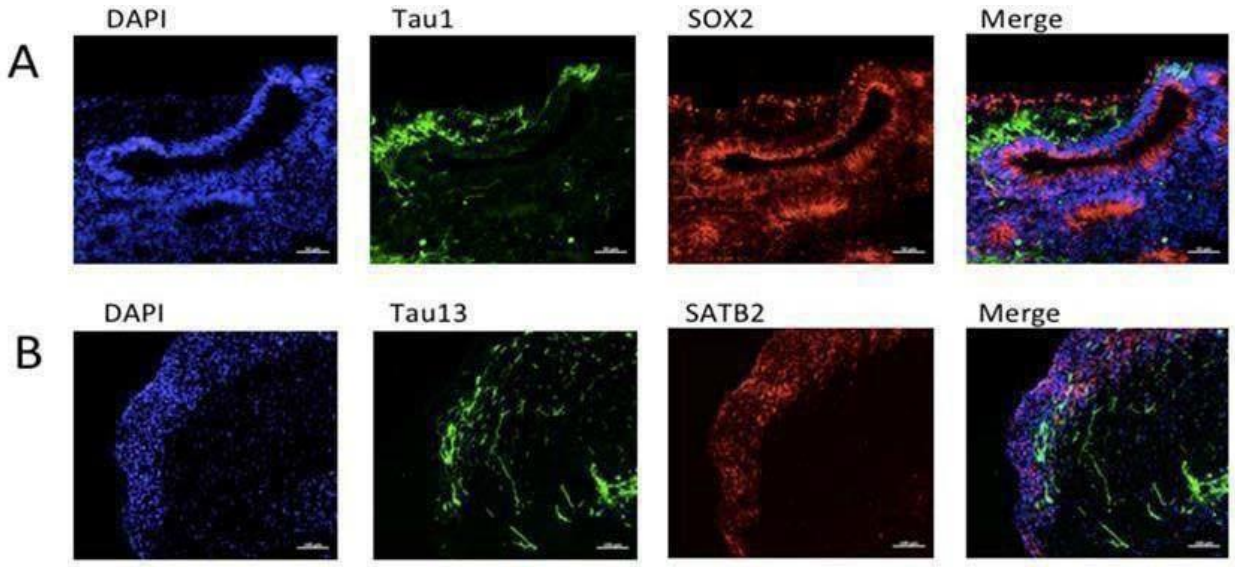


Figure 6. Neuronal validation of cerebral organoids. (A-B) Day 43 organoids. DAPI is a nuclear stain. Tau1 mark de- phosphorylated tau. SOX2 is a neural progenitor marker. Tau13 is a total tau marker. SATB2 stains surface layer neurons

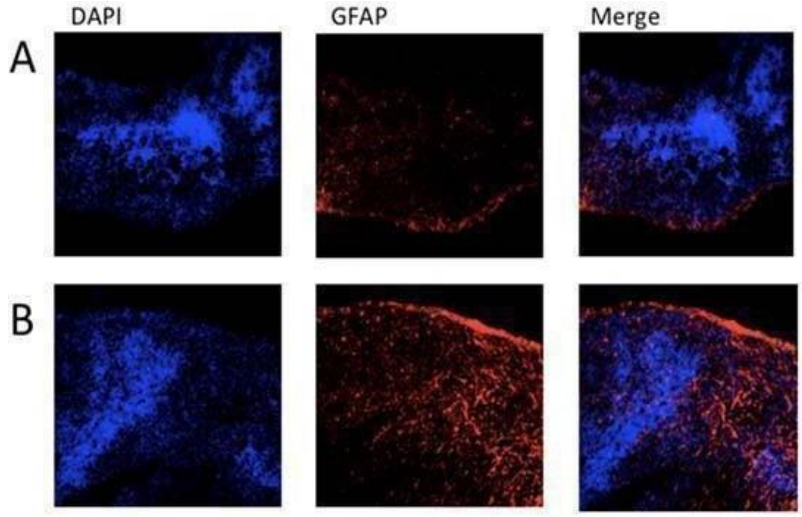


Figure7. Reactive astroglial neuroinflammation in cerebral organoids. Organoids were treated at 2 months with DFP+cortisol, then fixed 1 day after the end of neurotoxicant treatment. (A) Organoids treated with vehicle. (B) Organoids treated with DFP +cortisol. DAPI is a nuclear stain. GFAP is an astroglial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

Dr. Qiang's team is currently investigating whether the GWI neurotoxicants 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 organioids 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. They are examining changes in neurogenesis, cortical laminations, and astrogliosis.

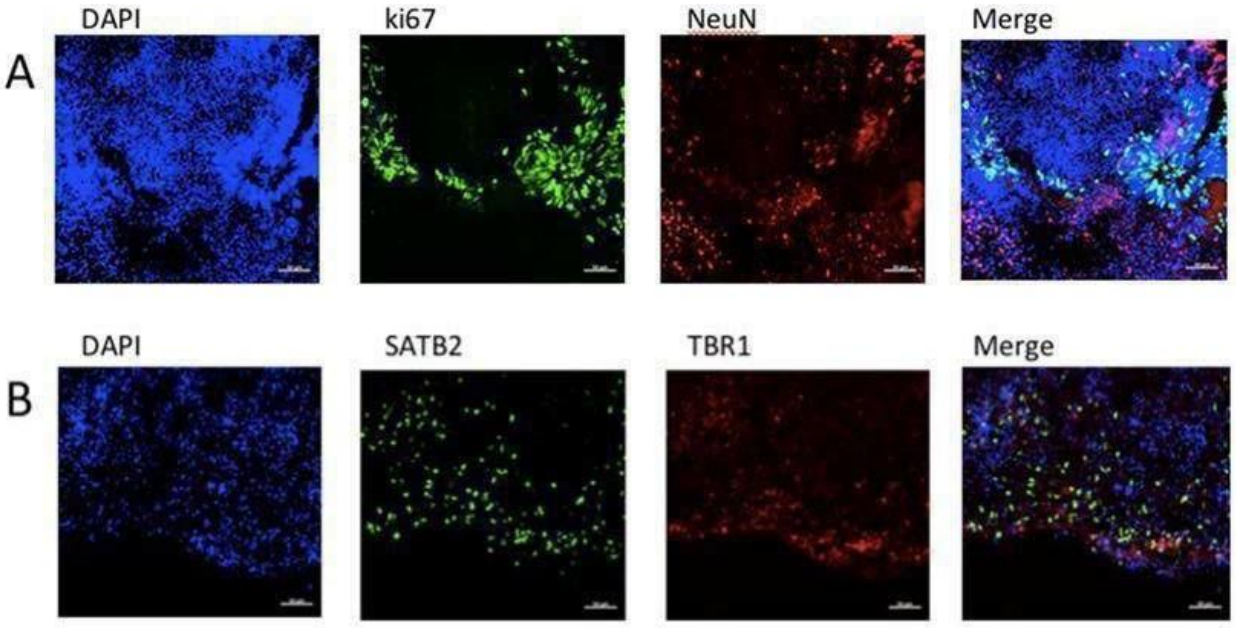


Figure 8. Neurogenesis in cerebral organoids. Organoids were treated at 2 months with DFP+cortisol, then fixed 1 day after the end of neurotoxicant treatment. DAP I is a nuclear stain. ki67 marks dividing cells. NeuN marks mature neurons. SATB2 and TBR1 stain for neurons in the developing cortical plate.

Previously, this team reported preliminary data for the microtubule therapy, tau pathology, and organoid projects, and now has performed multiple repeats for each experimental condition and technique. The team is now actively in the process of quantifying all the data.

We have tried several different protocols to optimize growth of our cerebral organoids, and we have settled on a protocol that produces more consistent organoids with ventricle-like structures as shown in Figure 9.

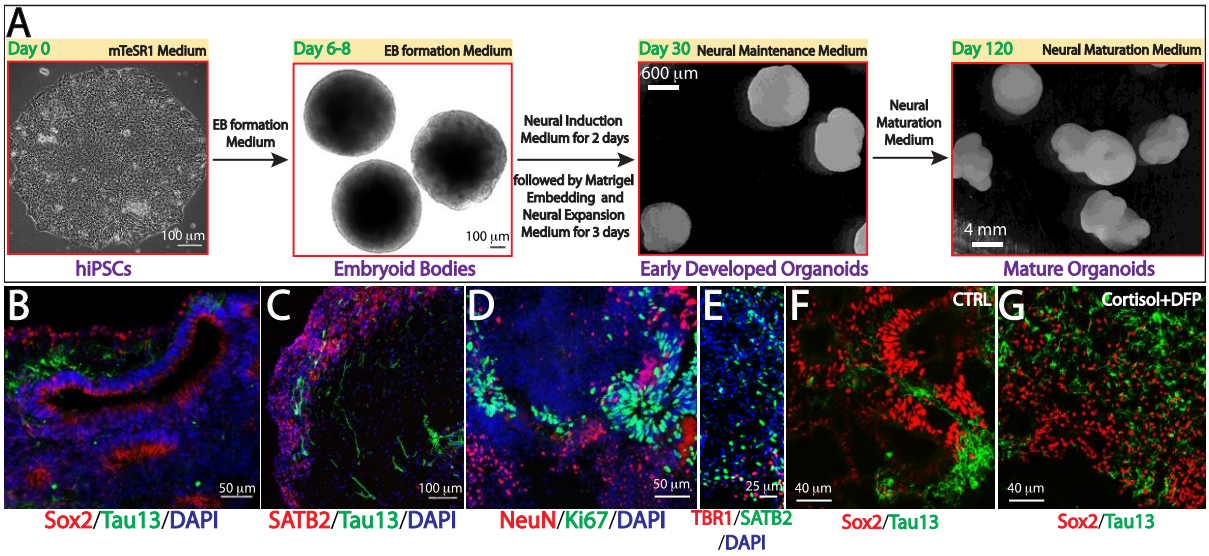


Figure 9. Generation (**A**) and characterization (**B-G**) of hiPSC-induced forebrain cortical cerebral organoids. **F-G.** Cortisol+DFP alters neurogenesis as exhibited by more disorganized ventricle-like structures.

4b. Assess for myelin integrity in rodent and cell models exposed to either GW-relevant neurotoxicants 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

1. We have characterized these findings in the O'Callaghan rat model of GWI and published two manuscripts (Fields et al., 2017; Belgrad et al., 2019). 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 publications (Belgrad et al., 2019; 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.

2. A poster was presented to the Society for Neuroscience summarizing our findings with cholinergic signaling in myelination is listed below and a recent paper published by Belgrad et al., 2019 is included in the appendixes:

   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 heterogenous 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.

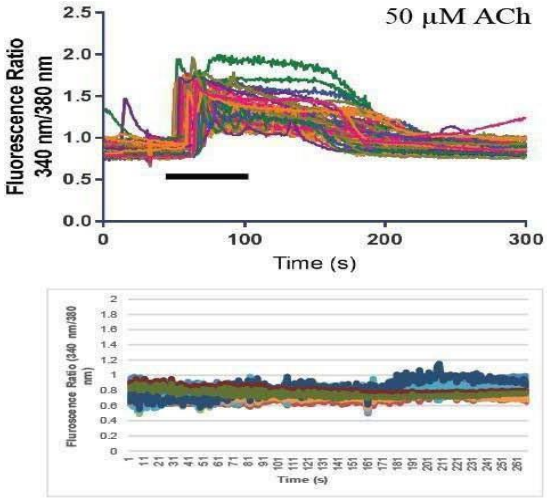


Fig 1. ACh induces OPC intracellular calcium response. a) Schematic for ACh treatment during live-cell calcium imaging. b) 50 µM ACh (N=5, n=56). Inset: inhibition with mAChR M1 inhibitor pirenzepine (PZP, 50 µM) to confirm specificity of ACh-induced calcium response (N=3, n=9). Left arrow at 40 seconds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

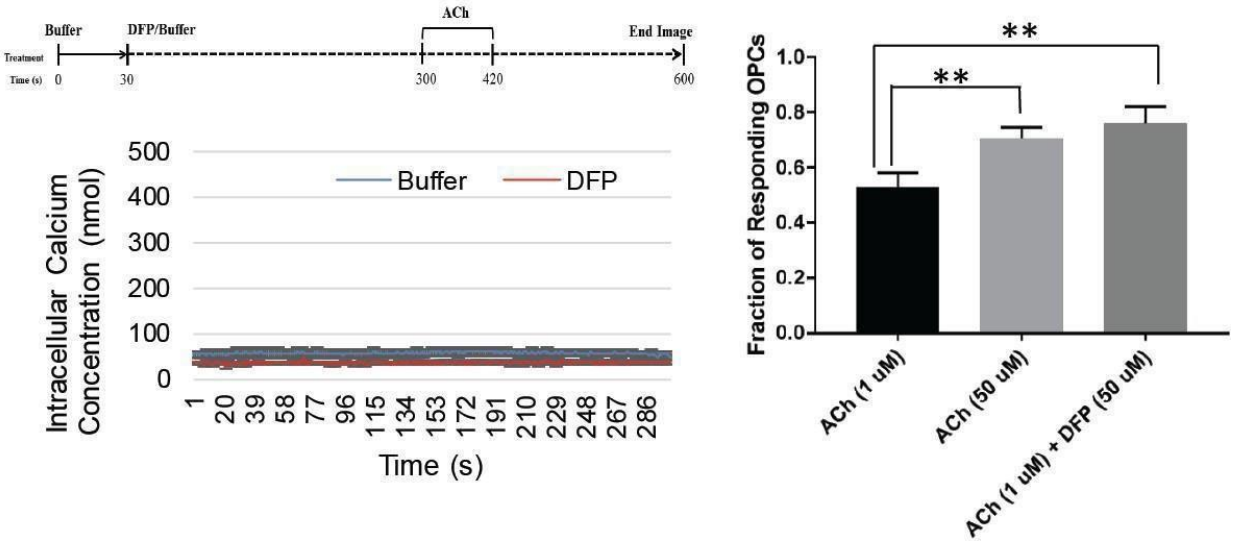


Fig 2. DFP increases fraction of OPCs responding to ACh. a) DFP (50 um) alone has no effect on intracellular calcium mobilization in OPCs. b) DFP pretreatment followed by ACh significantly increases fraction of cells that respond (t-test, p-value = 0.005). 50 µM ACh significantly increases number of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

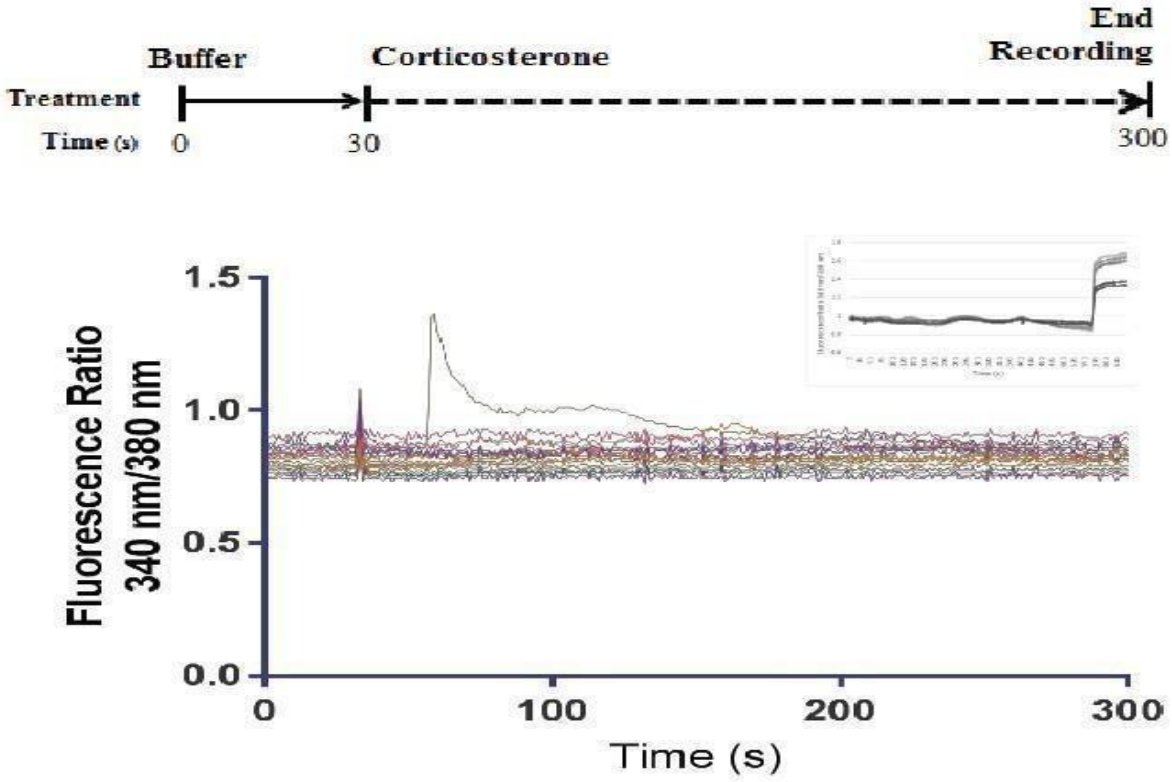


Fig 3. Corticosterone has no effect on intracellular calcium mobilization. a) Treatment paradigm used. b) intracellular calcium measurement. Each line indicates recording from one cell (N=3, n= 36). Inset box indicates positive control using calcium ionophore A23187.

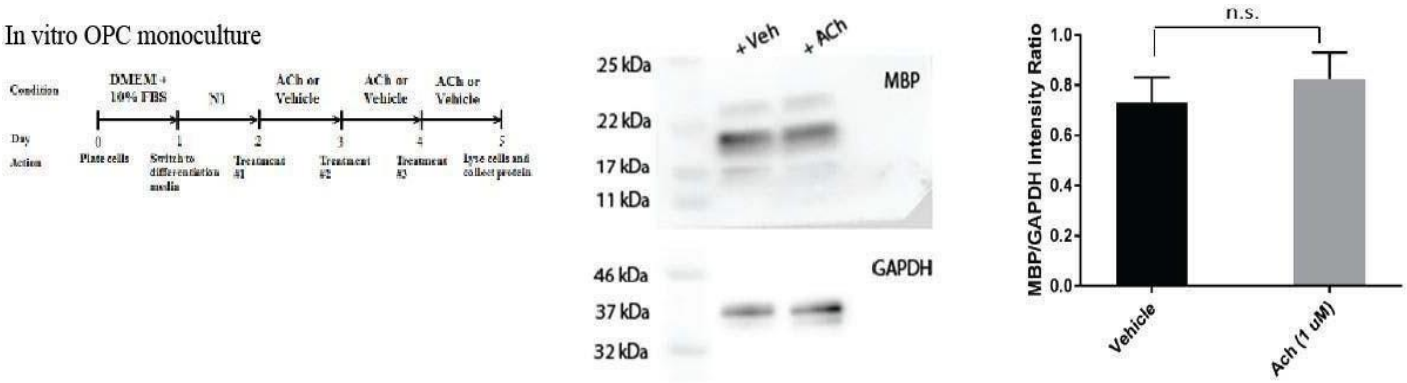
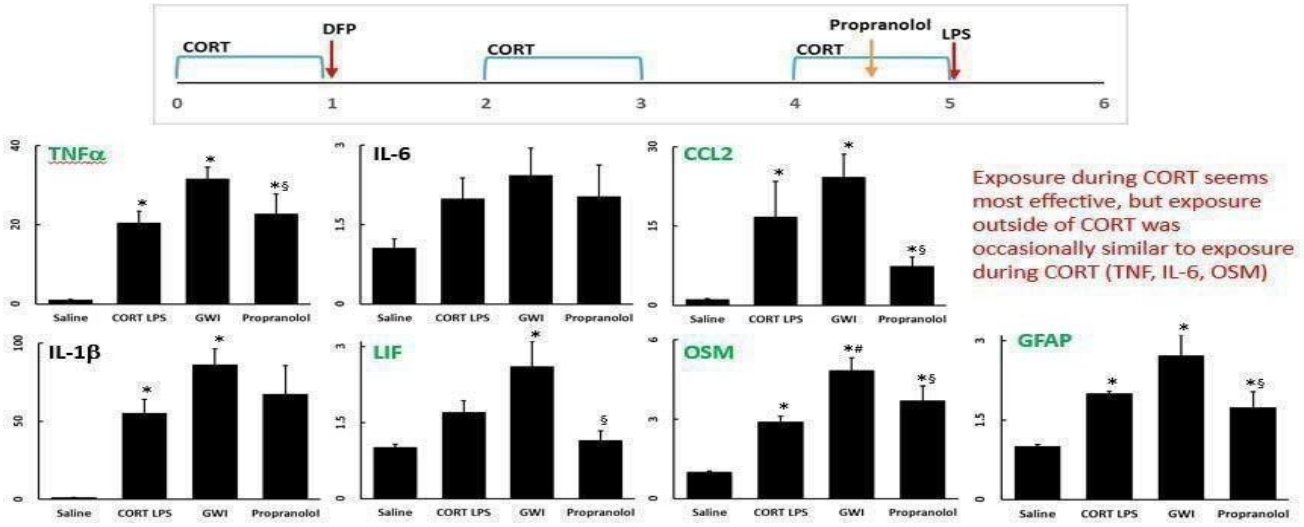


Fig. 4: ACh alone not sufficient to induce MBP changes in OPCs a) Treatment paradigm used for in vitro OPC monoculture experiments. b) Western blot for MBP and GAPDH on differentiating oligodendrocytes with Vehicle (differentiating media) and ACh (1 µM in N1 differentiation media). c) Quantified western 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).

a. 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).

Recently, we were able to compare sarin samples from Next Generation Sequencing to the DFP dataset we already have. Sarin data for neuroinflammation and phosphosignaling completely matched what we have for DFP as sarin surrogate, thus validating our use of DFP for GWI animal studies and for modeling GWI treatment studies.

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

CDC Accomplishments

Three papers were recently published: titled:

Kelly KA, Michalovicz LT, Ranpara A, Locker AR, Miller DB, O'Callaghan JP. 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, Toxicol Sci. 2018 May 28. doi:10.1093/toxsci/kfy130

Michalovicz, Lindsay T., Kimberly A. Kelly, Saurabh Vashishtha, Rotem Ben-Hamo, Sol Efroni, Julie V. Miller, and others. Astrocyte-specific Transcriptome Analysis Using the ALDH1L1 BacTRAP Mouse Reveals Novel Biomarkers of Astrogliosis in Response to Neurotoxicity, Journal of Neurochemistry, 150.4 (2019), 420–40. https://doi.org/10.1111/jnc.14800

O'Callaghan JP, Miller DB. Neuroinflammation disorders exacerbated by environmental stressors. Metabolism. 2019 Nov;100S:153951. doi: 10.1016/j.metabol.2019.153951.

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 "drugable" 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 now been published as Michalowicz et al., 2019. 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 and has completed rat brain imaging. 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 to help initiate and oversee the EAAT2 PET studies.

The imaging study has now been completed. Rats and drugs were obtained and brain imaging was completed. All of the Control Groups A-B arms and DFP arms of Groups B cohort of the study all underwent magnetic resonance and positron emission tomography (PET) imaging scanning followed by data analyses, along with post-mortem brain collecting. The Aim 1 rat dosing, PET-CT and MR scanning and also the acquisition of post-mortem brain tissues have been completed as of September 27, 2019.

The Aim 2 rat post-mortem brains have been catalogued and tissues have also now been analyzed for EAAT2 density and GFAP by the Rio contractor.

The Aim 3 PET imaging data has been processed and plots are completed. The temporal DFP and cortisterone+DFP data is initially thought to be highly consistent to the previously published findings of the GWI rodent model by J. O'Callaghan, et al. Once the Aim 2 post mortem brain data is in hand from the contractor it will be correlated to the EAAT2 PET imaging data.

We anticipate that a full pilot project report with all data sets and analyses will be reported shortly.

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 completed the two pilot studies for treatment of memory functioning and for treatment of pain with two IL10 based treatments that have been previously reported. These results are listed in the next section under treatments.
- 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 by Rao et al., in May 2017. Additional HDAC6 treatments that are FDA approved were submitted in a n o w f u n d e d 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 were used to determine an appropriate dose for testing in the 5 week GWI paradigm model that are now ongoing.

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 completed with results showing that it increased the pain threshold in rats exposed to the GWI animal model. This suggests IL10 therapies should be further explored in animal and clinical studies. Dr. Peter Grace is following up on this work with a funded grant to further assess these outcomes and treatment avenues.

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 last November. Thus, treatment development is occurring at the preclinical and clinical sites simultaneously. Importantly GWICTIC phase I and II trials will target GWIC identified TNF-alpha and microglial neuroinflammatory pathways.

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

Participant recruitment and screening is ongoing at only Boston University. Miami and Houston has completed recruitment and are no longer screening participants. Currently 633 participants have been screened and 264 have been assessed at the Boston, Miami and Houston. The Boston site currently has three additional participants in the process of being scheduled. Data collection began at the Baylor College of Medicine (BCM) site in Houston in October 2017 and continued through the current project year, with the final Houston GWIC participant evaluated in August 2019. In this current project year, a total of 42 veterans completed study appointments, providing an overall project total of 74 Gulf War veterans evaluated at the Houston site. In this final year of data collection, we have also continued to monitor our early findings concerning patterns of brain tissue abnormalities viewable on MRI scans of veterans with Gulf War illness (GWI). These changes have not been previously reported in the literature, but our initial assessments indicate they are potentially significant, clinically detectable, and associated with characteristics of Gulf War deployment.

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

Recruitment has been ongoing this quarter and 660 total subjects have contacted GWIC coordinators through print advertising, free newsletters to VSOs or social media outlets. From these contacts, 400 were found eligible to participate in the studies. Those not screened were largely not GW veterans. Of this group, 280 have been scheduled and 264 (224 case, 40 controls) have completed the study protocols at Boston, Miami or Houston sites. Miami and Texas site has completed recruitment. Boston site has completed case

recruitment of GW veterans. Recruitment is ongoing at the Boston site to recruit only GW controls. We are working with WeHealth, a media company to assist in connecting the GWIC research to Healthy Gulf War veterans who may be recruited to participate in the study.

	Total	Boston	Miami	Texas
Number of Subjects Contacted	660	382	79	199
Number of Subjects Screened	633 (95.9%)	366 (95.8%)	79 (100.0%)	188 (94.5%)
Number of Subjects Eligible	400 (63.2%)	214 (58.5%)	69 (87.3%)	117 (62.2%)
Number of Subjects with Appointments Made	281 (70.3%)	143 (66.8%)	64 (92.8%)	74 (63.2%)
Number of Subjects Assessed	264 (94.0%)	141 (98.6%)	50 (78.1%)	73 (98.6%)

	Total		Boston		Miami		Texas	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	575	58	333	33	70	9	172	16
Number of Subjects Eligible	350	50	185	29	60	9	105	12
Number of Subjects Assessed	224	40	117	24	42	8	65	8

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. 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 m TBI 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 m TBI exposure. Also, risk of other medical conditions was compared in the mTBI + C B W group when compared with the nonexposed group. These results were recently published in Janulewicz et al., 2018. 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 do main 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. Krengel, Sullivan, and Janulewicz (Yee et al., 2015; Yee et al., 2017).

Table 1. GWIC Subject Demographics

Subject Demographics	N=206	N=35
Demographics	Cases	Controls
Age at time of study (mean, SD)	52.1 (11.2)	54.5 (6.3)*
Years of education (mean, SD)	15 (2)	15 (2)
Female	18%	12%
Ethnicity		
Caucasian	77%	78%
African American	14%	15%
Other	9%	6%
GW-related Exposures		
Reported PB pill usage	73%	64%
Wore pesticide treated uniforms	56%	33%
Saw pesticides sprayed/fogged	43%	24%
Chemical/bio weapon exposed	45%	18%

Figure 1. Chi-square analyses of mTBI rates in GWI cases and controls pre, during and post Gulf War (Janulewicz et al., 2018)

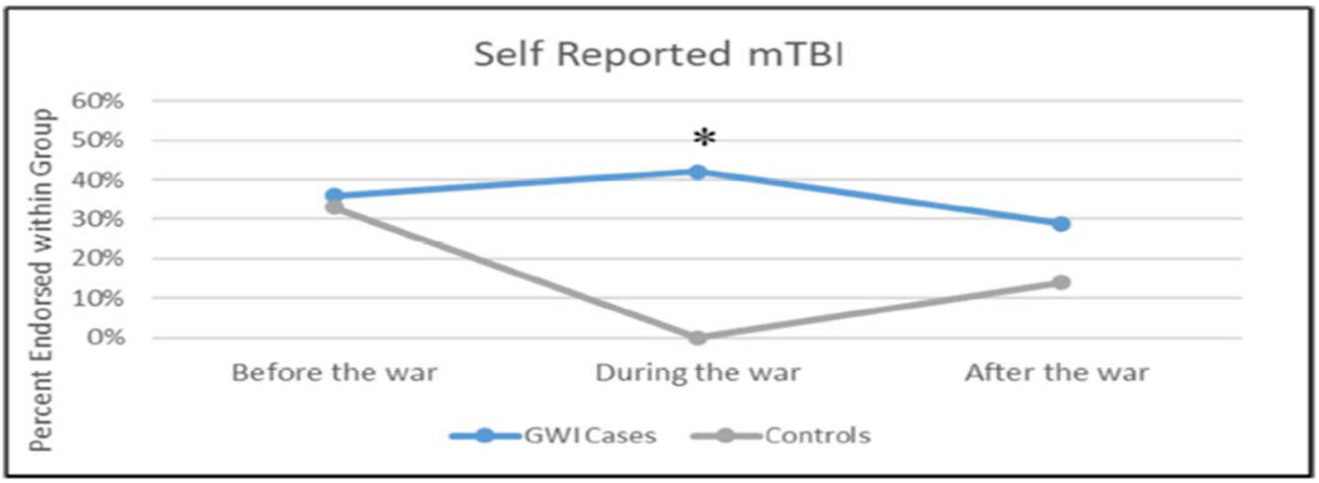


Figure 1. Percent of veterans with self-reported mTBI before, during, and after the war. * $p < 0.005$.

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

MRI scans were obtained from the first 161 study participants have been post-processed. The Baylor site has transferred 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 161 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 assisting 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.

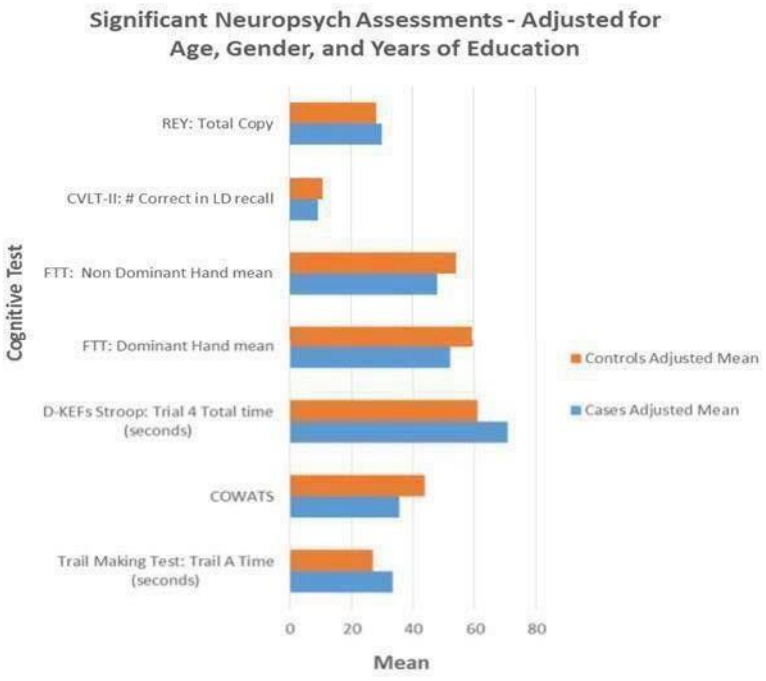
Task 5d. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.

Data from the first 186 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 conducted 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.

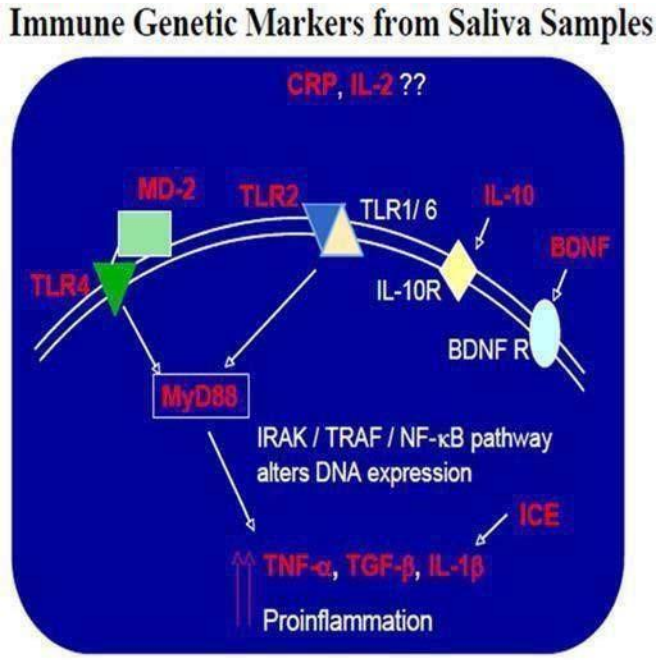
Task 5e. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements.

Blood and saliva samples have been sent to NOVA Southeastern University for each of the recently completed 264 study participants. The analysis of cytokine and chemokines has been completed for the first 184 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, IL1-b) 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).



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



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 alleletypes.

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 suggests that there may be important genetic-susceptibility in GWI cases and symptom severity that warrant further assessment. A grant was submitted by Drs. Coller, 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 GWIthat 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 Pittsburg sleep quality index was significantly correlated with TNF-RII levels. When compared with Kansas case criteria sympto m 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.

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

Brain Area	GWI 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

The Boston call-back studies are now completed. The microbiome pilot study has successfully recruited 30 of 30 participants 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 sodiumbutyrate 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 sodiumbutyrate 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. Chatterjee et al., has also recently published results of virome assessments of GWI animal models suggesting another potential treatment pathway for GWI (Kimono et al., 2019). This team has also published the results from the gut microbiome pilot study with GW veterans showing altered gut micrbiome ratios in veterans with GWI and those with GWI and GI problems compared with healthy controls. These results also corresponded with blood cytokines such that veterans with GWI +/- GI problems had nearly double the amount of TNF-RI levels in their blood compared with healthy GW veteran controls. See appendix for full publication by Janulewicz et al. 2019.

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

	Total	Boston	Miami	Texas
Number of Subjects Contacted	660	382	79	199
Number of Subjects Screened	633 (95.9%)	366 (95.8%)	79 (100.0%)	188 (94.5%)
Number of Subjects Eligible	399 (63.0%)	213 (58.2%)	69 (87.3%)	117 (62.2%)
Number of Subjects with Appointments Made	280 (70.2%)	142 (66.7%)	64 (92.8%)	74 (63.2%)
Number of Subjects Assessed	264 (94.3%)	141 (99.3%)	50 (78.1%)	73 (98.6%)
Subjects Participating in Stem Cell Call-Back Study	19	19	0	0
Subjects Participating in LP Call-Back Study	10	10	0	0
Subjects Participating in PET Scan Call-Back Study	30	30	0	0
Subjects Participating in Microbiome Call-Back Study	27	27	0	0

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

The first ten lumbar punctures have been completed but due to poor response for additional recruitment, we will now collaborate with other studies at Baylor College of Medicine (BCM) who will share their CSF samples to improve our numbers to start performing data analyses for this pilot study. IRB amendments have been submitted for this sample and data sharing and will now be sent to HRPO for approval before samples are shared with BCM investigators.

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 now been conducted as previously described and the final report from RIO staff is anticipated in the next quarter.

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 was conducted after a continuous performance test (CPT) of information processing and sustained attention task 30 GW veterans were assessed for differences in glucose utilization when compared with GW veteran healthy controls. Recruitment has been completed for this this pilot study and data analysis is in progress. 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 [^{18}F] 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

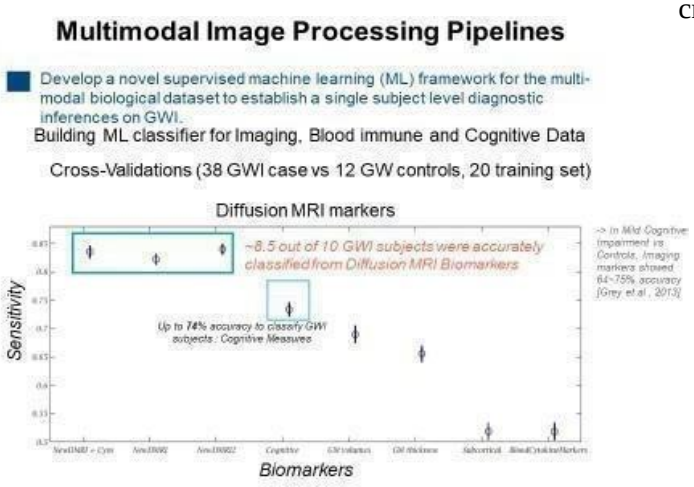
Data entry of all questionnaires and evaluations has been ongoing in as close to real time as possible. The Data Coordinating Center also tracks missing and inconsistent data. The latest data integrity report is listed below and shows very few missing or out of range data points.

GWIC: Gulf War Illness Consortium
Data Integrity Report
Data as of October 1, 2019

Site	*Out of Range Data*	*Missing Data Fields*	*Invalid Logic*
Boston	0.04% (29 of 77979)	0.12% (286 of 245377)	0.00% (0 of 4191)
Miami	0.03% (5 of 16214)	0.13% (67 of 50877)	0.00% (0 of 869)
Texas	0.03% (13 of 40926)	0.24% (307 of 128802)	0.00% (0 of 2200)
Total	0.03% (47 of 135119)	0.16% (660 of 425056)	0.00% (0 of 7260)

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

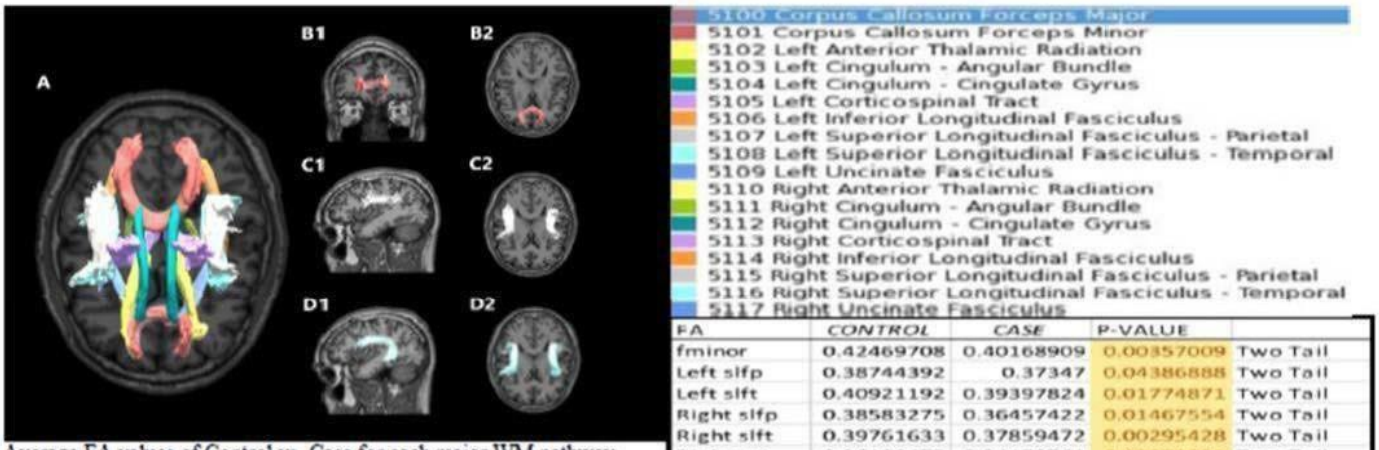
Interim analyses were presented above in Task 6 and additional analyses of machine learning multi-modal approach to brain connectomics is continuing to be conducted. Results to date are very promising and show that diffusion tensor imaging of WM microstructural outcomes predict Kansas GWI cases 85% of 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.



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. Little from the GWIC Houston site has observed a number of pathogical 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 maj or pathways in the brain of each subject. A single high resolution structural scan and a High Angular Resolution Diffusion-weighted Ima ging (HARDI) scan was input into TRACULA. For each pathway, we obtained measures of average Fractional Anisotrophy (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.



FA	CONTROL	CASE	P-VALUE	
fminor	0.42469708	0.40168909	0.00857009	Two Tail
Left slfp	0.38744392	0.37347	0.04386888	Two Tail
Left slft	0.40921192	0.39397824	0.01774871	Two Tail
Right slfp	0.38583275	0.36457422	0.01467554	Two Tail
Right slft	0.39761633	0.37859472	0.00295428	Two Tail
Right atr	0.36188475	0.34473761	0.03079892	Two Tail

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 year 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 will utilize 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 GWI'
4. 'Identifying Clinically Relevant Objective Brain Imaging Diagnostic Markers of GWI'
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 GWI'
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 6 years with corresponding quarterly reports in between.

Key Research Accomplishments: Twenty-five 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. Clinical results to date suggest that proinflammatory cytokines are increased in veterans with GWI and this correlates with behavioral outcomes including cognitive functioning and health symptoms of fatigue, pain and sleep problems. Brain imaging results indicate that veterans with GWI show atrophy in the frontal and parietal lobes and brain white matter. Microstructural integrity of the white matter is also impaired in veterans with GWI and correlates with proinflammatory cytokines.

Conclusion: Several promising therapeutic avenues have been developed to date in the preclinical studies and several more have been approved for ongoing 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 corrrelate with blood markers including cytokines and glutamate. These provide key areas to focus on for objective biomarkers of GWI and focused treatment targets.

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. Seth RK, Maqsood R, Mondal A, Bose D, Kimono D, Holland LA, Janulewicz Lloyd P, Klimas N, Horner RD, Sullivan K, Lim ES, Chatterjee S. Gut DNA Virome Diversity and Its Association with Host Bacteria Regulate Inflammatory Phenotype and Neuronal Immunotoxicity in Experimental Gulf War Illness. *Viruses*. 2019 Oct 21;11(10). pii: E968. doi: 10.3390/v11100968.

2. Joshi U, Pearson A, Evans JE, Langlois H, Saltiel N, Ojo J, Klimas N, Sullivan K, Keegan AP, Oberlin S, Darcey T, Cseresznye A, Raya B, Paris D, Hammock B, Vasylieva N, Hongsibsong S, Stern LJ, Crawford F, Mullan M, Abdullah L. A permethrin metabolite is associated with adaptive immune responses in Gulf War Illness. *Brain Behav Immun*. 2019 Oct;81:545-559. doi: 10.1016/j.bbi.2019.07.015. Epub 2019 Jul 17.

3. Janulewicz, Patricia A., Ratanesh K. Seth, Jeffrey M. Carlson, Joy Ajama, Emily Quinn, Timothy Heeren, and others, The Gut-Microbiome in Gulf War Veterans: A Preliminary Report, *International Journal of Environmental Research and Public Health*, 16.19 (2019), 3751. https://doi.org/10.3390/ijerph16193751

4. Zundel, Clara G., Maxine H. Krengel, Timothy Heeren, Megan K. Yee, Claudia M. Grasso, Patricia A. Janulewicz Lloyd, and others, Rates of Chronic Medical Conditions in 1991 Gulf War Veterans Compared to the General Population. *International Journal of Environmental Research and Public Health*, 16.6 (2019). https://doi.org/10.3390/ijerph16060949

5. Belgrad, Jillian, Dipankar J. Dutta, Samantha Bromley-Coolidge, Kimberly A. Kelly, Lindsay T. Michalovicz, Kimberly A. Sullivan, and others, Oligodendrocyte Involvement in Gulf War Illness. *Glia*, 67.11 (2019), 2107–24. https://doi.org/10.1002/glia.23668

6. Jeffrey, Mary G., Maxine Krengel, Jeffrey L. Kibler, Clara Zundel, Nancy G. Klimas, Kimberly Sullivan, and others, 'Neuropsychological Findings in Gulf War Illness: A Review', *Frontiers in Psychology*, 10 (2019). https://doi.org/10.3389/fpsyg.2019.02088

7. Chatterjee, Saurabh, Kimono, Diana, Sutapa Sarkar, Muayad Albadrani, Ratanesh Seth, Dipro Bose, Ayan Mondal, and others, 'Dysbiosis-Associated Enteric Glial Cell Immune-Activation and Redox Imbalance Modulate Tight Junction Protein Expression in Gulf War Illness Pathology', *Frontiers in Physiology*, 10 (2019). https://doi.org/10.3389/fphys.2019.01229

8. Latimer, Jean J., Abdullah Alhamed, Stefanie Sveiven, Ali Almutairy, Nancy G. Klimas, Maria Abreu, and others, 'Preliminary Evidence for a Hormetic Effect on DNA Nucleotide Excision Repair in Veterans with Gulf War Illness', *Military Medicine* (2019). https://doi.org/10.1093/milmed/usz177

9. Michalovicz, Lindsay T., Kimberly A. Kelly, Saurabh Vashishtha, Rotem Ben-Hamo, Sol Efroni, Julie V. Miller, and others, 'Astrocyte-specific Transcriptome Analysis Using the ALDH1L1 BacTRAP Mouse Reveals Novel Biomarkers of Astrogliosis in Response to Neurotoxicity', *Journal of Neurochemistry*, 150.4 (2019), 420–40. https://doi.org/10.1111/jnc.14800

10. O'Callaghan JP, Miller DB. Neuroinflammation disorders exacerbated by environmental stressors. *Metabolism*. 2019 Nov;100S:153951. doi: 10.1016/j.metabol.2019.153951.

11. Michalovicz LT, Locker AR, Kelly KA, Miller JV, Barnes Z, Fletcher MA, Miller DB, Klimas NG, Morris M, Lasley SM, O'Callaghan JP. Corticosterone and pyridostigmine/DEET exposure attenuate peripheral cytokine expression: Supporting a dominant role for neuroinflammation in a mouse model of Gulf War Illness. *Neurotoxicology*. 2019 Jan;70:26-32. doi: 10.1016/j.neuro.2018.10.006. Epub 2018 Oct 16.

12. 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.*

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15. 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*

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17. 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

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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.

K. 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.** Military Biorepositories. National Academy of Science (NAS) Workshop on Gulf War Respiratory Health Committee (Invited Speaker), Washington, DC, October 3, 2019.

K. **Sullivan.** Military Occupational Health and Toxicology. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.

N. Klimas. Can We "Reboot" Human Homeostasis to Cure Chronic Illness? What We Are Learning from Gulf War Illness and ME. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.

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, England July 6th- 8th 2017.

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 –

REPORTABLE OUTCOMES

Current/Newly Funded Studies

- Clarifying the Role Played by Microglia and Astrocyte Activation in Veterans with Gulf War Illness Using Positron Emission Tomography (PET) (PI: Killiany)(GW180103)
- Identifying Objective Diagnostic Markers of Gulf War Illness: Salivary and Plasma Autoantibodies Against Neural Proteins Validated With Brain Imaging (PI: Abou Donia) (GW180121)
- Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response (PI: Sullivan) (GW180099)
- 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, 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; 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: Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers of White Matter Integrity and Hemodynamic Response (PI: Sullivan)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180099)
Performance period: 09/15/19 – 08/31/22
Level of funding: $1,111,305

Brief description of project's goals/Specific aims: The study objectives are to confirm, validate and further define white matter (WM) microstructural integrity decrements in multiple imaging modalities (DKI and HARDI). This proposal also aims to assess the overlap of WM decrements with cerebral blood flow (CBF) alterations (pCASL) in GWI cases vs controls. Specific Aims include: 1) To compare, validate and further refine the pathobiology of WM microstructural pathways and crossing fibers in GWI 2) To compare longitudinal patterns of brain volumetric, microstructural and CBF differences in 50 GWI cases and 50 healthy control veterans. Aim 3: To perform machine learning advanced analytic data reduction analyses on GWIC and BBRAIN datasets to predict GWI case status and symptom severity over time.

Title: Clarifying the Role Played by Microglia and Astrocyte Activation in Veterans with Gulf War Illness Using Positron Emission Tomography (PET) (PI: Killiany)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180103)
Performance period: 10/01/19 – 09/30/22
Level of funding: $905,790

Brief description of project's goals/Specific aims: The primary objective of project is to assess the role played by Microglia and Astrocyte Activation in Gulf War Illness. The two group of subjects (10 GWI cases, 10 GW controls) will undergo a Fluorodeoxyglucose [18F] (FDG) PET scan at Boston Medical center and a second PET scan for astrocytes ([11C]-l-Deprenyl ligand) at Massachusetts General Hospital on their 3T MRI/PET scanner. GWI cases will be asked to undergo a third PET scan, this one using the ligand for microglia [11C]PBR28. Putting these data together will provide us with a better understanding of the role played by activated astrocytes in GWI and how readily this can be assessed using standard equipment and PET ligands that are readily available. Further, information from the [11C]PBR28 PET ligand binding will determine how much of the binding in the [11C]-l-Deprenyl may be coming from microglia activation.

Title: Identifying Objective Diagnostic Markers of Gulf War Illness: Salivary and Plasma Autoantibodies Against Neural Proteins Validated With Brain Imaging (PI: Abou Donia)
Supporting agency: Department of Defense (CDMRP/GWIRP GW180121)
Performance period: 9/30/19 – 9/29/22
Level of funding: $1,004,206

Brief description of project's goals/Specific aims: The project's specific aims are to (1) compare the levels of IgG-class autoantibodies for central nervous system (CNS) markers in the saliva, serum, and plasma of veterans with Gulf War illness (GWI) (100 cases) against healthy Gulf War (GW) veteran controls (50 controls); (2) compare and correlate brain volumetric and microstructural alterations on brain imaging with results of CNS autoantibodies in serum, plasma, and saliva from veterans with GWI and controls; (3) compare the levels of IgG-class autoantibodies for CNS markers in the blood, serum, and plasma; (4) determine the levels of IgG-class autoantibodies for CNS markers in the blood serum, plasma, and saliva of GWI cases with chronic fatigue syndrome (CFS) (50 cases) compared to GWI cases without CFS and healthy GW veteran controls (50 controls); and (5) compare and correlate brain volumetric and microstructural alterations on brain imaging with results of CNS autoantibodies in serum, plasma, and saliva from veterans with GWI and CFS compared with GWI only and healthy GW veteran controls.

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 1) 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, neuropoitetic 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 – 9/29/19
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 PON1192 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.

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APPENDICES

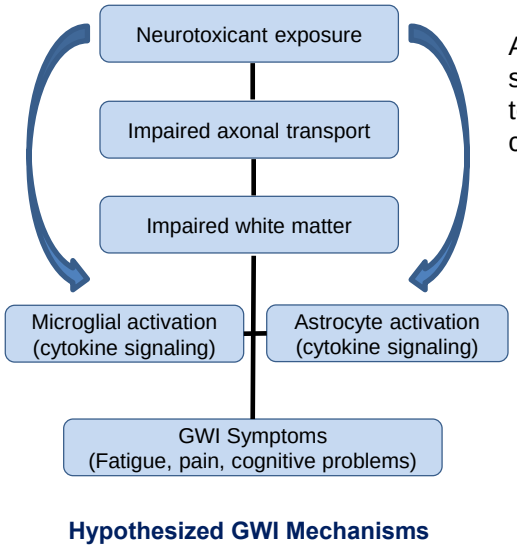
Appendix A – Quad Chart

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

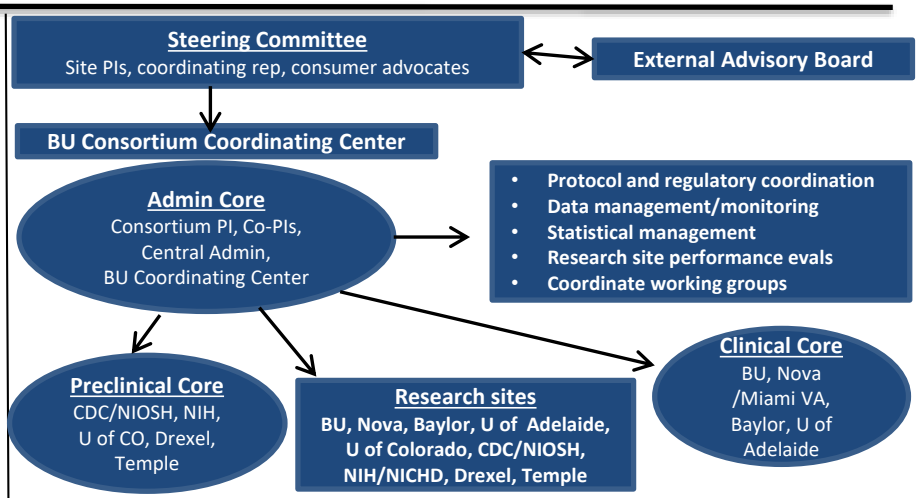


Hypothesized GWI Mechanisms

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 neurotoxicants 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 264 subjects completed. **7-** Twenty additional grant applications were funded for further collaborative research efforts. **8** -Conference symposia, 30 abstracts and 25 manuscripts published, multiple news stories published.

Timeline

Sept 2013 Start

Year

Year	1			2			3			4			Total
Months	1-4	5-8	9-12	13-16	17-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	
Task1													
Task 2													
Task 3													
Task 4													
Task 5			50	30	30	25	33	33	34	55	0		n=300 human subjects
Task 6						15	20	20	20	15	0		n=90 human subjects
Task 7													
Task 8													

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)**

Received: 28 November 2018 | Revised: 5 June 2019 | Accepted: 14 June 2019

DOI: 10.1002/glia.23668

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SPECIAL ISSUE ARTICLE

Oligodendrocyte involvement in Gulf War Illness

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Funding information
Center for Scientific Review, Grant/Award Number: ziahd000713-22; U.S. Department of Defense, Grant/Award Number: w81xwh-13-2-0072; U.S. Department of Defense, Grant/Award Number: 11162432

Abstract

Low level sarin nerve gas and other anti-cholinesterase agents have been implicated in Gulf War illness (GWI), a chronic multi-symptom disorder characterized by cognitive, pain and fatigue symptoms that continues to afflict roughly 32% of veterans from the 1990–1991 Gulf War. How disrupting cholinergic synaptic transmission could produce chronic illness is unclear, but recent research indicates that acetylcholine also mediates communication between axons and oligodendrocytes. Here we investigated the hypothesis that oligodendrocyte development is disrupted by Gulf War agents, by experiments using the sarin-surrogate acetylcholinesterase inhibitor, diisopropyl fluorophosphate (DFP). The effects of corticosterone, which is used in some GWI animal models, were also investigated. The data show that DFP decreased both the number of mature and dividing oligodendrocytes in the rat prefrontal cortex (PFC), but differences were found between PFC and corpus callosum. The differences seen between the PFC and corpus callosum likely reflect the higher percentage of proliferating oligodendroglia in the adult PFC. In cell culture, DFP also decreased oligodendrocyte survival through a non-cholinergic mechanism. Corticosterone promoted maturation of oligodendrocytes, and when used in combination with DFP it had protective effects by increasing the pool of mature oligodendrocytes and decreasing proliferation. Cell culture studies indicate direct effects of both DFP and corticosterone on OPCs, and by comparison with in vivo results, we conclude that in addition to direct effects, systemic effects and interruption of neuron–glia interactions contribute to the detrimental effects of GW agents on oligodendrocytes. Our results demonstrate that oligodendrocytes are an important component of the pathophysiology of GWI.

KEYWORDS
acetylcholine, activity-dependent myelination, cholinergic, corticosterone, Gulf War illness, myelin, organophosphate, plasticity, white matter

1 | INTRODUCTION

Gulf War Illness (GWI) is a chronic multi-symptom disorder that continues to afflict about a third of veterans who returned from the 1990–91 Gulf War (GW), a multi-nation coalition led by the U.S. against

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the Iraqi invasion of Kuwait (Steele, Lockridge, Gerkovich, Cook, & Sastre, 2015; White et al., 2016). The cause of GWI is unknown, but exposure to low-level sarin nerve gas, pesticides and pyridostigmine bromide (PB), all acetylcholinesterase (AChE) inhibitors, have been linked to the etiology of GWI (Sullivan et al., 2018; White et al., 2016). PB, a reversible AChE inhibitor, was ingested by GW veterans as a prophylactic against potential exposure to sarin nerve gas, an irreversible and lethal AChE inhibitor (Sullivan et al., 2003; White et al., 2016). Pesticides, predominantly organophosphates like chlorpyrifos and dichlorvos, were applied, often in excess, to protect against insects in the battle lines along the Mesopotamian marshes (Sullivan et al., 2018). Naturally, the primary focus of GWI research has been on the consequences of disrupting cholinergic synaptic neurotransmission. However, it is unknown how transient disruption of cholinergic synaptic neurotransmission can lead to chronic neurological deficits that have persisted for over two decades in GWI patients. Recent research has shown that neurotransmitters are also involved in communication between axons and myelinating glia. Here, we test the hypothesis that disrupting cholinergic neuro-glial communication via AChE inhibitors can impair oligodendrocyte development and function, and thereby contribute to the chronic pathophysiology of GWI.

In addition to synaptic release, neurotransmitters are released non-synaptically along axons through exocytosis at axonal varicosities (Wake et al., 2015) and through ion channels (Fields, 2011; Vizi & Lendvai, 2008). Recent research indicates that oligodendrocyte development and myelination are impaired when this axo-glial signaling is disrupted. For example, blocking vesicular release of the neurotransmitter glutamate from axons by Botulinum toxin treatment, inhibits local protein synthesis of the major protein in myelin, myelin basic protein (MBP), and impairs induction of myelination on electrically active axons (Wake, Lee, & Fields, 2011). Activity-dependent myelination has been primarily studied in the context of glutamatergic signaling (Fields, 2015; Kukley, Capetillo-Zarate, & Dietrich, 2007; Wake et al., 2011; Wake et al., 2015), but the neurotransmitter acetylcholine (ACh) has recently been suggested to influence oligodendrocyte progenitor cell (OPC) proliferation, differentiation, and myelination (De Angelis, Bernardo, Magnaghi, Minghetti, & Tata, 2012; Fields, Dutta, Belgrad, & Robnett, 2017). In the CNS, cholinergic neurons in the basal forebrain extend long-range axons to broadly innervate the entire cerebral cortex (Luchicchi, Bloem, Viaña, Mansvelder, & Role, 2014; Wu, Williams, & Nathans, 2014). Associated with arousal, focus, and emotional salience, ACh signaling has been found to modulate plasticity of synapses across brain regions including the hippocampus, hypothalamus, and visual cortex (Luchicchi et al., 2014; Picciotto, Higley, & Mineur, 2012). Receptors in cholinergic signaling include the ionotropic nicotinic acetylcholine receptors, and the G-protein coupled muscarinic receptors. The acetylcholinesterase enzyme (AChE) degrades ACh at cholinergic synapses to terminate synaptic transmission and thereby prevents neuronal hyperactivity and excitotoxicity. In contrast to synaptic transmission, far less is known about the effects of acetylcholine and AChE inhibitors on oligodendrocyte development and function.

The severity of GWI correlates with exposure to pesticides and PB in a dose-dependent manner (Steele, Sastre, Gerkovich, & Cook,2012; Wolfe, Proctor, Erickson, & Hu, 2002). Butyrylcholinesterase (BChE) is a nonspecific cholinesterase that hydrolyzes choline-based esters including toxicants such as organophosphate pesticides. A genetic variant of BChE, that encodes a less active form of BChE and hence is less adept at neutralizing GW toxicants, is one of the reported genetic risk factors for GWI (Steele et al., 2015). GW veterans with the atypical BChE gene are more susceptible to developing GWI upon exposure to GW pesticides. Together these findings highlight the important role of AChE inhibitors in the pathophysiology of GWI (Golomb, 2008).

Recent studies provide support for this previously unexplored hypothesis of involvement of myelinating glia in the pathophysiology of GWI. Brain imaging studies have reported white matter abnormalities in GW veterans (Chao, Zhang, & Buckley, 2015; Heaton et al., 2007) and such disruption has been associated with the key diagnostic symptoms of GWI: musculoskeletal pain (Rayhan et al., 2013; Van Riper et al., 2017), impaired attention (Janulewicz et al., 2017), disturbances of mood (Van Riper et al., 2017), and chronic fatigue (Rayhan et al., 2013). However, white matter is a complex tissue comprised of axons, astrocytes, oligodendrocytes, vascular cells, and microglia. Therefore, alterations in white matter detected by MRI could result from many types of cellular perturbations, including changes in axon number, diameter, tortuosity, vascular changes, alterations in astrocyte number or morphology, as well as direct effects on myelin. Furthermore, loss of myelin could be secondary to loss of axons, rather than a direct effect on oligodendrocytes.

Myelination proceeds in different brain regions at different times, but the process continues through adolescence into early adulthood. In the prefrontal cortex (PFC), myelination continues during the early 20s (Miller et al., 2012), a demographic accounting for roughly 50% of deployed GW soldiers (Veterans Affairs, 2011). Additionally, there is a reserve pool of proliferative NG2+ cells in the adult brain, which have the potential to generate oligodendrocyte lineage cells throughout life (Nishiyama, Suzuki, & Zhu, 2014; Kang, Fukaya, Yang, Rothstein, & Bergles, 2010). Epidemiological data indicate that GW veterans who report impaired cognition as their prominent symptom were significantly younger than their GW veteran counterparts with no-symptoms or who experience primarily sensory symptoms (Gopinath et al., 2012). This pattern is consistent with possible involvement of disrupted PFC myelination in GWI and presents a compelling hypothesis for the neurological and cognitive impairments of GWI.

Here we test the hypothesis that acute exposure to AChE inhibitors affects oligodendrocyte proliferation, differentiation, survival, and myelination. These studies were carried out in an established Center for Disease Control (CDC) rat model of GWI (Koo et al., 2018) in combination with studies in cell culture. Key diagnostic features of GWI include musculoskeletal pain, impaired cognitive functioning, disturbances of mood, and debilitating fatigue; symptoms that have persisted over time (Binns et al., 2008; Maule et al., 2018; White et al., 2016). GWI animal models replicate many of these symptoms (Zakirova et al., 2016), including impaired working memory (Phillips & Deshpande, 2018) and social memory (Zakirova et al., 2016). The established animal model of GWI includes treatment with corticosterone (Cort) for 7 days before exposure to diisopropyl fluorophosphate (DFP), an irreversible AChE inhibitor used as

a proxy for sarin nerve gas (Koo et al., 2018; O'Callaghan, Kelly, Locker, Miller, & Lasley, 2015; Zakirova et al., 2016). This necessitates studying the effects of Cort exposure independently, and together with DFP, on oligodendrocyte development and function. Interaction between these two agents is possible in influencing oligodendroglial biology. Stress and corticosterone have been shown to influence oligodendrocyte and myelin biology outside of the context of GWI. Corticosterone treatment has been shown to inhibit OPC proliferation (Alonso, 2000), promote OPC differentiation (Mann et al., 2008), and shorten the node of Ranvier length (Miyata et al., 2016). Inhibition of ACh signaling promotes remyelination in experimental autoimmune encephalomyelitis studies, an animal model of Multiple Sclerosis (MS), and in human MS clinical trials (Abiraman et al., 2015; Green et al., 2017; Li, He, Fan, & Sun, 2015; Liu et al., 2016; Mei et al., 2014; Welliver et al., 2018). Although this treatment is therapeutic for a demyelinating disease, disrupting ACh signaling may be detrimental to oligodendroglia in other contexts. In studies reported here, effects of DFP and Cort were investigated in adult rats in the GWI model, in the PFC, which is still undergoing myelination, and in subcortical white matter (corpus callosum), which in comparison to PFC is undergoing less active myelination.

Both DFP and Cort may act directly on oligodendroglia and indirectly by disrupting neuron–glia interactions. In addition to disrupting cholinergic signaling, these agents could have non-cholinergic actions or produce systemic effects, such as vascular and immune responses, that could have detrimental effects on myelinating glia. These alternatives were investigated using a combination of in vivo and in vitro studies. The results indicate that exposure to DFP, with and without Cort, disrupts oligodendrocyte development in the GWI animal model. In vitro experiments using purified oligodendrocyte lineage cell monocultures, in the absence of detectible ACh, indicate that DFP and corticosterone have direct effects on oligodendroglial cell proliferation and survival, but these effects differ in important respects from those seen in the animal model of GWI. This finding distinguishes the consequences of the systemic and non-cholinergic effects of GW agents from their role in disrupting cholinergic signaling between axons and oligodendroglia as AChE inhibitors. The results of this study support the conclusion that oligodendrocyte biology is an important contributor to the pathophysiology of GWI and that GW agents impair cholinergic signaling between axons and myelinating glia but also have direct non-cholinergic effects on these cells. Cort treatment in the GWI animal model has additional and, in some respects, counteracting effects to DFP on oligodendrocytes. The findings suggest possible therapeutic approaches to alleviate the chronic neurological symptoms in GW veterans from exposure to anticholinesterase agents during the GW.

2 | MATERIALS AND METHODS

2.1 | Mixed glial cell culture preparation

Primary rat mixed glial cell cultures were generated from P1-2 day old wild-type Sprague–Dawley rat pups. Briefly, pups were decapitated, and their cerebral cortices were isolated, minced, separated into a single cell suspension, and plated in T75 flasks. Mixed glial cultures were grown in Dulbecco's Modified Enriched Media (DMEM, ThermoFisher Scientific, Waltham, MA, Cat. No. 11995–065) that contained high glucose, L-glutamine, phenol red, and sodium pyruvate with 10% Fetal Bovine Serum (FBS, ThermoFisher Scientific, Cat. No. 16000–044) for 3 weeks at 37°C and 10% CO_2.

2.2 | Oligodendrocyte progenitor cell purification

At 3–4 weeks post-dissection, flasks were shaken (180 rpm) for an hour at 37°C to remove microglia and dead cells, followed by a compete media change and an overnight shake under the same conditions. Media was collected from shaken flasks and plated onto two 6 cm tissue culture dishes per flask, for 15 min, to separate OPCs from heavier endothelial and astrocyte cells. Supernatant from 6 cm dishes was collected and centrifuged for 10 min at 1200 rpm. Cells were then plated onto 25 mm glass coverslips coated with 0.1 mg/mL poly-L-lysine (PLL) (Sigma-Aldrich, P9155) and 0.1 mg/mL poly-L-ornithine (PLO) (Sigma-Aldrich, P3655). Coverslips were used for calcium imaging 1–3 days post-plating. 80–90% of the cells on coverslips used for experiments were oligodendrocytes as confirmed by immunocytochemistry with the pan-oligodendrocyte marker, Olig2. Purified OPCs were grown in DMEM+10% FBS (described above) without additional growth factors.

2.3 | Calcium imaging

Calcium imaging was performed on OPCs 2–3 days post-plating using the fluorescent calcium chelator dye, Fura2 AM (Invitrogen, Carlsbad, CA Cat. No. F1221). Fifty microgram Fura-2 AM was added to 50 μL Pluronic Acid (Invitrogen, Cat. No. P3000-MP). Fifteen microliter of the Fura-2 Pluronic acid solution was added to HEPES buffer and brought to a total volume of 1.5 mL. HEPES buffer (pH 7.4) consisted of 8 g/L Sucrose, 1 g/L D-glucose, 20 mM HEPES stock (150 nM NaCl, 10 nM HEPES, 3 mM KCl), 2 mM $CaCl_2$, 2 mM $MgCl_2$. One mililiter of the diluted Fura-2 was added to a 35 mm dish containing the 25 mm coverslip containing OPCs for 15 min in the dark at 37°C. After 15 mins, 1 mL of HEPES buffer was added and the cells were then incubated a second time for 15 min in the dark at room temperature. Coverslip was washed one time for 10 min with HEPES buffer before use. MetaFluor Software (Molecular Devices) was used to image and measure fluorescence emission at 340 and 380 nm excitation wavelengths. Acetylcholine was diluted in HEPES buffer to 1 and 50 μM concentrations. Intracellular Fura-2 levels were calibrated using 10 μM A23187 with and without EGTA or Ca^{2+} in buffer. Calcium concentrations were calculated from fluorescence levels using the equation derived by Grynkiewicz, Poenie, and Tsien (1985). While sampling, N was defined as a coverslip while n was defined as a cell.

2.4 | Oligodendrocyte differentiation

OPCs were differentiated in N1 media with 0.2% FBS. OPCs were plated from flasks, as described above, in DMEM + 10% FBS for 24 hr and then switched to N1 media + 0.2% FBS for the remainder of the experiment.

2.5 | Astrocyte cultures

At 3–4 weeks post dissection, flasks were shaken overnight at 37°C and 1200 rpm to remove microglia and dead cells. Media was removed, and flasks were washed twice with sterile Earle's Balanced Salt Solution (EBSS). Trypsin, warmed to 37°C, was added to the flasks and trypsinization was stopped 10 min later with 1:1 addition of DMEM + 10% FBS. Cells were collected and centrifuged at 1200 rpm and then plated on PLL/PLO coated 25 mm glass coverslips.

2.6 | In vitro cell culture treatments

OPCs isolated from postnatal day 2 (P2) cerebral cortex were treated 24 hr after plating. Cells were fixed with 4% paraformaldehyde and immunocytochemistry was performed 72 hr following treatment.

2.7 | Immunocytochemistry

Coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in Phosphate Buffered Saline (PBS, Sigma-Aldrich, St. Louis, MO) for 20 min followed by the addition of 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 5 min. The coverslips were washed three times with PBS and blocked for 1 hr in 5% Goat Serum in PBS (Fisher Scientific). The coverslips were then incubated overnight at 4°C with the primary antibody. The coverslips were then washed three times with PBS and incubated with secondary antibody for 2 hr at room temperature. Coverslips were washed three times and plated on microscope slides with Vectorshield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA) for imaging. For immunocytochemistry, the primary antibodies used were: Olig2 (EMD Millipore, Rabbit) at 1:500, Olig2 (EMD Millipore, Mouse, Cat. No. MABN50) at 1:500, Myelin Basic Protein (EMD Millipore, Chicken) at 1:200, AChE (Invitrogen, Mouse) at 1:200, Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Rabbit) at 1:200. The secondary antibodies used were: Alexa Fluor 488 (Thermo Fisher Scientific, Goat Anti-Rabbit IgG) at 1:1000, Alexa Fluor 488 (Thermo Fisher Scientific, Goat Anti-Mouse) at 1:1000, Alexa Fluor 568 (Thermo Fisher Scientific, Goat Anti-Mouse) at 1:1000, Alexa Fluor 568 (Thermo Fisher Scientific, Goat Anti-Rabbit) at 1:1000, Alexa Fluor 555 (Thermo Fisher Scientific, Goat Anti-Chicken) at 1:1000.

2.8 | Pharmacological agents

Acetylcholine (Sigma), DFP (Sigma), Corticosterone (Sigma) and Calcium Ionophore A23187 (Sigma) were used in the study. Ethanol (0.6%) was used as the corticosterone vehicle for in vivo and in vitro experiments. Doses used for each experiment are described in the results section.

2.9 | GWI animal model

Adult male Sprague Dawley rats aged 6–8 weeks received Corticosterone (Cort, 200 mg/L in 0.6% ethanol) in drinking water for days 1–7, followed by a single subcutaneous (s.c.) injection of DFP (1.5 mg/kg) on the morning of day 8. Animals were sacrificed 12, 24, 72 hr, or 21 days after DFP exposure.

2.10 | Tissue sectioning

Rats were sacrificed by decapitation and brains were rapidly removed. One hemisphere was frozen for protein analysis and the other was post-fixed in 4% paraformaldehyde overnight and cryopreserved in 30% sucrose for up to 4 days. Following adequate cryopreservation, brains were embedded in optimal cutting temperature (OCT) embedding media (Fisher Healthcare). Embedded tissue was cyrosectioned into 14 μm thick sagittal sections.

2.11 | Immunohistochemistry

Tissue sections were brought to room temperature and then rinsed one time with PBS to dissolve the embedding medium OCT. The sections were then incubated in Citrate buffer, pH 6, at 94°C for 10 min to promote epitope retrieval. The tissues sections were then rinsed three times with PBS containing 0.1% TritonX-100 and blocked for 1 hr in blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS). Sections were incubated overnight at 4°C with the primary antibodies. Primary antibodies used include: APC/CC1 (Millipore, Mouse) at 1:500; Olig2 (Millipore, Rabbit) at 1:500; Ki67 (Abcam, Rabbit) at 1:500; Olig2, (Millipore, Mouse) at 1:100. The following day, the tissue sections were rinsed three times with PBS containing 0.3% TritonX-100 and then incubated for 2 hr at room temperature with secondary antibodies (listed in the Immunocytochemistry methods section) at 1:200 dilution. Tissue sections were then rinsed three times with PBS and mounted on coverslips using mounting medium (Vectashield Antifade Mounting Medium with DAPI). Ki67 staining was performed on animals fixed 24 hr after treatment. CC1 staining was performed on animals fixed 21 days after treatment. A fluorescent light microscope with AxioCam MRm was used to acquire 10 images per region per animal at 40 X magnification.

2.12 | Acetylcholine release assay

Cells were plated onto coverslips as described above and incubated with 150 μL buffer or DFP at 37°C. Conditioned buffer was collected, and flash frozen after 4 hr. Buffer was analyzed with a Choline/Acetylcholine fluorometric assay kit (Abcam). To measure total levels of acetylcholine and choline ([Ach + Ch]), AChE enzyme was used in the buffer. To measure choline levels excluding acetylcholine, ([ACh]), DFP was added to the buffer with no AChE.

2.13 | Immunoblotting

To extract proteins from tissue and cell culture, samples were lysed in RIPA buffer (Sigma Aldrich) with protease inhibitor cocktails (Complete Mini EDTA-free Protease Inhibitor Cocktail, Sigma Aldrich). Lysate was mixed with LDS sample buffer (Thermo Fisher Scientific)

and electrophoresed in a 4–12% Bis-Tris Gel (Invitrogen) for 2 hr at 150 V in MOPS-SDS running buffer (Thermo Fisher Scientific). The samples were transferred to PVDF membrane (Immobilon-P, Millipore) overnight at 4°C in Tris-Glycine transfer buffer (Thermo Fisher Scientific). Membranes were blocked in blocking buffer, containing TBS (10 mM Tris–HCl, pH 7.5, 0.9% NaCl), 0.1% (vol/vol) Triton X-100 and 5% (wt/vol) bovine serum albumin (MP Biomedicals) or 5% nonfat dry milk (American Bio), for 1 hr at room temperature (RT). The appropriate primary antibody was diluted in blocking buffer and incubated overnight with the PVDF membrane at 4°C. Primary antibodies used were: GAPDH (Cell Signaling Tech, Rabbit) used at 1:4000; NSE (Abcam, Rabbit) used at 1:2000; GAPDH (Encor, Mouse) used at 1:4000 dilution; MBP (Millipore, Rabbit) used at 1:1000; GFAP (Invitrogen, Rabbit) used at 1:500, GAP43 (Millipore Sigma Aldrich, Rabbit) used at 1:1000, NG2 (Abcam, Mouse) used at 1:1000. The PVDF membrane was washed four times, 15 min each, in washing buffer, TBS (10 mM Tris–HCl, pH 7.5, 0.9% NaCl) and 0.1% (vol/vol) Triton X-100. The corresponding secondary antibody, diluted in blocking buffer, was then incubated with the PVDF membrane for 2 hours at RT. Secondary antibodies that were used include ECL Anti-Mouse IgG Horseradish Peroxidase-linked F(ab')2 fragment or ECL Anti-Rabbit IgG Horseradish Peroxidase-linked F(ab')2 fragment. Chemiluminescent substrate was applied for 10 min (SuperSignal West Pico Plus, Thermo Scientific). Membranes were quantified with densitometry using Image J software and normalized to NSE loading control. In reported bar graphs of data, each treatment condition was normalized to saline control levels.

2.14 | Animal protocol

All animal studies for in vivo experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, and the animal facility was certified by AAALAC International. All animal studies for in vitro experiments were performed at the Section on Nervous System Development and Plasticity, Bethesda, MD, according to Animal Study Protocol #15–007, and were approved by the Institutional Animal Care and Use Committee (IACUC), *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH). All animals for in vivo and in vitro studies were approved through the Department of Defense Animal Care and Use Review Office (ACURO).

2.15 | Statistics

Statistics were performed with Minitab 18 (Minitab Inc., State College, PA) and GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). Immunoblot data were analyzed with One-Way ANOVA (Analysis of Variance) followed by Dunnett's multiple comparison post-test. Cell counting data from histology and cell-culture experiments were analyzed using the Chi-Squared statistical test. Figures were made in SigmaPlot 14.0 (Systat Software Inc., San Jose, CA). While sampling for immunoblotting, N was defined as an animal. For histology, *N* was defined as an animal and *n* was defined as a microscopic field of view. For in vitro imaging, *N* was defined as biological experimental replicates and n was defined as a microscopic field of view. For calcium imaging, *N* was defined as a coverslip and *n* was defined as an individual cell. Grouped data are mean ± *SEM* unless stated otherwise.

3 | RESULTS

3.1 | OPCs engage in cholinergic signaling

To understand the etiology of white matter disruption present in veterans with GWI, we investigated how Cort exposure and disrupting cholinergic signaling through AChE inhibition may interfere with oligodendrocyte biology. To do so, we first determined the capacity for OPCs to engage in cholinergic signaling across development. Immunocytochemistry indicated that all cells of the oligodendrocyte lineage express muscarinic acetylcholine receptors (mAChRs) M1–M5 in vitro and expression of these receptors varied in abundance and localization across oligodendrocyte development from immature progenitors to mature myelinating cells (Figure 1a, Table 1). Similarly, AChE was expressed on the membrane of OPCs grown for 24 hr in growth medium (Figure 1b) and oligodendrocytes grown for 3 days in differentiation medium (Figure 1c). The function of AChE in oligodendrocytes is unclear (Fields et al., 2017), but sarin gas and other AChE inhibitors would act on both neuronal and glial AChEs.

We performed live-cell calcium imaging to determine if the mAChRs expressed on OPCs were functional. M1, M3, and M5 receptors signal via activating intracellular Ca^{2+}, but M2 and M4 receptors signal through cAMP. Live-cell calcium imaging of OPC monoculture demonstrated that OPCs respond to 50 μM ACh (Figure 2a, $N = 5$, $n = 56$) and to concentrations as low as 1 μM ACh, (Figure 2b, $N = 5$, $n = 91$) with robust and heterogenous calcium kinetics indicating mAChRs were functional on OPCs. ACh doses of 50 and 1 μM were chosen because similar ACh concentrations have been used previously in studies of the cholinergic neuromuscular synapse (Vianney, Miller, & Spitsbergen, 2014). The response was mediated by mAChR activation, as pretreatment with the m1AChR inhibitor, Pirenzepine (50 μM), eliminated intracellular ACh-mediated Ca^{2+} signaling (Figure 2c, $N = 3$, $n = 9$). The dynamics of intracellular calcium responses varied in different cells in response to ACh (1 μM). The responses included prolonged oscillations, dampened oscillations, and a sharp rise to peak which plateaued and partially recovered (Figure 2d). These varied waveforms suggest multiple underlying intracellular Ca^{2+} release, extrusion, and sequestration processes, as well as heterogeneity in mAChR type and expression levels, in the cell population stimulated with ACh.

The calcium imaging experiments were performed in HEPES Buffer (ingredients defined in Materials and Methods), which does not contain ACh. DFP treatment alone ($N = 4$, $n = 95$) or Cort alone ($N = 3$, $n = 36$) had no effect on intracellular calcium levels measured over a 300 s treatment (Figure 2e). Thus, DFP and Cort do not influence intracellular Ca^{2+} signaling in OPCs in the absence of ACh. No ACh was detected in buffer conditioned by astrocyte or OPCs for 4 hr using an acetylcholine fluorometric assay with a threshold sensitivity of 100 pmol ($N = 3$). The lack of measurable ACh in OPC and astrocyte monocultures suggests that ACh

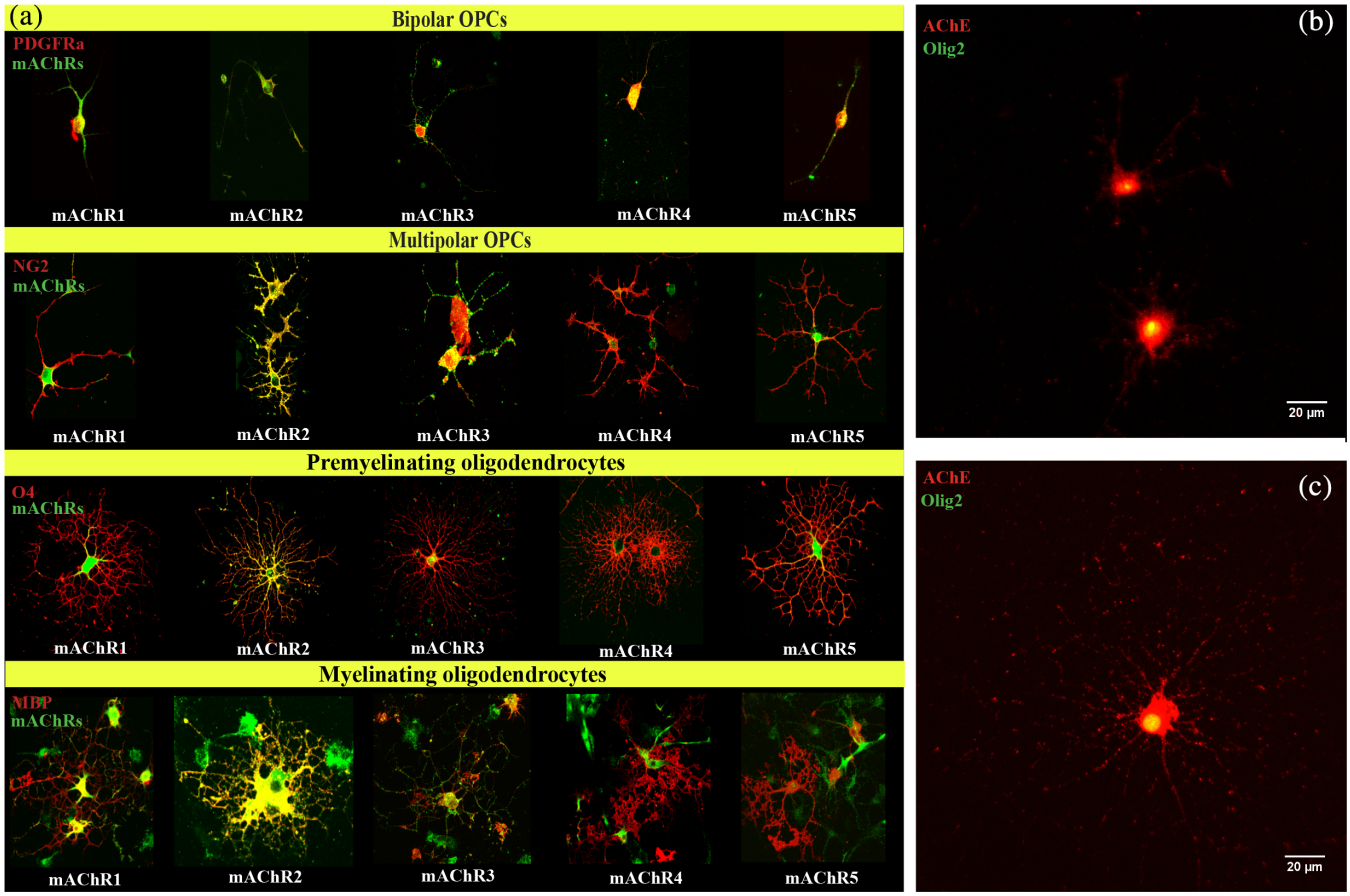


FIGURE 1 Expression of muscarinic receptors and acetylcholinesterase in oligodendrocyte lineage cells. (a) Expression of muscarinic acetylcholine receptors (mAChRs) 1–5 at various stages of oligodendrocyte development, from immature bipolar progenitors to highly branched mature oligodendrocytes, in primary cultures of oligodendrocyte lineage cells. Red are oligodendrocyte markers; green are muscarinic ACh receptors. (b) AChE expression in immature OPCs. Red is AChE; green is Olig2. (c) AChE expression in mature oligodendrocytes. Red is AChE; green is Olig2

TABLE 1 Expression of mAChRs on oligodendrocyte lineage cells

		Muscarinic receptors				
Stages of the oligodendrocyte lineage	**Cellular compartments**	**mAChR1**	**mAChR2**	**mAChR3**	**mAChR4**	**mAChR5**
Bipolar OPCs (PDGFRa+)	Cell body	+++	+++	++	+	+++
	Cell processes	+++	+	++	-	++
Multipolar OPCs (NG2+)	Cell body	+++	+++	++	+	+++
	Cell processes	+	+++	++	-	++
Premyelinating OLs (O4+)	Cell body	+++	+++	++	+	+++
	Cell processes	+	+++	++	-	++
Mature OLs (MBP+)	Cell body	+++	+++	++	+	++
	Cell processes	+	+++	++	-	+

Note: Plus (+) sign indicates relative qualitative levels of receptor expression; dash (–) sign indicates no observable expression.

is not secreted from either OPCs or astrocytes. This supports the hypothesis that release of ACh by neurons signal to oligodendrocytes and that disruption of this neuro-glial signaling could disrupt oligodendroglial development and function.

We therefore applied low concentrations of ACh to OPC cultures, together with DFP, to test whether inhibiting AChE activity on oligodendrocytes would alter their Ca^{2+} responses. The results showed that DFP treatment in the presence of 1 μM ACh increased the percentage of cells that responded to ACh (Figure 2f, 52.8 ± 5.37% vs. 75.9 ± 6.21%, t[15 dishes] = 2.131, p = 0.005). This suggests that inhibition of AChE on OPC cell membrane by DFP increased the concentration of ACh in the extracellular environment, thus eliciting responses from more OPCs. A larger percentage of cells also responded to 50 μM ACh compared with the 1 μM treatment (Figure 2f, 52.8 ± 5.37% vs. 70.6 ± 3.92%, t [15 dishes] = 2.947, p = 0.0118; 50 μM: N = 5, n = 101).

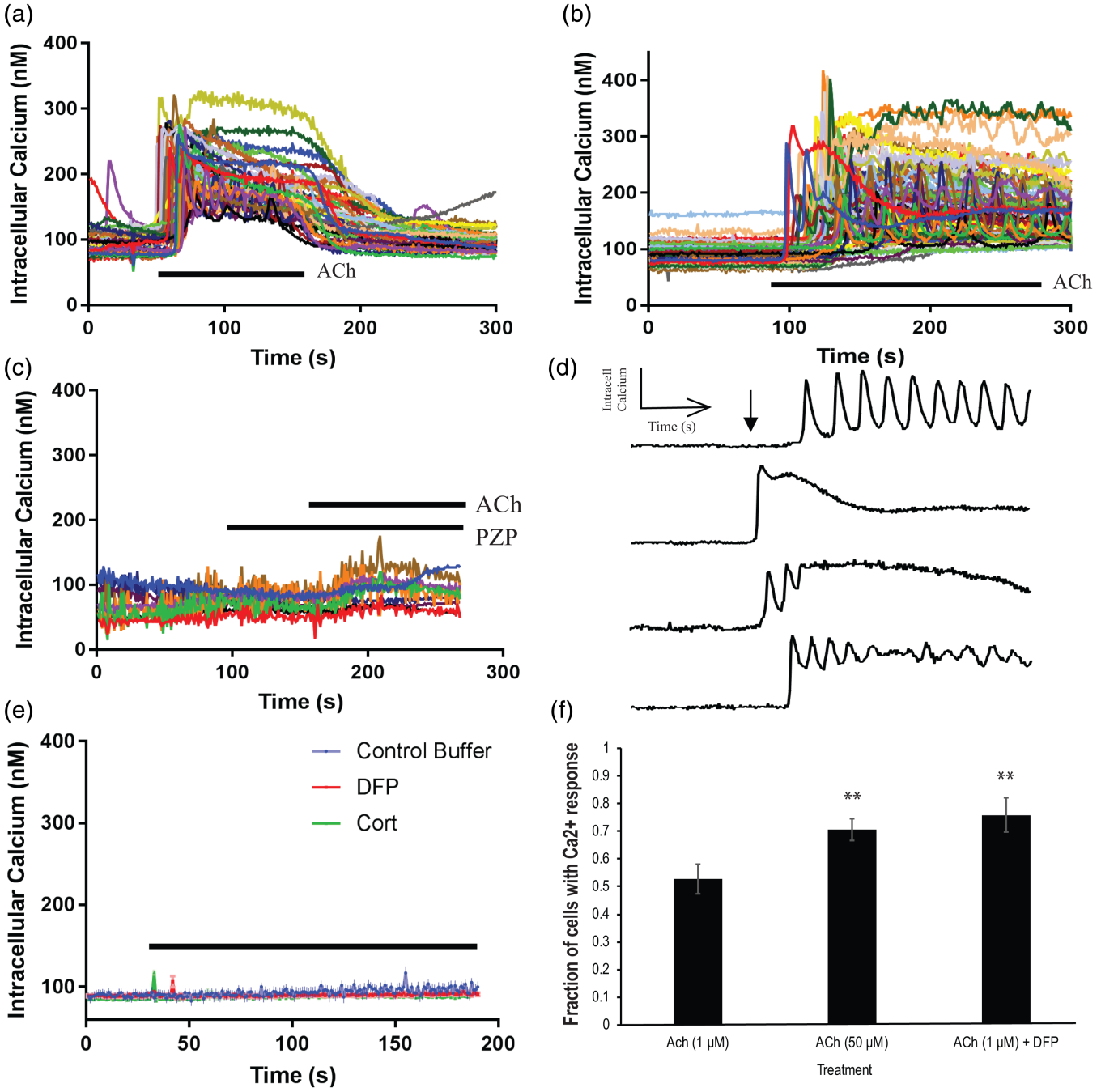
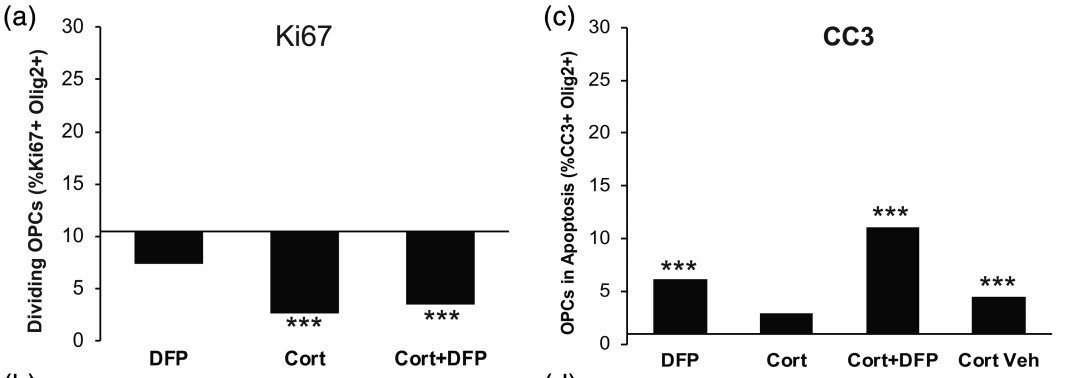


FIGURE 2 ACh induces OPC intracellular calcium response. (a) 50 μM ACh ($N = 5$, $n = 56$). Line indicates duration of treatment. (b) 1 μM ACh ($N = 5$, $n = 91$). Line indicates duration of treatment. (c) Inhibition with mAChR M1 inhibitor, Pirenzepine (PZP, 50 μM), to confirm specificity of mAChR induced calcium response ($N = 3$, $n = 9$). Line indicates duration of treatment. PZP pretreatment onset at 40 s and co-treatment of PZP with 50 μM ACh beginning at 160 s. (d) Representative waveform traces of data presented in b. Arrow indicates onset of ACh treatment. (e) DFP (50 μm) alone and Cort (5 μM) alone had no effect on intracellular calcium mobilization in OPCs. (DFP: $N = 4$, $n = 95$; Cort: $N = 3$, $n = 36$). (f) DFP pretreatment followed by ACh significantly increased the fraction of cells that respond to ACh (52.8 ± 5.37% vs. 75.9 ± 6.21%, t[15 dishes] = 2.131, $p = 0.005$). Fifty micromolar ACh significantly increased the number of cells that respond to ACh compared to 1 μM ACh (52.8 ± 5.37% vs. 70.6 ± 3.92%, t [15 dishes] = 2.947, $p = 0.0118$). Student's t-test was performed comparing ACh (1 μM) and ACh (1 μM) vs. DFP. * indicates $p < 0.05$; ** indicates $p < 0.01$

We conclude that AChE expressed on OPCs is functional and that its inhibition in the presence of ACh leads to increased ACh-dependent Ca^{2+} signaling, which could influence OPC development and function.

3.2 | Direct effects of GW agents on oligodendrocyte biology

Any effect on oligodendrocyte development and function in the GWI animal model or in GW veterans could be due to direct effects on

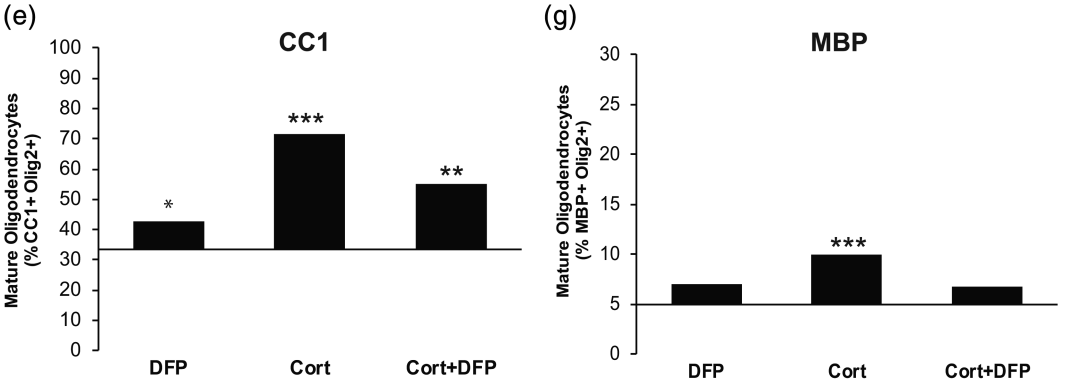


(b)

	Control	DFP	Cort	Cort+DFP
Ki67+Olig2+	60	40	12	14
Total Olig2+	568	541	466	405
Percentage	**10.6**	**7.4**	**2.6**	**3.5**

(d)

	Control	DFP	Cort	Cort+ DFP	Cort Veh
CC3+Olig2+	4	20	13	53	20
Total Olig2+	407	323	445	480	445
Percentage	**0.98**	**6.19**	**2.92**	**11.04**	**4.49**



(f)

	Control	DFP	Cort	Cort+DFP
CC1+Olig2+	324	262	325	352
Total Olig2+	967	611	453	639
Percentage	**33.5**	**42.9**	**71.7**	**55.1**

(h)

	Control	DFP	Cort	Cort+DFP
MBP+Olig2+	50	43	45	43
Total Olig2+	967	611	453	639
Percentage	**5.2**	**7.0**	**9.9**	**6.7**

FIGURE 3 In vitro exposure to GW agents disrupts OPC development. (a) Immunocytochemical staining of OPC monoculture using Ki67 and Olig2 primary antibodies. Cort (χ^2 [1, n = 466] = 26.604, $p < 0.001$) and Cort+DFP (χ^2 [1, n = 405] = 11.150, $p = 0.001$) significantly reduced OPC proliferation, identified as Ki67+ Olig2+ cells, compared to Cort condition. DFP alone had no effect on oligodendrocyte proliferation (χ^2 [1, n = 541] = 2.436, $p = 0.119$). Baseline indicates control value. (b) Table of raw Ki67+ Olig2+ cell counts used to generate part (a). (c) Immunocytochemical staining of OPC monoculture using cleaved caspase 3 (CC3) and Olig2 primary antibodies. DFP (χ^2 [1, n = 323] = 14.316, $p < 0.001$) and Cort+DFP (χ^2 [1, n = 480] = 11.663, $p = 0.001$) treatments significantly increased OPC apoptosis, identified as cleaved caspase 3+ cells. Cort had no effect on apoptosis (χ^2 [1, n = 445] = 1.432, $p = 0.23$). Baseline indicates control value. (d) Table of CC3+ Olig2+ cell counts were used to generate percentages in part (c). (e) Immunocytochemistry staining of oligodendrocyte monoculture (grown in differentiation media for 5 days total) using CC1 and Olig2 primary antibodies. DFP alone, (χ^2 [1, n = 611] = 6.371, $p = 0.012$), Cort alone (χ^2 [1, n = 453] = 28.962, $p < 0.001$) and Cort+DFP (χ^2 [1, n = 639] = 11.133, $p = 0.001$) increased CC1+ mature oligodendrocytes. Baseline indicates control value. (f) Table of CC1+ Olig2+ cell counts used to generate percentages in part (e). (g) Immunocytochemical staining of oligodendrocyte monoculture (grown in differentiation media for 5 days total) using MBP and Olig2 primary antibodies. Cort alone increased MBP+ mature oligodendrocytes (χ^2 [1, n = 453] = 8.419, $p = 0.004$). DFP (χ^2 [1, n = 611] = 2.083, $p = 0.149$) and Cort+DFP (χ^2 [1, n = 639] = 2.449, $p = 0.118$) had no significant effect on counts of MBP+ mature oligodendrocytes. Baseline indicates control value. (h) Table of MBP+ Olig2+ cell counts used to generate percentages in part (g). * indicates $p < 0.05$; ** indicates $p < 0.01$, *** indicates $p < 0.001$. Significance is determined by comparing all treatment conditions with the control condition. DMEM + 10% FBS (used in experiments for parts a–d) or differentiation media (used in experiments for parts e–h). DFP values were compared to control. Cort and Cort+DFP values were compared relative to the "Cort vehicle" control

biology of oligodendrocyte lineage cells, or due to interruption of cholinergic neuron-oligodendrocyte communication or caused by indirect systemic effects resulting from exposure to Cort and DFP. Cholinergic signaling and stress hormone signaling have diverse biological effects in the CNS, PNS, the cardiovascular, and the immune system. Therefore, to test for direct, non-systemic effects of the GW agents on OPCs, we exposed OPCs in vitro to the AChE inhibitor, DFP, and to Cort. We performed a dose response study

with DFP (Figure S1a) and Cort (Figure S1b) to determine the most appropriate treatment concentration to avoid toxicity (defined by Olig2+ cell counts). Based on the dose response curves, we adopted an OPC treatment paradigm using 1 μM Cort, 1 μM DFP, and a combined Cort+DFP condition, as used in the GWI animal model. OPCs in growth medium were treated 24 hr after plating and were examined via immunocytochemistry 72 hr following treatment.

The data showed that Cort treatment significantly decreased OPC proliferation (Ki67+ Olig2+ cells) (Figure 3a, 10.6 vs. 2.6% respectively, $\chi^2[1, n = 466] = 26.604$, $p < 0.001$). DFP alone had no effect on OPC proliferation (Figure 3a, 10.6 vs. 7.4%, respectively, $\chi^2[1, n = 541] = 2.436$, $p = 0.119$). Cort+DFP co-treatment, as used in the GWI animal model, also decreased OPC proliferation due to the effect of Cort (Figure 3a, 10.6 vs. 3.5%, respectively, $\chi^2[1, n = 405] = 11.150$, $p = 0.001$). Cell counts used to determine Ki67+ Olig2+ frequency are reported in Figure 3b.

Apoptosis, identified by cleaved caspase 3 (CC3) expression, was increased by DFP treatment (Figure 3c, 0.98 vs. 6.19%, respectively, χ^2 [1, $n = 323$] = 14.316, $p < 0.001$) and by Cort+DFP (Figure 3c, 0.95 vs. 11.04%, respectively, χ^2 [1, $n = 480$] = 11.663, $p = 0.001$) treatment due to the effect of DFP. The vehicle used to dissolve Cort, (0.00004% ethanol) caused a small, but statistically significant increase in apoptosis when used alone (Figure 3c, 0.98 vs. 4.49% respectively, $\chi^2[1, n = 480] = 37.06$, $p < 0.001$), but in combination with Cort had no measurable effect (Figure 3c, 0.98 vs. 2.92%, respectively, $\chi^2[1, n = 445] = 1.432$, $p = 0.23$). Cell counts used to determine CC3 + Olig2+ frequency are reported in Figure 3d. Thus, DFP reduces OPC survival and this effect is not prevented by Cort in the Cort+DFP condition.

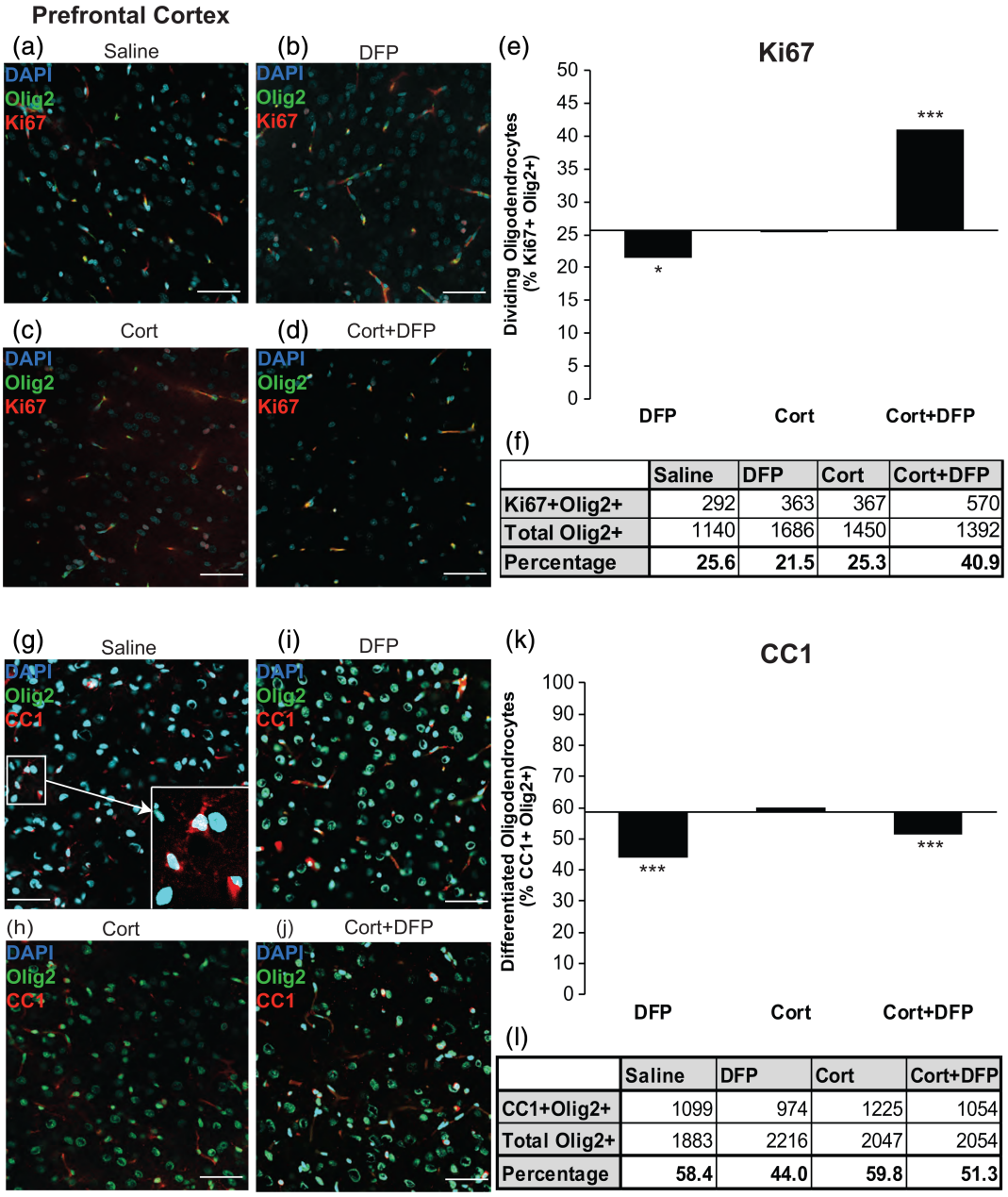
To determine the effects of AChE inhibition and Cort on oligodendrocyte lineage cell maturation, OPCs were differentiated into oligodendrocytes using N1 differentiation media for 3 days in the presence of Cort alone, DFP alone, and in the presence of both Cort and DFP. The data show that Cort (Figure 3e, 33.5 vs. 71.7%, respectively, $\chi^2[1, n = 453] = 28.962$, $p < 0.001$), DFP (Figure 3e, 33.5 vs. 42.9%, respectively, χ^2 [1, $n = 611$] = 6.371, $p = 0.012$), and Cort+DFP (Figure 3e, 33.5 vs. 55.1%, respectively, $\chi^2[1, n = 639] = 11.133$, $p = 0.001$) conditions increased the number of Olig2+ cells that were post-mitotic and pre-myelinating (CC1+). Cell counts used to determine CC1 + Olig2+ frequency are reported in Figure 3f. Cort treatment alone also increased the number of MBP+ mature myelinating oligodendrocytes (Figure 3g, 5.2 vs. 9.9%, respectively, $\chi^2[1, n = 453] = 8.419$, $p = 0.004$). DFP (Figure 3g, 5.2 vs. 7.0%, χ^2 [1, $n = 611$] = 2.083, $p = 0.149$) and Cort+DFP (Figure 3g, 5.2 vs. 6.7%, respectively, $\chi^2[1, n = 639] = 2.449$, $p = 0.118$) had no significant effect on the number of MBP+ mature oligodendrocytes. Cell counts used to determine MBP+ Olig2+ frequency are reported in Figure 3h.

To interpret whether the effects of DFP were due to cholinergic or non-cholingeric signaling we measured the levels of ACh in the culture serum. We previously showed that neither OPCs nor astrocytes secrete ACh. We measured no detectable ACh in the media containing 10 or 0.2% FBS used for OPC proliferation and differentiation, respectively, using a fluorometric assay with a sensitivity of ≥100 pmol. Since there was no detectable ACh in either culture medium, the observed responses were due to direct and non-cholinergic effects of Cort and DFP. Taken together, the data demonstrates that Cort inhibits proliferation and drives maturation of OPCs by direct action on these cells, while DFP increases maturation marginally, but also stimulates apoptosis. In combination with Cort, the toxicity of DFP in vitro acts to counter the pro-maturation effects of Cort.

To test if ACh alone could directly affect MBP expression, we treated oligodendrocytes with ACh in vitro. We found that elevated ACh was not sufficient to increase MBP expression in cell-culture (Figure S2a, b, 0.731 ± 0.0995 (vehicle) vs. 0.824 ± 0.105 (treated) MBP/GAPDH ratio, respectively, $t[2] = 0.816$, $p = 0.55$).

3.3 | AChE inhibition decreases frequency of mature oligodendrocytes in the PFC

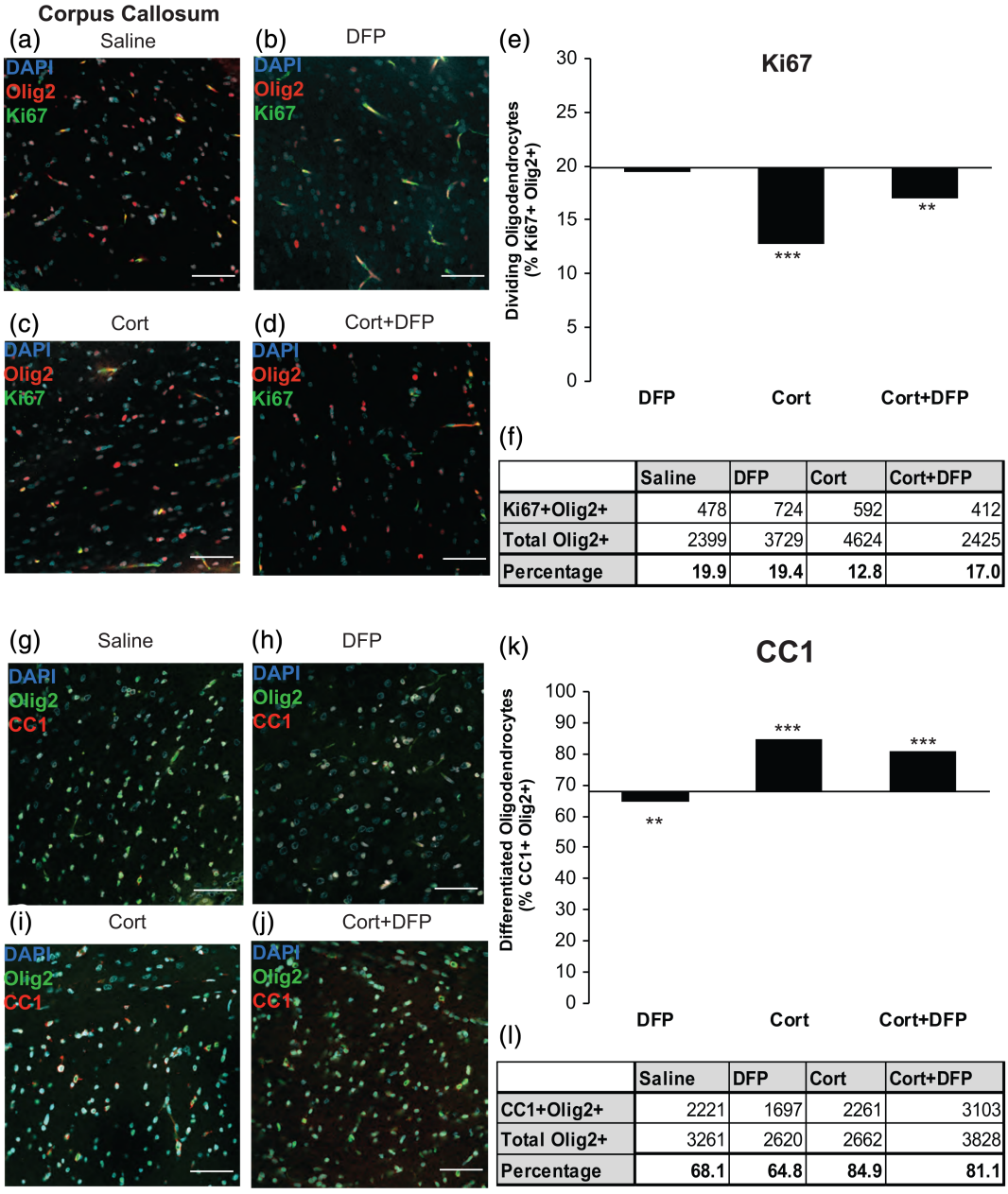
Cell culture studies indicate that Cort and DFP have complex effects on OPC proliferation, survival, and maturation. Oligodendrocytes in vivo are likely affected by these agents, making it important to determine how oligodendrocytes may be affected in the GWI animal model. Any differences found in the animal model from the results in cell culture will indicate systemic effects and neuron–glia interactions that are not modeled in culture. In addition, the exposures in vivo could be different due to metabolic breakdown of the compounds. We examined the expression of CC1, a nuclear marker for postmitotic but pre-myelinating oligodendrocytes (Bin, Harris, & Kennedy, 2016), in the PFC of GWI rat model brains at 21 days post treatment with DFP. The PFC was chosen for analysis because this brain region is implicated in GWI and the PFC is still undergoing myelination in humans in the third decade of life (Miller et al., 2012). Representative immunohistochemistry images from the PFC used for quantification are reported in Figure 4a–d. Exposure to the AChE inhibitor, DFP, and Cort had different effects on oligodendrocyte cell proliferation, with DFP being inhibitory (Figure 4b, e, f, 25.6 vs. 21.5%, respectively, $\chi^2[1, n = 1686] = 6.370$, $p = 0.0119$) and Cort having no significant effect (Figure 4c, e, f, 25.6 vs. 25.3%, respectively, $\chi^2[1, n = 1450] = 0.031$, $p = 0.860$). Paradoxically, in combination, DFP + Cort increased the number of proliferating oligodendrocytes (Figure 4d–f, 25.65 vs. 40.9%, respectively, $\chi^2[1, n = 1392] = 65.6309$, $p < 0.00001$). Cell counts of frequencies of proliferating oligodendrocytes (Ki67+ Olig2+) are reported in Figure 4f. In terms of maturation in the PFC, DFP also inhibited maturation to the CC1+ stage, (Figure 4i, k, l, 58.4 vs. 44.0%, respectively, $\chi^2[1, n = 2216] = 27.611$, $p < 0.001$) and Cort with DFP (Figure 4j–l, 58.4 vs. 51.3%, respectively, $\chi^2[1, n = 2054] = 5.763$, $p = 0.0163$) had a similar effect, but Cort treatment alone did not affect maturation of oligodendrocytes to the CC1+ stage (Figure 4h, k, l, 58.4% vs. 59.8%, respectively, χ^2 [1, $n = 2047$] = 0.888, $p = 0.346$). Representative immunohistochemistry images from the PFCare shown in Figure 4g–j. Tables of cell counts of mature oligodendrocytes (Olig2+ CC1+) for the PFC are reported in Figure 4l.



	Saline	DFP	Cort	Cort+DFP
Ki67+Olig2+	292	363	367	570
Total Olig2+	1140	1686	1450	1392
Percentage	**25.6**	**21.5**	**25.3**	**40.9**

	Saline	DFP	Cort	Cort+DFP
CC1+Olig2+	1099	974	1225	1054
Total Olig2+	1883	2216	2047	2054
Percentage	**58.4**	**44.0**	**59.8**	**51.3**

FIGURE 4 AChE inhibition decreases the frequency of mature oligodendrocytes in the prefrontal cortex (PFC) of the GWI animal model. Data are cell counts from immunohistochemistry of GWI animals at 24 hr postexposure for proliferation analysis and 21 days postexposure for maturation analysis. (a–d) Representative images of Olig2+ Ki67+ cells across treatment conditions. (e) Fraction of proliferating oligodendrocytes (Olig2+ Ki67+) compared to total oligodendrocytes (Olig2+) in the PFC varied with treatment condition (χ^2 [1, N = 3] = 158.86, p < 0.00001). DFP decreased the frequency of proliferating oligodendrocytes (χ^2 [1, n = 1686] = 6.3703, p = 0.01195). Cort had no effect on Ki67+ oligodendrocyte frequency (χ^2 [1, n = 1450] = 0.031, p = 0.8602). Cort+DFP condition had significantly more proliferating oligodendrocytes than saline control (χ^2 [1, n = 1392] = 65.6309, p < 0.00001) Bar graphs are total cell counts (N = 5, n = 50). X-axis is drawn at the saline control value. (f) Table of proliferating (Ki67+ Olig2+) cell counts in the PFC for each condition. (g–j) Representative images of CC1+ Olig2+ cells across treatment conditions. Inset in (g) illustrates CC1+ (red) Olig2+ (green) and CC1+ Olig2+ (yellow) cell identification. (k) Fraction of mature oligodendrocytes (Olig2+ CC1+) compared to total oligodendrocytes (Olig2+) in the PFC varied with treatment condition (χ^2 [1, N = 3] = 135.04, p < 0.001). Bar graphs are total cell counts (N = 3, n = 30). X-axis is drawn at the saline control value. DFP treatment resulted in significantly fewer mature oligodendrocytes than saline condition. (χ^2 [1, n = 2216] = 27.611, p < 0.001). Cort alone had no effect on CC1+ oligodendrocytes in the PFC (χ^2 [1, n = 2047] = 0.888, p = 0.346). Cort+DFP was associated with significantly fewer mature oligodendrocytes than saline condition (χ^2 [1, n = 2054] = 5.7633, p = 0.0163). (l) Table of mature (Olig2+ CC1+) cell counts in the PFC for each condition. Scale bar on all representative images is 40 μM. * indicates p < 0.05, *** indicates p < 0.001, significance is determined by comparing all treatment conditions with the control condition, saline



	Saline	DFP	Cort	Cort+DFP
Ki67+Olig2+	478	724	592	412
Total Olig2+	2399	3729	4624	2425
Percentage	**19.9**	**19.4**	**12.8**	**17.0**

	Saline	DFP	Cort	Cort+DFP
CC1+Olig2+	2221	1697	2261	3103
Total Olig2+	3261	2620	2662	3828
Percentage	**68.1**	**64.8**	**84.9**	**81.1**

FIGURE 5 Corticosterone increases the frequency of mature oligodendrocytes in the corpus callosum of the GWI animal model. Data are cell counts from immunohistochemistry of GWI animals at 24 hr postexposure for proliferation analysis and 21 days postexposure for maturation analysis. (a–d) Representative images of Ki67+ Olig2+ cells across treatment conditions. (e) Fraction of proliferating oligodendrocytes (Olig2+ Ki67+) compared to total oligodendrocytes (Olig2+) in the corpus callosum was dependent on treatment condition (χ^2 = 88.142, p < 0.001). Bar graphs are total cell counts (N = 5, n = 50). X-axis is drawn at the saline control value. DFP had no effect on the frequency of proliferating oligodendrocytes in the corpus callosum (χ^2 [1, n = 3,729] = 0.240, p = 0.624). Cort treatment was associated with significantly fewer proliferating oligodendrocytes (χ^2 [1, n = 4,624] = 60.080, p < 0.001). Cort+DFP treatment was associated with significantly fewer proliferating cells than saline condition (χ^2 [1, n = 2425] = 6.910, p = 0.009). (f) Table of mature (Olig2+ CC1+) cell counts in the corpus callosum for each condition. (g–j) Representative images of Olig2+ CC1+ cells across treatment conditions. (k) Fraction of mature oligodendrocytes (Olig2+ CC1+) compared to total oligodendrocytes (Olig2+) in the corpus callosum was dependent on treatment condition (χ^2 = 444.328, p < 0.001). Bar graphs are total cell counts (N = 5, n = 50). X-axis is drawn at the saline control value. Cort treatment resulted in significantly more mature oligodendrocytes than saline condition. (χ^2 [1, n = 2,662] = 225.445, p < 0.001). Cort+DFP was also associated with significantly more mature oligodendrocytes than saline condition, (χ^2 [1, n = 3,828] = 157.995, p < 0.001). DFP alone had significantly fewer mature oligodendrocytes (χ^2 [1, n = 2,620 = 7.275, p = 0.007). (l) Table of mature (Olig2+ CC1+) cell counts in the corpus callosum for each condition. Scale bar on all representative images is 40 μM. ** indicates p < 0.01; *** indicates p < 0.001, significance is determined by comparing all treatment conditions with the control condition, saline

3.4 | Effects of GW agents on oligodendrocytes in corpus callosum

The PFC is implicated in GWI and it is still undergoing myelinating in young adults. The major white matter tract in rodents is the corpus callosum. We therefore analyzed the effects of GW agents on oligodendrocyte lineage cells residing in grey matter PFC versus white matter corpus callosum. The effects of DFP and Cort on proliferation and maturation of OPCs differed in the corpus callosum compared with the PFC. This is not unexpected, given the differences in the cellular composition and environment in these two regions. In the corpus callosum, 19.9% of Olig2+ cells were proliferating, immature OPCs, but in the PFC more cells were in this state (25.6%), as this grey matter region is still undergoing active myelination. In the corpus callosum, Cort (Figure 5c, e, f, 19.9 vs. 12.8%, respectively, χ^2 [1, n = 4624] = 60.080, $p < 0.001$), but not DFP (Figure 5b, e, f, 19.9 vs. 19.4%, respectively, χ^2[1, n = 3729] = 0.240, $p = 0.624$) decreased OPC proliferation and this effect persisted when Cort was delivered together with DFP (Figure 5d–f, 19.9 vs. 17.0%, respectively, χ^2[1, n = 2425] = 6.910, $p = 0.009$). Cell counts for corpus callosum oligodendrocyte proliferation frequencies are reported in Figure 5f. In the corpus callosum, differentiation to the CC1+ stage was also promoted by Cort (Figure 5i, k, l, 68.1 vs. 84.9%, respectively, χ^2[1, n = 2662] = 225.445, $p < 0.001$) and Cort + DFP (Figure 5j, k, l, 68.1 vs. 81.1%, respectively, χ^2[1, n = 3828] = 157.995, $p < 0.001$), but DFP alone decreased maturation (Figure 5h, k, l, 68.1 vs. 64.8%, respectively, χ^2(1, n = 2620 = 7.275, $p = 0.007$). Thus, Cort has opposite effects in the PFC and corpus callosum on proliferation and maturation. DFP decreased proliferation and differentiation in both regions, but the effects in the corpus callosum are either small or not statistically significant.

3.5 | GWI animals have increased myelin basic protein levels in subcortical white matter

Changes in the integrity of white matter tracts has been found to underlie the key cognitive and sensory impairments in GW veterans (Rayhan et al., 2013; Van Riper et al., 2017). Therefore, we sought to understand next how the changes in oligodendrocyte development observed in the corpus callosum of the GWI animal model translates to myelin formation. By using immunoblot to measure myelin basic protein (MBP) levels in subcortical white matter, we found that GWI animals co-treated with Cort and DFP had significantly increased levels of MBP in subcortical white matter as early as 12 hr posttreatment (Figure 6a, e, 1.0 ± 0.071 vs. 1.600 ± 0.078 relative MBP expression, respectively, ANOVA F[3, 8] = 10.97, $p = 0.0033$, Dunnett's post hoc analysis: saline vs. Cort +DFP $p < 0.01$) and this effect persists at 24 hr (Figure 6b, e, 1.0 ± 0.070 vs. 2.015 ± 0.134 relative MBP expression, respectively, ANOVA F[3, 8] = 11.16, $p = 0.0031$, Dunnett's post hoc analysis: saline vs. Cort +DFP $p < 0.01$), 72 hr (Figure 6c, e, 1.0 ± 0.103 vs. 1.7 ± 0.0384 relative MBP expression, respectively, ANOVA F[3, 8] = 8.81, $p = 0.0065$, Dunnett's post hoc analysis: saline vs. Cort+DFP $p < 0.01$) and 21 days (Figure 6d, e, 1.0 ± 0.136 vs. 1.67 ± 0.165 relative MBP expression, respectively, ANOVA F[3, 16] = 5.258, $p = 0.0102$, Dunnett's post hoc analysis: saline vs. Cort+DFP $p < 0.01$). Subcortical white matter was the ideal medium for the immunoblot experiments because it is enriched in white matter, including the corpus callosum, which allowed for robust testing of the hypothesis. For the histology experiments, we chose a specific area, the corpus callosum, to eliminate heterogeneity of each subcortical region.

Interestingly, DFP treatment alone produced a significant increase in MBP expression at 12 hours (Figure 6a, e, 1.0 ± 0.071 vs. 1.522 ± 0.0928 relative MBP expression, respectively, Dunnett's multiple comparison: saline vs. DFP, 12 hours: $p < 0.01$) and 24 hours (Figure 6b, e, 1.0 ± 0.070 vs. 1.795 ± 0.226 relative MBP expression, respectively, Dunnett's multiple comparison: saline vs. DFP $p < 0.05$). Histological analysis showed that at later time points, Cort and Cort + DFP increased the number of mature oligodendrocytes in the corpus callosum (Figure 5k), which is consistent with increased subcortical MBP levels measured by immunoblot. The observed changes in protein expression in vivo were detected as early as 12 hr following treatment, which is too early to be caused by changes in OPC proliferation and differentiation, as the cell cycle of OPCs is ~25–30 hr (Durand, Gao, & Raff, 1997). Thus, the early increase in MBP protein must reflect an increase in its synthesis. Immunoblot analysis of a marker of OPCs supports this conclusion. We measured OPC membrane protein marker NG2 by immunoblot analysis after 12, 24, and 72 hr (Figure S3a–d) and abundance of the transcription factor Olig2 at 12 and 24 hr (Figure S3e–g). GW agents significantly altered NG2 levels 12 hr postexposure but post-hoc analysis revealed no significant pairwise difference between saline and individual GW agents treatment conditions (Figure S3a,d, 1.0 ± 0.1369 vs. 0.643 ± 0.1788 vs. 1.09 ± 0.211 vs. 1.95 ± 0.365, ANOVA F[3, 8] = 5.398, $p = 0.0252$, Dunnett's multiple comparisons, saline vs. Cort $p > 0.05$; saline vs. DFP $p > 0.05$; saline vs. Cort+DFP $p > 0.05$). At 1 day postexposure GW agents significantly altered NG2 levels (Figure S3b, d, 1.0 ± 0.335 vs. 0.816 ± 0.0738 vs. 1.84 ± 0.0734 vs. 1.226 ± 0.174 relative NG2 levels, ANOVA F[3, 8] = 5.2, $p = 0.0277$) and post-hoc analysis reveals DFP treatment significantly increased NG2 levels compared to saline (1.0 ± 0.335 vs. 1.226 ± 0.174, respectively, Dunnett's multiple comparisons, saline vs. Cort+DFP $p < 0.05$). This could imply an early spurt in proliferation due to increased availability of ACh, which promotes proliferation, via DFP mediated inhibition of AChE, however NG2 is expressed by perivascular and other cells (Smyth et al., 2018), and the amount of protein can be regulated without changes in number of types of cells. At 3-days postexposure (Figure S3c, d, 1.0 ± 0.0859 vs. 0.676 ± 0.0545 vs. 1.073 ± 0.056 vs. 0.994 ± 0.176 relative NG2 levels, ANOVA F[3, 8] = 2.825, $p = 0.1068$), there were no significant differences between the treatment conditions, suggesting an increase in the proliferative pool of OPCs was likely not the sole source of the persistently increased MBP across early and later time-points. Abundance of transcription factor Olig2 at 12 hr (Figure S3e, g, 1.0 ± 0.283 vs. 0.965 ± 0.158 vs. 0.790 ± 0.0430 vs. 0.460 ± 0.05 relative Olig2 levels, ANOVA F [3,4] = 1.48, $p = 0.3472$) and 24 hr (Figure S3f, g, 1.0 ± 0.122 vs. 0.8255 ± 0.170 vs. 1.6876 ± 0.326 vs. 1.365 ± 0.0256 relative Olig2 levels, ANOVA F[3,4] = 2.618, $p = 0.1877$) was not significantly

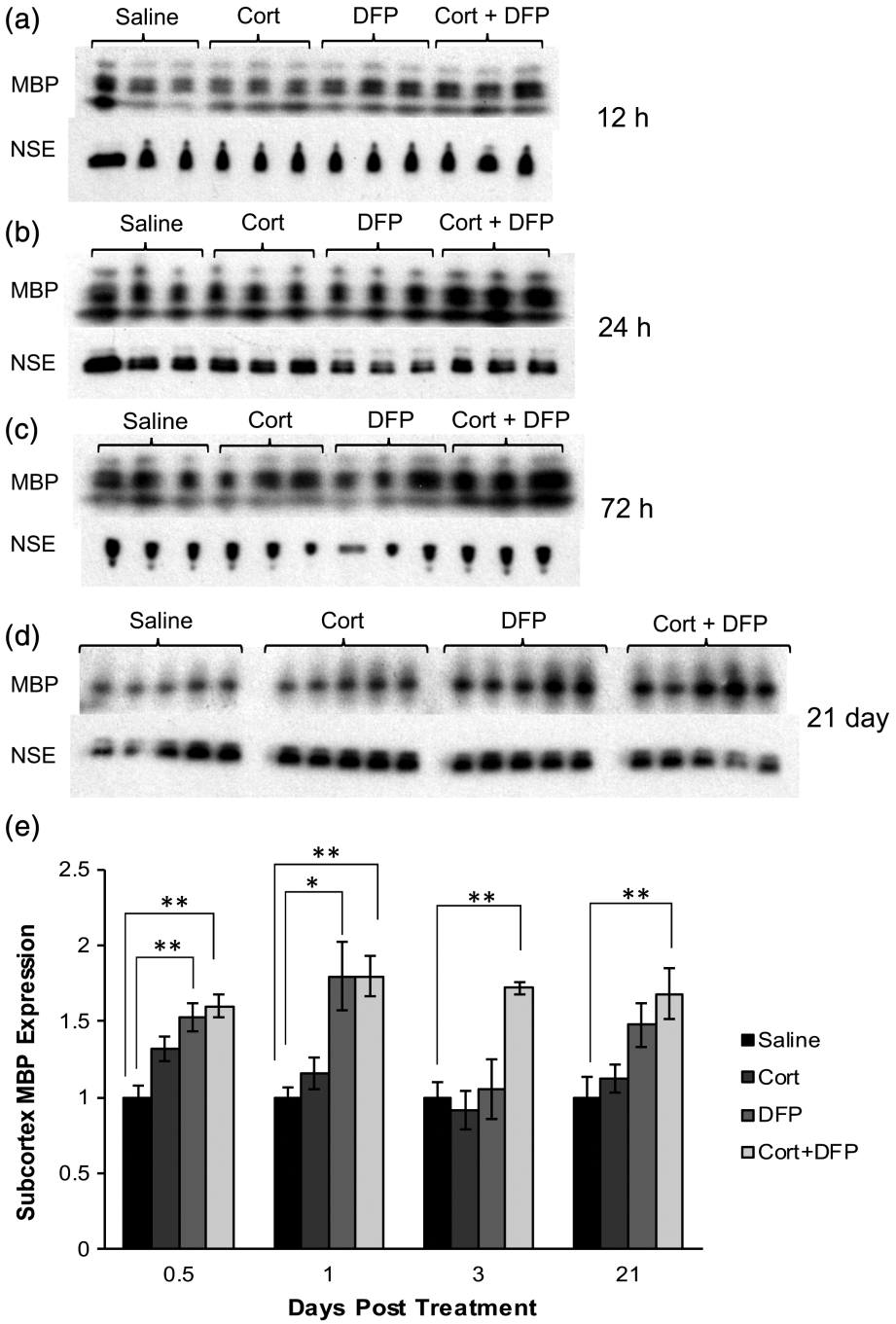


FIGURE 6 GWI treatment paradigm increases levels of myelin basic protein (MBP) in subcortical white matter. Immunoblots of subcortical white matter homogenates indicate MBP levels after treatment with saline, Cort, DFP, and Cort +DFP at (a) 12 hr, (b) 24 hr, (c) 72 hr, and (d) 21 days posttreatment. MBP isoforms correspond to four immunoblot bands with molecular weights of 21, 18, 17, and 14 kDa. NSE band occurs at 47 kDa. (e) MBP expression was quantified with densitometry and normalized to neuron-specific enolase (NSE). Each treatment was compared to the control condition, saline, at each time point (12 hr: ANOVA $F[3, 8] = 10.97$, $p = 0.0033$; 24 hr: ANOVA $F(3,8) = 11.19$, $p = 0.0031$; 72 hr: ANOVA $F(3,8) = 8.81$, $p = 0.0065$; 21 days: ANOVA $F(3,16) = 5.258$, $p = 0.0102$. Dunnett's multiple comparison: Saline vs. DFP. $p < 0.01$; saline vs. Cort+DFP, $p < 0.01$). Cort+DFP cotreatment significantly increased MBP levels beginning at 12 hr and persisting at 1, 3, and 21 days postexposure (Dunnett's multiple comparison: Saline vs. Cort+DFP, $p < 0.01$ each time point). Data are reported from $N = 3$ or 5 animals per condition. * indicates $p < 0.05$, ** indicates $p < 0.01$

different among any treatment condition at these early timepoints, consistent with the transient changes in NG2 levels resulting from factors other than the number of oligodendrocytes.

Alternatively, neuroinflammation and injury can alter axonal sprouting (Chen & Zheng, 2014), which would create new axonal branches to be myelinated, thus stimulating synthesis of MBP. To test this hypothesis, we measured the levels of growth associated protein 43 (GAP43), a marker for axonal sprouting (Benowitz & Routtenberg, 1997) and found that GAP43 levels in subcortical white matter did not change after any treatment condition at 12 hr (Figure S4a, d, 1.0 ± 0.0126 vs. 1.224 ± 0.126 vs. 1.169 ± 0.131 vs. 0.877 ± 0.114 relative GAP43 levels, ANOVA $F[3,8] = 2.179$, $p = 0.1684$), 24 hr (Figure S4b, d, 1.0 ± 0.017 vs. 2.249 ± 0.6929 vs. 2.342 ± 0.354 vs. 0.885 ± 0.263 relative GAP43 levels, ANOVA $F[3,8] = 3.638$, $p = 0.0640$), or 72 hr (Figure S4c, d, 1.0 ± 0.299 vs. 0.674 ± 0.152 vs. 0.807 ± 0.0355 vs. 0.472 ± 0.123 relative GAP43 levels, ANOVA $F[3,8] = 1.534$, $p = 0.2790$) postexposure to DFP and Cort, either alone or in combination. Therefore, the increase in MBP is not explained by myelination of new axons.

Additionally, to test if changes in astrocyte biology were responsible for increased subcortical MBP levels, we measured the levels of the astrocytic protein, GFAP, by immunoblot. GFAP levels remained unaffected by treatment conditions at 12 hr (Figure S5a, d, 1.0 ± 0.200 vs. 0.920 ± 0.080 vs. 1.094 ± 0.059 vs. 0.815 ± 0.064 relative

GFAP levels, ANOVA $F[3,8] = 1.039$, $p = 0.426$), 24 hr (Figure S5b, d, 1.0 ± 0.187 vs. 1.142 ± 0.025 vs. 1.53 ± 0.149 vs. 1.383 ± 0.151 relative GFAP levels, ANOVA $F[3,8] = 2.826$, $p = 0.1067$) and 72 hr (Figure 5c,d, 1.0 ± 0.044 vs. 1.385 ± 0.216 vs. 1.525 ± 0.09 vs. 1.23 ± 0.150 relative GFAP levels, ANOVA $F[3,8] = 2.543$, $p = 0.1295$) posttreatment. This is consistent with studies showing GFAP expression is not altered up to 3 days posttreatment with GW agents (O'Callaghan et al., 2015). However, at 21 days post treatment, GW agents significantly affected GFAP expression (Figure S5d, e, 1.0 ± 0.0877 vs. 0.697 ± 0.066 vs. 0.627 ± 0.0417 vs. 1.040 ± 0.113 relative GFAP levels, ANOVA $F[3,16] = 6.638$, $p = 0.0040$). GFAP expression was significantly reduced with Cort alone (Dunnett's multiple comparisons: saline vs. Cort, 1.0 ± 0.0877 vs. 0.697 ± 0.066, respectively, $p < 0.05$) and DFP alone (Dunnett's multiple comparisons: saline vs. DFP, 1.0 ± 0.0877 vs. 0.627 ± 0.0417, respectively, $p < 0.05$) treatment conditions. These observations are consistent with decrease in GFAP expression with chronic exposure to Cort (O'Callaghan et al., 1991; Nichols, Osterburg, Masters, Millar, & Finch, 1990) and DFP (Gupta & Abou-Donia, 1995) reported in other studies.

The results do not support axonal sprouting, early OPC proliferation or gliosis as the source of increased MBP. In conclusion, immunoblot of subcortical white matter indicates persistently elevated MBP protein levels in subcortical white matter, potentially via elevated MBP protein production at early timepoints (i.e., 24 hr) and by an increase in the number of mature oligodendrocytes in the corpus callosum at later timepoints (21 days). Further, the finding that exogenous ACh on an in vitro monoculture of OPCs is insufficient to change MBP protein levels (Figure S2) suggests the necessity of interactions with axons as axons provide physical support and an appropriate substrate for myelination to promote MBP production in oligodendrocytes (Wake et al., 2011).

Together these results show effects on OPC proliferation, survival, maturation, and increased myelin basic protein expression in the

TABLE 2 Summary of the effects of GW agents on development of oligodendrocyte lineage cells based on drug treatments and experimental methods

A.					
		Cort			
		Sample			
		In vitro	**PFC**	**CC**	**Subcortical WM**
Developmental marker	Ki67	↓	∅	↓	X
	CC3	∅	X	X	X
	CC1	↑	∅	↑	X
	MBP	↑	X	X	∅
B.					
		DFP			
		Sample			
		In vitro	**PFC**	**CC**	**Subcortical WM**
Developmental marker	Ki67	∅	↓	∅	X
	CC3	↑	X	X	X
	CC1	↑	↓	↓	X
	MBP	∅	X	X	∅
C.					
		CORT+DFP			
		Sample			
		In vitro	**PFC**	**CC**	**Subcortical WM**
Developmental marker	Ki67	↓	↑	↓	X
	CC3	↑	X	X	X
	CC1	↑	↓	↑	X
	MBP	∅	X	X	↑

Note: Effects of (A) Cort alone, (B) DFP alone, or (C) Cort+DFP on developmental markers for oligodendrocyte lineage cells. in vitro category is data obtained from OPC monoculture. PFC and CC indicate data from cell counts from prefrontal cortex (PFC) and corpus callosum (CC) respectively. Subcortical WM indicates data from immunoblots of subcortical white matter. Up arrows (↑) indicate increase and down arrows (↓) indicate decrease in cell frequency or protein abundance. Null sign (∅) corresponds to no significant effects, while "X" denotes experiments that were not preformed.

subcortical white matter resulting from treatment with Cort and DFP in the GWI animal model. These effects differ in different brain regions and in cell culture because of the differential contribution of cholinergic and non-cholinergic effects of GW agents and differences in cellular environments of grey and white matter regions where these oligodendroglial populations reside (Table 2).

4 | DISCUSSION

In this study we have investigated the plasticity of myelinating glia in the context of white matter abnormalities in GWI and demonstrated that biology and development of oligodendrocyte lineage cells are significantly affected by exposure to AChE inhibiting agents and the stress hormone, corticosterone. We conclude that DFP, a sarin nerve gas surrogate, decreases maturation of OPCs when acetylcholine signaling is present. DFP also decreases proliferation of oligodendrocytes in the PFC, a region with a higher percentage of proliferating oligodendrocytes than in the corpus callosum.

Our finding that DFP decreases maturation of oligodendrocytes is consistent with the current literature about the role of ACh signaling on oligodendrocyte development (Fields et al., 2017). In MS human clinical trials and MS animal models, inhibition of muscarinic receptors and consequent inhibition of ACh signaling is shown to promote remyelination (Abiraman et al., 2015; Green et al., 2017; Li et al., 2015; Liu et al., 2016; Mei et al., 2014; Welliver et al., 2018). Therefore, our finding that elevated ACh signaling decreases the number of mature oligodendrocytes is consistent with the literature. In our non-cholinergic in vitro studies, DFP increased the frequency of mature oligodendrocytes and also significantly increased apoptosis in OPCs. In previous in vitro studies, organophosphates preferentially affected the maturation and survival of immature neuronal cells (Monnet-Tschudi, Zurich, Schilter, Costa, & Honegger, 2000). DFP has various non-cholinergic effects, including neurotoxicity (Qian et al., 2007), immunogenicity (Chaubey et al., 2019), and disruption of axonal transport (Naughton et al., 2018; Rao et al., 2017).

Another factor to consider, is that the CDC GWI animal model includes chronic Cort exposure prior to DFP exposure. In terms of oligodendrocyte and white matter biology, we find that Cort alone has robust protective effects by promoting maturation of oligodendrocytes and decreasing proliferation. These findings are consistent with previous literature exploring the effects of Cort on oligodendrocyte biology (Alonso, 2000; Miyata et al., 2016).

Given the somewhat antagonistic interactions of DFP and Cort, we find that the Cort+DFP condition reveal region specific effects on oligodendrocyte biology. With Cort+DFP treatment, we find that in the PFC, OPCs are pushed into a more proliferative and less mature state. In contrast, with the same treatment paradigm in the corpus callosum, OPCs are pushed to a less proliferative and more mature state. This could be due to the relatively more mature population of oligodendrocytes in the corpus callosum as well as differences in ACh availability in the two regions. The importance of regional differences is highlighted by the reported abundance of cognitive rather than sensory symptoms in the younger GW veterans (Gopinath et al., 2012), implicating the involvement of PFC, a region where myelination continues into the third decade of life (Miller et al., 2012). The decrease in mature oligodendrocytes identified in the PFC suggests that the PFC is especially vulnerable to GW agents; a finding that is consistent with the neuropsychological impairments presented by GW veterans (Janulewicz et al., 2017; Sullivan et al., 2003; Sullivan et al., 2018). Our cell-culture studies more closely match the data from the corpus callosum where Cort+DFP promotes maturation of oligodendrocytes. Given that there is no detectable ACh in our OPC monocultures, we find that promotion of maturation by Cort overwhelms the anticholinesterase effect of DFP.

MBP protein was elevated in subcortical white matter. The increase in MBP levels with Cort+DFP co-treatment, could be due to promotion of the oligodendrocyte lineage toward a more mature state by Cort in the corpus callosum. However, an increase in MBP does not necessarily imply a healthier and more functional white matter (Kristensson et al., 1986). Increased MBP mRNA transcripts have been reported during periods of demyelination, providing evidence for a compensatory increase in MBP levels in response to pathology (Kristensson et al., 1986). A transient increase in MBP mRNA, occurring within 6 hr, has been previously reported as an oligodendroglial cellular response to injury (Bartholdi & Schwab, 1998). This argument is underscored by studies showing that DFP exposure of rats increases myelin decompaction while having no effect on the g-ratio or white matter volume (Naughton et al., 2018). Therefore, it is important to note the complicated relationship between MBP levels, oligodendrocyte maturity, and myelin integrity.

The results of the quantitative histological analysis of oligodendroglia in the PFC of the GWI rat model is consistent with RNA-seq data in mice showing that combined Cort and DFP treatment decreases the fraction of mRNA transcripts associated with mature oligodendrocytes in the PFC (Ashbrook et al., 2018), but our results are not consistent with this gene profiling study in other respects. Cort treatment alone did not alter the number of mature oligodendrocytes in the PFC, but mRNA transcripts associated with oligodendrocytes are reportedly reduced in this condition (Ashbrook et al., 2018). Also, the frequency of mature oligodendrocytes in the PFC decreased in response to DFP treatment, but mRNA transcripts associated with mature oligodendrocytes remained unchanged under this condition (Ashbrook et al., 2018). These discrepancies may be explained by the fact that gene and protein abundance are indirect indicators of cell numbers, and gene expression and protein levels are influenced by physiological conditions. Alternatively, methodological or species differences could also account for discrepancies between mRNA profiling and histological analysis.

Based on our findings we predict that GW veterans would have decreased white matter integrity varying by brain region. These predictions are supported by the brain imaging studies of GW veterans (Bierer et al., 2015; Rayhan et al., 2013; Van Riper et al., 2017). The published neuroimaging data reflect both increased and decreased myelin integrity depending on the myelin track analyzed and methodological differences in measurement. For example, previous GWI

imaging data with MRI has shown that axial diffusivity in the right inferior fronto-occipital fasciculus, a white matter tract that links cortical regions involved in fatigue, pain, emotional and reward processing, and the right ventral attention network in cognition, is significantly increased in GW veterans and correlate with the severity of pain and fatigue (Rayhan et al., 2013). In veterans with post-traumatic stress disorder (PTSD), increased structural integrity has been reported in the cingulum bundle, a white matter tract connecting the right amygdala and anterior cingulate cortex (Bierer et al., 2015). Importantly, an equal number of studies have identified decreased myelin integrity depending on the brain region. It has been shown in veterans with GWI and chronic pain that there is a lower white matter integrity across multiple brain regions including the frontal gyrus, corpus callosum, and precentral gyrus (Van Riper et al., 2017). GW veterans with PTSD also display significantly reduced mean diffusivity in the right, but not left cingulum (Bierer et al., 2015). Our studies show that DFP treatment of OPC monoculture also promotes toxicity. Taken together, the data and available evidence suggest that the effects of AChE inhibition, corticosterone exposure, and their combined treatment, on oligodendrocyte biology and white matter vary depending on the brain region and cell environment, reflecting underlying differences in ACh availability and cellular composition between regions.

5 | CONCLUSION

Our study shows that impairment of oligodendrocyte biology is an important aspect of the pathophysiology of GWI. We have identified that DFP, an analog to sarin nerve gas, reduces the frequency of differentiated oligodendrocytes across multiple brain regions. Our data shows that Cort, used in the CDC animal model, antagonizes the effects of DFP, as Cort alone increases the frequency of differentiated oligodendrocytes. With co-treatment of Cort and DFP, we find a lower frequency of CC1+ oligodendrocytes in the PFC and higher frequency of CC1+ oligodendrocytes in the corpus callosum. The cell count data in the corpus callosum is supplemented and corroborated by elevated MBP levels in the sub cortex. These differences highlight the heterogenous responses of oligodendrocytes to agents implicated in GWI and used in the GWI animal model. Similar heterogeneity is reflected in brain imaging studies and in the wide range of symptoms experienced in GWI. Taken together, these findings suggest therapeutic avenues where restoring the endogenous cholinergic signaling required for normal oligodendrocyte cell biology and function may potentially alleviate the chronic symptoms of veterans with GWI. This study also suggests that civilian exposure to AChE inhibitors, such as commercial pesticides, may have chronic effects on white matter, especially during childhood and early adolescence, when the brain is at its most plastic.

ACKNOWLEDGMENTS

The authors thank the Department of Defense's CDMRP Gulf War Illness Research Program. The authors also recognize and thank Maya Robnett for help with immunocytochemistry and immunoblotting studies, and Anika Schipma for performing cell counting with enthusiasm and diligence.

DISCLAIMER

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Henry M. Jackson Foundation for the Advancement of Military Medicine, and U.S. Department of Defense.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Belgrad J, Dutta DJ, Bromley-Coolidge S, et al. Oligodendrocyte involvement in Gulf War Illness. *Glia*. 2019;67:2107–2124. https://doi.org/10.1002/glia.23668

frontiers in Psychology

REVIEW
published: 26 September 2019
doi: 10.3389/fpsyg.2019.02088

Neuropsychological Findings in Gulf War Illness: A Review

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This review paper summarizes the accumulation of research investigating neuropsychological outcomes in veterans with Gulf War illness (GWI). Earlier research focused on Gulf War veterans (GW) who were deployed versus non-deployed, as well as those who were symptomatic versus asymptomatic, or compared neuropsychological test results to published norms. Further research became more sophisticated, investigating specific GWI criteria, as well as the result of neurotoxicant exposure and the relationship to possible neurocognitive outcomes. As the early research supported both psychological and physiological effects on GWI; current research as summarized in this literature review supports the presence of neuropsychological deficits, particularly in the domains of attention, executive functioning, memory, and motor functioning related to chemical exposures that can be exacerbated by comorbid mood-related conditions. The same test battery has not been used consistently making it difficult to compare results among studies. Therefore, researchers created a resource to provide recommendations for the recently listed Neuropsychological Tests for Common Data Elements (CDEs) for use in all future GWI studies. Future research is necessary to further understand patterns of neuropsychological test data and how these decrements may relate to immunological or other biological markers, and the impact of trauma from physical and psychological stressors. In conclusion, there is consistent evidence that GWI is characterized by neuropsychological decrements – with future research these findings may aid in the diagnosis and assessment of treatment trial efficacy of GW veterans.

Keywords: Gulf War illness, neurotoxicant, neuropsychology, posttraumatic stress disorder, veterans, review

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Psychology for Clinical Settings, a section of the journal Frontiers in Psychology

Received: *12 December 2018*
Accepted: *28 August 2019*
Published: *26 September 2019*

Citation:
Jeffrey MG, Krengel M, Kibler JL, Zundel C, Klimas NG, Sullivan K and Craddock TJA (2019) Neuropsychological Findings in Gulf War Illness: A Review. Front. Psychol. 10:2088. doi: 10.3389/fpsyg.2019.02088

NEUROPSYCHOLOGICAL FINDINGS IN GWI: A REVIEW

Gulf War illness (GWI), also known as chronic multi-symptom illness (CMI; Fukuda et al., 1998), has impacted approximately a third of the veterans deployed to the 1990–1991 Gulf War (Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI], 2008; White et al., 2016). By definition, GWI includes self-reported cognitive complaints indicative of neuropsychological impairment (Fukuda et al., 1998; Steele, 2000). Research examining the neuropsychological profile of GWI is necessary given that cognitive problems remain one of the most prevalent and distressing

symptoms of GW veterans (Smith et al., 2012; Yee et al., 2016). Early on these cognitive symptoms resulted in veterans being referred for neuropsychological evaluations soon after their return from deployment and spurred research in this area. There have now been 25 papers specifically comparing objective neuropsychological performance in GW veterans with different comparison groups (deployed vs. non-deployed, symptomatic vs. non-symptomatic veterans, toxicant vs. non-toxicant). Here we present an overview of all 25 papers and describe the trajectory of research sophistication over time.

Neuropsychological findings in veterans with GWI have varied because of the use of different comparison populations (i.e., asymptomatic versus symptomatic GW veterans, different GWI cohorts) and different neuropsychological test batteries. A recent meta-analysis of the neuropsychological decrements associated with GWI provided some clarity to the question of which neuropsychological decrements were present in GW veterans and which tests were most sensitive to identifying these decrements. This was accomplished by combining multiple study results and using aggregate data when three or more studies used the same neuropsychological test. Studies were added into the meta-analysis when GW veterans served in the war from 1990 to 1991, had neuropsychological results reported in a manner conducive to meta-analysis, when comparison groups were deployed versus non-deployed or ill versus non-ill veterans and contained a unique sample (Janulewicz et al., 2017). The meta-analysis showed that the neuropsychological domains of visuospatial abilities, attention/executive functioning, and learning/memory were significantly different in GW veterans compared with two other comparison groups (Janulewicz et al., 2017). These findings remained significant when study results were adjusted for possible effects of publication bias (Janulewicz et al., 2017). In addition, analyses indicated that the following specific tests were most sensitive in discriminating between cohorts, including Block Design from the Wechsler Adult Intelligence Scale- Third Edition (WAIS-III; Wechsler, 1997), the Trail Making Test (Reitan, 1992), the Continuous Performance Test (CPT; Letz, 1991), and the California Verbal Learning Test (CVLT). The focus of the meta-analysis was not on the methodological strengths and weaknesses of the papers across the range of publications, but rather was to combine results to increase power and effect size across studies. These statistical findings were then used to determine which neuropsychological tests were most sensitive to GWI and were recommended in the recently listed neuropsychological component of the Common Data Elements (CDEs) for use in GWI studies[1].

Recently, through the Congressionally Directed Medical Research Program (CDMRP) Gulf War Illness Research Program (GWIRP), a collaborative effort of GWI researchers was conducted to identify the CDEs or sensitive measures of cognitive functioning to guide future research and treatment trial efficacy (Gulf War Illness Research Program [GWIRP], 2019). Measures are presented in **Table 1**. These tests were chosen based on their sensitivity in distinguishing between groups in three or more prior studies with GW veterans. These tests were recommended so that future studies can compare biomarker and treatment trial outcomes between studies in a consistent manner and to use tests that are known to be sensitive to GWI.

TABLE 1 | Gulf War illness common data elements module: neuropsychological test measures.

Supplemental – Highly Recommended
Word Reading Subtest of the Wide Range Achievement Test (WRAT-4) – (Wilkinson, 1993).
Continuous Performance Test-3 (CPT) – (Conners, 2014)
Wechsler Adult Intelligence Scale-IV (WAIS-IV) – (Weschler, 2008) Recommended tests: Digit Spans, Block Design
Profile of Mood States (POMS) – (McNair et al., 1971)
Davidson Trauma Scale (DTS) – PTSD - (Davidson et al., 1997).
Delis-Kaplan Executive Function System (D-KEFS) – (Delis et al., 2001) Recommended modules: Color-Word-Interference Test, Trail Making Test, Verbal Fluency
California Verbal Learning Test – Second Edition (CVLT-II) – (Delis et al., 2000)
Rey-Osterrieth Complex Figure Test (RCFT) – (Meyers and Meyers, 1995).
Supplemental
Finger Tap Test – (Reitan and Wolfson, 1993)
Grooved Pegboard Test – (Matthews and Klove, 1964)
Hopkins Verbal Learning Test (HVLT-R)* - (Brandt and Benedict, 2001)
Brief Visual Memory Test (BVMT)* - (Benedict, 1997)
PTSD Checklist for DSM-5 (PCL-5) – (Weathers et al., 1993)
Center for Epidemiological Studies Depression Scale (CES-D) – (Radloff, 1977)
Clinician Administered PTSD Scale (CAPS-5) – (Blake et al., 1990)
Structured Clinical Interview for DSM-5 (SCID-5) – (First, 2015)

* *denotes multiple test versions available for treatment trial use.*

In addition to facilitating neuropsychological outcomes research, researchers have also been learning more about the potential risk factors leading to objective neuropsychological decrements in GW veterans, including exposure to neurotoxicants (e.g., pesticides, nerve agents, and pyridostigmine bromide [PB] anti-nerve gas pills) as well as exposure to traumatic events during the war. These risk factors also include a history of mild traumatic brain injury and psychological trauma (Posttraumatic stress disorder (PTSD or mood disorder) (Sullivan et al., 2003, 2018; Yee et al., 2016, 2017; Janulewicz et al., 2017; Chao and Zhang, 2018).

More recent studies have also focused on biomarkers that are etiologically related to the neuropsychological deficits in GW veterans. These include toxicant induced neuroinflammation as well as war-time stressors (Brimacombe et al., 2002; Sullivan et al., 2003). Relevant to these factors are the rodent studies showing increased neuroinflammation when neurotoxicants were combined with simulated war-time stressors in the models (O'Callaghan et al., 2015; Ashbrook et al., 2018; Koo et al., 2018).

REVIEW OF GENERAL NEUROPSYCHOLOGICAL FINDINGS

The neuropsychological literature has been reviewed multiple times by the RAC-GWVI (Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI], 2008, 2014;

[1]https://cdmrp.army.mil/gwirp/research_highlights/19gwi_cde_initiative_highlight.aspx

White et al., 2016). Additionally, review papers were published between 2000 and 2009, including Axelrod and Milner (2000), Vasterling and Bremner (2006), and White et al. (2016). More recently, a meta-analysis of the research data was published (Janulewicz et al., 2017). The current paper reviews the methodological strengths and limitations of the neuropsychological outcome studies to date. This includes 25 papers of which 14 were included in the meta-analysis. All 25 papers included assessments with validated neuropsychological instruments and were not case studies. **Table 2** illustrates the increased sophistication in the field over time regarding case definitions and sensitive comparison groups and subsequent progress in understanding GW veterans' neuropsychological profiles.

TABLE 2 | Review papers on neuropsychological outcomes.

References	Summary and recommendations
Axelrod and Milner, 2000	• Concluded that methodological issues limited the ability to understand the data. • Recommended that future studies include more sophisticated cohort comparisons, including exposure data.
Vasterling and Bremner, 2006	• Concluded that there was no clear pattern in neuropsychological outcomes and insufficient neuroimaging evidence to draw conclusions at this point. • The impact of mood and the discrepancy between subjective reports and objective measurements made it more difficult to determine the etiology of any deficits observed. • Recommended that results need replication, objective measures of exposure should be used when applicable, baseline data should be used to investigate pre-existing vulnerabilities. • Future research should be built on more complex models that incorporate individual vulnerabilities, environmental factors and their physiological and emotional consequences and immunologic functioning.
Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI], 2008	• Concluded that symptomatic veterans have a subtle "sub-clinical" CNS damage. This included deficits in attention, executive function, memory, visuospatial skills, psychomotor functioning, and mood. • Recommended that analyses of veteran subgroups, i.e., those with more pronounced cognitive deficits and those with differing exposure histories, would be most informative.
White et al., 2016	• Concluded that GW exposures are associated with decrements in cognitive function. • Future research should investigate the mechanisms and etiology of GW health problems so that biomarkers of exposure and illness may be discovered.
Janulewicz et al., 2017	• Concluded with meta-analytic methods that GW deployment is associated with deficits in visuospatial, attention, executive function, and learning and memory but not simple motor function. • Future research developing treatments or investigating biomarkers of GWI should include neuropsychological outcomes in the domains of visuospatial, attention and executive function, and learning and memory. Particularly, Block Design, Trail Making Test, Digit Span, and CVLT, were sensitive measures to use with veterans with GWI.

Axelrod and Milner (2000) summarized the neuropsychological literature to date and reported that methodological problems limited the ability to understand the data. It was recommended that neuropsychological literature would be better served by including not only analyses based on normative data, non-deployed control comparisons, or self-reported medical concerns, but by also including relative risk for neurotoxicant exposures and hypothesis driven data collection.

Vasterling and Bremner (2006) found in their review of the literature, that there was no clear pattern in neuropsychological outcomes. Additionally, the impact of mood and the discrepancy between reported symptoms and objective performance made it more difficult to elucidate etiology of any deficits that were found.

In addition, the Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI] (2008) review concluded that symptomatic veterans had subtle "sub-clinical" CNS damage. This included deficits in attention, executive function, memory, visuospatial skills, psychomotor functioning, and mood. The RAC-GWVI Committee recommended that analyses of veteran subgroups, i.e., those with more pronounced cognitive deficits or those with differing exposure histories, would be most informative. When the general data regarding these studies were reviewed by the Institute of Medicine (Institute of Medicine [IOM], 2006), it was determined that overcorrecting for mood may have diminished the power to detect differences in neuropsychological variables in some prior studies.

White et al. (2016) concluded that exposures were associated with decrements in cognitive functioning in GW veterans and future research should investigate the mechanisms and etiology of GW health problems so that biomarkers of exposure and illness may be identified. In the recent GW meta-analysis by Janulewicz et al. (2017), it was reported that there were difficulties assessing domain specific findings given the sparse information reported in included studies, and the overlap between studies that prevented a more diverse sample. In addition, data were too limited to assess toxicant exposure in relation to neuropsychological deficits. Even with limitations across studies, it was found that deployed GW veterans and symptomatic GW veterans demonstrated levels of cognitive impairment, particularly in visuospatial abilities, attention/executive functioning, and learning/memory domains.

SUBJECTIVE MEMORY

Subjective memory has long been one of the most reported and debilitating symptom complaints of GW veterans. However, it has been unclear if this relates to objective memory deficits vs. attentional variability or the fatigue symptoms and sleep difficulties of those with GWI. It may also be that one-time objective neuropsychological testing in a quiet room does not fully capture functional memory concerns. The three studies that have addressed this topic to date include Binder et al. (1999), Lindem et al. (2003b), and Chao (2017).

Binder et al. (1999) incorporated measures of subjective cognitive complaints (e.g., Symptom Check List-90- Revised [SCL-90-R; Derogatis, 1992]) and affective distress (e.g., Beck

Depression Inventory [BDI; Beck and Steer, 1993], Beck Anxiety Inventory [BAI; Beck et al., 1988]) in addition to a computerized test battery (Anger et al., 1996). With a sample of 100 symptomatic GW veterans, results showed higher correlations between subjective memory complaints and affective distress versus between subjective memory complaints and objective neuropsychological results. Therefore, Binder et al. (1999) concluded that affective distress was a necessary component of GW evaluations and provided additional explanation for worse cognitive outcomes in some GW veterans.

Lindem et al. (2003b) studied the relationship between neuropsychological symptom reporting and outcomes on objective tests in GW veterans. Symptom reporting was done with the Expanded Health Symptom Checklist (HSC, Proctor et al., 1998) which included five neuropsychological symptoms (e.g., difficulty concentrating, difficulty learning new material, forgetfulness, memory lapses, and confusion). Based on responses, participants were divided into groups of no complaints, a moderate level of complaints, and a high level of complaints. The researchers predicted that higher endorsement of neuropsychological symptoms would be associated with poorer performance on measures of attention and memory. Mood–related diagnosis was assigned using the following measures: Structural clinical interview for DSM (SCID; Spitzer et al., 1990), Clinical-Administered PTSD Scale (CAPS; Blake et al., 1990), the Mississippi Scale for Desert Storm, and Brief Symptom Inventory (BSI; Derogatis, 1993). Analyses were conducted to evaluate the ability of neuropsychological performance to categorize those with no, moderate, or high neuropsychological self-reported symptoms while controlling for covariates. Analyses indicated that subjective complaints did not show a pattern consistent with predicted performance on cognitive domains; however, they were more associated with mood complaints, which aligned with findings in Binder et al. (1999). Veterans with high levels of neuropsychological symptoms also reported tension, fatigue, confusion, and decreased vigor on the Profile of Mood States (POMS). Therefore, researchers concluded that these deficits are best measured by both objective neuropsychological testing and mood assessment to elucidate a clinical picture of GWI.

More recently, Chao (2017) conducted a study aimed at examining how subjective memory complaints (1 query of difficulty remembering) correspond with the likelihood of objective test results using the CVLT-II with a sample of 428 deployed GW veterans. Chao (2017) found significant impairment in verbal learning, retention, and recall in veterans with subjective complaints, even when accounting for age, sex, years of education, and mood-related diagnoses (e.g., major depressive disorder [MDD], PTSD, and anxiety). However, those with subjective memory complaints were more likely to have a PTSD diagnosis. Regression analyses also demonstrated poorer retention in association with subjective memory complaints. These results contrast with previous research (Binder et al., 2001; White et al., 2001) that did not find a connection between subjective complaints and objective impairment. Chao (2017) concluded that subjective memory complaints are sensitive to neuropsychological deficits and, as subjective memory complaints are linked to dementia risk, a necessary component of GW neuropsychological assessment.

These three studies did not show consistency in regard to objective tests (**Table 3**). Binder et al. (1999) and Lindem et al. (2003b) found results that linked subjective complaints to more mood-related factors, whereas Chao (2017) found evidence of objective memory impairment with subjective complaints. Given the discrepancy between subjective complaints and objective test performance, more validation research is needed with tests sensitive to memory impairment in GW veterans as delineated in the CDE protocol (**Table 1**). Also, none of these studies used the same subjective question of memory functioning making comparisons with objective measures difficult. Future studies should incorporate a validated subjective measure of cognitive functioning such as the Everyday Cognition Scale. In addition, careful use of statistical measures must be implemented to understand the unique contribution that mood and cognitive factors play in neuropsychological performance.

NEUROPSYCHOLOGICAL PERFORMANCE AS COMPARED BY NORMATIVE DATA

The following two early studies (Axelrod and Milner, 1997; Sillanpaa et al., 1997) examined those deployed in the GW in comparison to normative data. Axelrod and Milner (1997), tested 44 male GW veterans on a comprehensive neuropsychological exam (**Table 3**). Compared to normative data, deficits were found on only a motor test; Grooved Pegboard and a test of executive function; Stroop Color and Word Test (Matthews and Klove, 1964; Heaton et al., 1992). The researchers attributed the neuropsychological issues to elevations on selected subtests of a personality measure the Minnesota Multiphasic Personality Inventory Second Edition (MMPI-2; Graham, 1990). However, Janulewicz et al. (2017) found through examination of effect sizes, that cognitive flexibility as measured by the Trail Making Test- Trail B had a large effect size, while a small to medium effect was seen in motor tests, which may show some deficits that were masked by a small sample size. Other limitations were the lack of a control group (i.e., comparison to normative data collected from a non-military population), and lack of control regarding covariates of cognitive performance (i.e., age, gender, developmental history), and psychopathology (i.e., PTSD).

Sillanpaa et al. (1997) investigated neuropsychological and neurological functioning in 49 GW veterans from an Army Reserve Military Police unit. Each veteran completed personality and neuropsychological testing (**Table 3**). Neuropsychological performance was evaluated in comparison to normative data and models were created to test variables associated with a syndrome and to test variables associated with mood. The syndrome model included demographic factors, self-reported exposure to toxicants and a composite score of subjective complaint (i.e., composed of scores from the SCL-90-R and MMPI-2), and a clinical signs index (i.e., composite score of laboratory tests for liver and immune functioning or infection presence). The model of mood-related issues included indices of trait anxiety, subjective

TABLE 3 | Neuropsychological studies with Gulf War veterans.

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Goldstein et al., 1996	29 GWV, 39 non-veterans	WAIS-R Information WAIS-R Similarities WAIS-R Digit Span WAIS-R Arithmetic WAIS-R Digit Symbol WAIS-R Picture Completion WAIS-R Block Design COWAT Incidental Memory Verbal Associative Learning Short-Term Memory Symbol Digit Learning Trail Making Test CPT Grooved Pegboard	– GWVs compared to demographically matched controls on neuropsychological tests – Test battery included some measurements sensitive to GWI (i.e., Block Design)	– No case designation based on GWI – Limited measurement of mood	– Found that GWVs performed worse on impairment index in compared to controls – However, the level of impairments (1 SD) was not consistent with subjective cognitive complaints – Effect size Cohen's d calculation* show small effect for Trails B (d = 0.25) and pegboard dominant (d = 0.18).
Axelrod and Milner, 1997	44 male GWV from Army Guard unit	Reitan-Indiana Aphasia Screening Test WAIS-R AVLT Stroop* Trail Making Test WMS-R Finger Tapping Grooved Pegboard* Grip Strength COWAT Category Fluency PIAT-R WCST	– Veterans compared via normative data and grouped by both objective (i.e., Grooved Pegboard, Stroop) and subjective (i.e., health complaint) measures	– Small sample size – Lack of correction for Type 1 error – Volunteer sample – No hypotheses	– No evidence of deficits – Differences in subjective complaints in psychological measures – Effect size Cohen's d calculation* show large effect for Trails B (d = 1.28), and small/medium effect for motor tests (d = −0.63 to −0.48)
Hom et al., 1997	26 GWV with Haley Syndromes, 10 GWV and 10 non-deployed veteran controls	WAIS* Halstead Category Test* Tactual Performance Test Seashore Rhythm Test Speech-Sounds Perception Test Finger Oscillation Test Trail Making Test* Reitan-Indiana Aphasia Screening Examination, Reitan-Klove Sensory Perceptual Examination Reitean-Klove Lateral Dominance Examination Reitan Word Finding Test WMS-R* WRAT3*	– Matched GWV group – GWI criteria used with a factor derived technique	– Small sample size – Limitations of those with fitting factor criteria – Multiple hypothesis testing Initial differences between control group and cases	– Differences between GWI and GWVs in global neurocognitive functioning – Cohen's d calculation* showed a large effect size for Block Design (d = −1.57) and a medium effect size for Trail Making Test- Trail B (d = 0.69) – Psychological responding was consistent with other medical patients
Sillanpaa et al., 1997	49 GWV from a single Army reserve military police unit	NES-2 CPT* Grip Strength Grooved Pegboard* Neurological Screen Fingertip Number Writing perception* WCST* AVLT WAIS-R *	– Examiner blind to participant's medical history – Exposure to toxins measured via self-report – Models tested to mimic GWI and psychological functioning	– Small sample size – Low variance and range in scores – Multicollinearity problems present	– Psychological model accounted for neuropsychological performance with a R^2 of at least a 0.03 (at or above a small effect) for all domains – At least a small effect for exposure and symptoms seen in nearly all domains – Cohen's d calculation* showed a medium effect for motor functioning (d = 0.76)
Vasterling et al., 1998	43 GWVs: 19 with PTSD and 24 without	Letter Cancelation Stroop CPT* WCST WAIS-R Digit Span WAIS-R Arithmetic* Rey-AVLT* CVMT*	– Investigated GWVs with PTSD	– Small sample size – Lack of comparison sample of participants with differing mental disorders – Unable to manipulate trauma exposure	– Veterans with PTSD had deficiencies in sustained attention, mental manipulation, information acquisition, and retroactive interference

(Continued)

TABLE 3 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Anger et al., 1999	66 GWV with unexplained symptoms from WA and OR, 35 GWV controls	Behavioral Assessment and Research System (BARS): Simple Reaction Time* Selective Attention Test Digit Span* Symbol Digit* Serial Digit Learning ODTP*	– Compared those with GWI with controls – Physician blind to participant status	– Volunteer sample Self-selection bias	– Specific problems demonstrated in processing speed – Individuals compared based on processing speed differences also found that those with slower processing speed had deficits in memory and attention
Binder et al., 1999	100 GWV with unexplained symptoms	CPT ODTP*	– Investigated self-report of cognitive ability and affective distress in conjunction with objective cognitive performance	– Cognitive measures may lack sensitivity	– Subjective complaints associated with psychological distress over objective cognitive performance
Bunegin et al., 2001	8 symptomatic GWV, 8 GWV controls	NES-2: Hand-Eye Coordination Simple Reaction Time Visual Digit Span Forward and Backward* Horizontal Addition Pattern Memory* Switching Attention*	– Compared symptomatic GWVs with non-symptomatic GWVs – Investigated blood flow	– Small sample size – Less sensitive measures used	– Symptomatic GWVs had worse performance in memory and executive function tasks – Exposure to acetone also impacted cognitive performance in GWVs – Cohen's d calculation* showed a small effect size for CPT, reaction time (d = −0.14)
Lange et al., 2001	48 symptomatic GWV, 39 GWV controls	NES* PASAT* WAIS-R Digit Span* CVLT RCFT Trails Making Test Category Test* Judgment of Line Orientation Test WAIS-R Block Design Grooved Pegboard	– Compared those with GWI with matched controls	– Volunteer sample of health-care seeking veterans – Small sample size CFS sample – Unequal cells comparisons	– Impairment found in attention (R^2 = 0.12–0.19) and executive functioning tasks (R^2 = 0.07) even after controlling for mood – Cohen's d calculation* showed a large effect size for CPT reaction time (d = 0.85).
White et al., 2001	193 GWV, 47 Germany deployed veterans	WAIS-R CPT Trail Making Test PASAT WCST Digit Span CVLT* WMS-R* Finger Tapping Purdue Pegboard POMS* TOMM	– Compared deployed and non-deployed veterans – Detailed account of toxin exposure – Stratified Random sample	– TOMM scores evidenced possible poor effort in some participants – Multiple comparisons	– Initially, mood was only significant with adjustment for multiple comparisons – Comparing those with and without exposure, had worse performance in short term memory (R^2 = 0.315–0.399), attention (R^2 = 0.381), and mood ((R^2 = 0.202–0.315) – Cohen's d calculation* showed small effect sizes for all neuropsych tests (d = −0.47 to 0.22
David et al., 2002	209 British GWV, 132 non-deployed era veterans	WAIS-R NART WAIS-III Letter Sequencing PASAT SART Stroop Trail Making Test WMS-R Purdue Pegboard	– Compared Gulf War deployment and medical status against other deployments (Bosnia) and controls – Stratified Random sample Blind raters Statistical analyses	– Cross-over effects Self-report symptoms	– Significance only found in PTSD measure – Cohen's d calculation* showed a large effect size for Block Design (d = −2.53)

(Continued)

TABLE 3 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Lindem et al., 2003a	193 GWV, 47 Germany deployed veterans	WAIS-R Information subscale* WAIS-R Digit Span* WMS-R Digit span CPT* Trail Making Test WCST PASAT* Finger Tapping* Purdue Pegboard* WAIS-R Block Design WMS-R Verbal Paired Associate Learning CVLT* Visual Reproduction* POMS*	– Investigated PTSD in relation to exposure to chemical agents – Investigated symptom severity – Better generalization with use of overall cohorts in Gulf War – Large sample size	– Correlational analyses – Lack of baseline performance or known preexisting conditions	– PTSD symptoms severity correlated with greater deficits in a wide array of neuropsychological measures in GW deployed veterans (Partial R^2 = 0.02–0.10) – CBW exposure and PTSD severity in GWVs associated with deficits in sustained attention (Partial R^2 = 0.0004–0.0015), motor speed/motor coordination (0.0000–0.0007)
Lindem et al., 2003b	193 GWV, 47 Germany deployed veterans	WAIS-R Information subscale WAIS-R Digit Span WMS-R Digit span CPT Trail Making Test A Trail Making Test B WCST PASAT Finger Tapping Purdue Pegboard WAIS-R Block Design WMS-R Verbal Paired Associate Learning CVLT Visual Reproduction POMS*	– Investigated GWVs discrepancy between subject complaints and objective performance	– Multiple comparisons	– Subjective complaints more associated with mood symptoms
Lindem et al., 2003c	58 GWV and 19 Germany-deployed veterans	WAIS-R Information subscale WAIS-R Digit Span WMS-R Digit span CPT Trail Making Test* WCST* PASAT Finger Tapping Purdue Pegboard WAIS-R Block Design WMS-R Verbal Paired Associate Learning* CVLT* Visual Reproduction* TOMM* POMS	– Investigated motivation in GWVs	– Small sample size – Difficult to ascertain the reason behind lower TOMM scores – Low amount of those with low TOMM scores	– Variability was seen in those with lower TOMM scores particularly in attention, executive functioning, and memory
Proctor et al., 2003	Danish GWVs (215), comparing deployed (143) and non-deployed veterans (72)	WAIS-R Information CPT Trail-making Test Wisconsin Card Sorting Test Purdue Pegboard WAIS-R Block Designs California Verbal Learning Test WMS Visual Reproductions POMS* TOMM	– Blind to categorization of "higher or lower" symptom status during all phases of recruitment, testing, and interviewing – Differences in deployment missions between Danish and American groups	– Significant mean age difference between deployed (38.8 years) and non-deployed (34.8 years) – Self-report of exposure	– Evidence of increased mood complaints related to GW service – no significant domain-specific evidence of CNS dysfunction was found – No associations between reported GW Environmental exposures related to the Danish GW deployment mission and objective measures of cognitive functioning were observed

(Continued)

TABLE 3 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Sullivan et al., 2003	207 treatment seeking GWV (120 referred for neuropsych evaluation), 53 treatment seeking non-deployed veterans	WAIS-R Information WAIS-R Digit Span* Trail Making Test NES CPT Stroop Test Paced Auditory Serial Addition Test Wisconsin Card Sort Test* CVLT WMS-R Paired Associate Learning WMS-R Visual Reproductions* Hooper Visual Organization Test WAIS-R Block Design* Finger tapping Purdue Pegboard RCFT* POMS* TOMM	– Investigated deployment, treatment seeking, use of pyridostigmine bromide (PB) and PTSD on cognitive functioning – Matched by control group that was also treatment seeking	– Self-report of exposure – Sample size small for comparisons	– GW deployed worse than controls on attention, visuospatial skills, visual memory, and mood – PB use in GWVs worse in executive system tasks – GWVs with PTSD versus those without PTSD showed no differences – Cohen's d calculation* showed a large effect sizes for block design and digit span forward (d = −2.43 to −1.00), small effect sizes for Trails A and B, digit span backward, CVLT, WMS, immediate recall, and finger tapping (d = −0.090 to 0.43), and a medium effect size for WMS, delay recall (d = −0.55).
Vasterling et al., 2003	72 GWVs deployed and 33 non-deployed GWVs	WAIS-R Digit Span WCST AVLT CVMT Purdue Pegboard WAIS-R Information	– Selection of a non-treatment seeking group of GWVs – Comparison of deployed GWVs to a group of GWVs mobilized but no deployed – Use of olfactory and neurocognitive measures with demonstrated sensitivity to neurotoxic exposures	– Sample was regionally recruited	– No evidence that performance on olfactory or neurocognitive measures were related to war-zone duty or to self-reported exposure to GW toxicants – Symptoms of emotional distress were positively correlated with self-report of health and cognitive complaints
Proctor et al., 2006	140 Army GWV with modeled estimates of nerve agent exposure	CPT Trail Making Test WAIS-R Digit Span WCST Finger Tapping Purdue Pegboard* WAIS-R Block Design* CVLT WMS-R verbal paired associate learning WMS visual reproduction	– Stratified random sampling – Examined performance by exposure to sarin and cyclosarin – Sample was unaware of sarin and cyclosarin components, analyses were conducted a prior to exposure knowledge	– Etiology undetermined given the risk of another illness between exposure and measurement (i.e., no baseline health information) – Limited objective information about exposures	– Exposure associated with poor fine psychomotor dexterity (d = 0.44) and visuospatial abilities (d = 0.43)
Barrash, 2007	301 GWV, 99 era veterans deployed elsewhere	WAIS-III Similarities* Block Design* Digit Symbol Digit Span North American Reading Test – Revised Starry Night Test COWAT AVLT* Benton Visual Retention Test* RMT-Words and Faces* Stroop Grooved Pegboard*	– Study of effort and neurocognitive performance in GWVs – Grouped by credible or non-credible impairment	– Small sample of non-credible group – Decreased statistical power – Lack of measures investigating reason behind low effort	– Non-credible impairment associated with more variability in tests and worse emotional/cognitive functioning

(Continued)

TABLE 3 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Wallin et al., 2009	41 GWVs: 25 with GWI and 16 controls	WRAT reading Block Design Trail Making Test CVLT Pegboard	– Stratified random sampling – Used GWI criteria to divide groups	– Small sample size – Gap between deployment and time of study – Multiple analyses	– Differences only seen in mood and health measures – Cohen's d calculation* showed a medium effect size for block design, Trails B, and CVLT long delay, (d = −0.73 to 0.51), and a small effect size for WRAT reading, Trails A, and Pegboard (d = −0.13 to 0.39).
Toomey et al., 2009	1061 deployed GWV and 1128 non-deployed GWV	WAIS-III Digit Span Trail Making Test* PASAT CPT* WCST CVLT* RCFT* Finger Tapping* Purdue Pegboard* TOMM WRAT-III	– Investigated differences in deployment, toxin exposure, and GWI status – Large sample size, stratified random sampling method – Use of factor analysis – Use of Khamisiyah exposure data	– Low study participation rates – Cross-sectional design – Neuropsychology raters were not blind to condition	– Deployed veterans had worse performance on motor speed (OR = 2.35) and sustained attention (OR = 2.64) – Those with Khamisiyah exposure showed poor motor speed after controlling for mood – Cohen's d calculation* showed small effect sizes for all neuropsych tests (d = −0.09 to 0.06).
Chao et al., 2010	40 GWV with a history of DOD notified sarin cyclosarin exposure risk and 40 non-exposed matched GW veteran controls	CPT Trail Making Test WAIS-III Digit Span Short Category Test COWAT Grooved Pegboard WAIS-III Digit Symbol, matching WAIS-III Block Design WAIS-III Verbal Comprehension Index CVLT-II WMS-III Logical Memory BVMT-R TOMM*	– Used matched cohort sample – Use of Khamisiyah exposure data	– Lack of information regarding the unit and rank of veterans – Lack of information regarding symptom severity (i.e., CMI, smoking status, head injuries) – Lack of cumulative exposure for all GW veterans – Plume estimates only by unit	– No differences in cognitive measures after controlling for poor effort (i.e., failure of TOMM). – Cohen's d calculation* showed a small effect sizes for all neuropsych tests (d = 0.22 to 0.26).
Chao et al., 2011	64 sarin and cyclosarin exposed GWVs and 64 "matched" unexposed GWVs	CPT* WAIS-III Digit Span* Trail Making Test Short Category Test CVLT-II* Grooved Pegboard TOMM	– Used matched controls to compare structural and functional differences in veterans with suspected neurotoxicant exposure – Use of more sensitive MRI (4T) – Use of some sensitive tests for neuropsychological and mood outcomes	– Lack of information regarding veteran's unit, severity of GWI symptoms, smoking status, or history of head injury – Neurotoxicant exposure measured at unit over individual level	– Reduced gray matter and white matter in exposed veterans which was linked to neurotoxicant exposure – Exposed veterans made more omission errors and had slower responses times; omission errors was also linked to neurotoxicant exposure – Cohen's d calculations* showed a medium effect size for Trails A (d = −0.64), and small effect sizes for CPT, Trails B, CVLT, and pegboard (d = −0.36 to 0.38).
Chao et al., 2016	136 GWVs: 106 who reported hearing chemical alarms sound	WAIS III Block Design* Digit Span* CVLT		– Had to rely on self-reports of deployment-related exposures – Lack of pre-GW measurements of brain structure and function – Small sample size – Lack of a non-deployed GW-era veteran control group	– Self-reported frequency of hearing chemical alarms was inversely associated with and significantly predicted performance on the Block Design visuospatial task. – This effect was partially mediated by the relationship between hearing chemical alarms and lateral occipital cortex volume.

(Continued)

TABLE 3 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Conclusions
Chao, 2017	428 deployed GWVs: 272 which met CDC criteria for CMI	CVLT-II*	– Tested verbal memory with GWVs presenting with subjective memory complaints – Large sample size	– Only measured one domain to control for Type 1 error	– Worse performance on verbal memory associated subjective complaints over and above mood, however, there was higher endorsement of PTSD symptoms
Sullivan et al., 2018	159 GW-deployed preventative medicine personnel who had varying levels of pesticide exposure	WAIS-III information subtest Boston Naming Test Trail Making Test* CPT* WCST Finger Tapping Grooved Pegboard HVOT RCFT* Stanford-Binet Copying Test CVLT II POMS* TOMM	– Grouped veterans by exposure (low/high) to PB and pesticides – Sample had sophisticated knowledge of exposure as they were part of the medical team	– Multiple analyses – Exposures of PB and pesticide may be correlated – Classifications of groups based on self-report	– High pesticide/high PB had worse information processing speed, attention (i.e., errors), visual memory, and increased mood complaints

*See original journal articles in first column for test references. *Denotes significance of $p < 0.05$.*

complaints, depression, and state anxiety. Results indicated that mood-related factors (i.e., anxiety, depression) accounted for more variance in neuropsychological performance measuring attention, motor coordination, and executive functioning in comparison to the syndrome model. However, limitations of the study included a small sample size and the use of a syndrome model that does not represent the current case criteria for GWI (i.e., CMI or Kansas GWI criteria).

These two studies (Axelrod and Milner, 1997; Sillanpaa et al., 1997) were similar in that they attributed more mood-altering factors to neuropsychological functioning as measured by the MMPI-2. However, comparison of effect sizes may point toward a trend in relatively impaired motor coordination and executive functioning.

DEPLOYMENT STATUS AND NEUROPSYCHOLOGICAL FINDINGS

Goldstein et al. (1996) tested 21 GW deployed veterans with a battery of neuropsychological tests and compared their performance to results from 38 demographically matched non-military controls. Cognition was measured via an extended version of the Pittsburgh Occupational Exposure Test battery (**Table 3**). Psychological distress was measured via the SCL-90-R. An impairment index was composed of 14 total neuropsychological tests (**Table 3**). Differences were found in the overall impairment index with significantly poorer performance in deployed compared to the control group. When controlling for mood, the impairment index difference was no longer significant. Specific impairments were found on the Controlled Oral Word Association Test (COWAT) and the Continuous Performance Test (CPT) reaction time. Notable limitations of this study are the small sample size utilized as well as the use of matched controls from a non-military population. Effect sizes noted in the recent meta-analysis by Janulewicz et al. (2017) reported a small effect (0.25) in the Trail Making Test -Part B between the groups, while the Grooved Pegboard (dominant) score approached a small effect size (0.18).

White et al. (2001) performed neuropsychological testing and compared the outcomes in those with specific self-reported neurotoxicant exposures. Veterans (n = 240) were recruited from 2 deployed and one non-deployed cohorts (Proctor et al., 1998). Veterans underwent an environmental interview, mood surveys, a full neuropsychological test battery (**Table 3**) and a psychological diagnostic interview. Neuropsychological outcomes showed differences in CPT when mood covariates were not controlled for; however, no individual measure achieved statistical significance when controlling for mood. Of note, additional tests showed moderate effect sizes in measures of attention, executive, and motor function (Paced Auditory Serial Addition Test (PASAT), Wisconsin Card Sort Test (WCST), Trail Making Test- Part A, Purdue Pegboard) which suggest that those deployed in the GW had poorer cognitive performance.

David et al. (2002) investigated neuropsychological patterns among 341 veterans who served in the United Kingdom military forces. Out of 341 participants, 98 were designated "Gulf well," 111 were designated "Gulf ill," 78 were designated "Era ill" and 54 were designated "Bosnia ill." David et al. (2002) assessed general functioning through a complete neuropsychological battery (**Table 3**). In regard to neuropsychological test performance, the GW ill group had poorer performance on WAIS-R Performance IQ, the digit symbol test, the Trail Making Test, and Sustained Attention to Response Task (SART) accuracy. After adjusting for the BDI score and multiple comparisons, no significant differences were found between healthy and GW ill on cognitive performance measures. David et al. (2002) found that the ill group had higher scores on the Mississippi Combat Related

PTSD Scale. When testing the main effect of deployment, it was found that participants in the Gulf group had significantly lower Verbal IQ and Performance IQ scores (i.e., most notably, in Block Design) compared to the Era ill Group. Additionally, the Gulf ill group had the lowest pegboard performance compared to the other groups. After controlling for BDI scores, there were still significant differences in Verbal IQ and the Purdue Pegboard when comparing the Gulf ill group to other groups. However, these contrasts were not significant after adjusting for multiple comparisons. Therefore, David et al. (2002) concluded that there was no major neuropsychological impairment, but rather, more associations with mood related impairment in deployed veterans which may better account for poor performance on neuropsychological measures. However, by controlling these factors, they may have discounted the mood symptoms may have resulted from neurological impairment and/or neurotoxicant exposures. Additionally, before correction, there was indication that individuals who were GW ill may have difficulties associated with performance in Performance IQ, Digit Symbol, Trail Making Test- Part A and B, and SART errors. Additionally, GW ill was also associated with poorer performance in Verbal IQ, Performance IQ, Block Design, and the Purdue Pegboard. Furthermore, these studies highlight the importance of overcorrection for emotional symptoms that may lead to underestimating true neuropsychological deficit that can also lead to mood symptoms as stated by Institute of Medicine [IOM] (2006).

Lindem et al. (2003a) investigated neuropsychological performance in conjunction with chemical exposure and severity of trauma symptoms with a sample of 225 deployed and non-deployed participants. Participants were administered the CAPS to determine the level of trauma symptoms. In addition, the veterans underwent a full neuropsychological test battery White et al. (2001). Chemical exposure was assessed through self-report measures and a clinical interview. Results indicated that the severity of PTSD symptoms in the full sample after controlling for covariates was directly correlated with poorer performance in general intellectual ability, attention, motor, memory, and mood measures. In GW deployed veterans, partial correlations were significant for those with PTSD and worse performance on general intellectual ability, sustained attention, motor functioning, verbal learning, and all mood scales.

Proctor et al. (2003) studied neuropsychological measures in deployed (n = 143) and non-deployed veterans (n = 72) Danish GW veterans. Researchers compared groups across neuropsychological measures (White et al., 2001), controlling for age. It was found that there were significant differences for neuropsychological domains; such that individual tests of executive functioning and verbal memory showed poorer performance in the deployed veterans. There was significant difference on the POMS Fatigue and Confusion scales, with deployed groups reporting a moderate to high number of symptoms. Therefore, the researchers concluded that, as there was no connection between deployed and non-deployed groups on neuropsychological measures, there was no evidence in this study of toxicant exposure leading to neurocognitive deficits. Rather, mood related symptoms were more likely to be reported. However, this study was composed of Danish soldiers who were not exposed to combat and were not in chemical warfare areas indicating that they likely differed from other cohorts (e.g., British, American) given differential exposure to GW neurotoxicants (less endorsement of exposure to chemical warfare agents and no use of anti-nerve gas pills) and less trauma. However, further investigation of the effect sizes via Janulewicz et al. (2017) found small effects in the Trail Making Test (d = 0.22 to 0.31) and in a memory measure (CVLT; d = −0.32 to −0.20). Block Design approached a small effect as well (d = −0.18).

Sullivan et al. (2003) evaluated a sample of 260 veterans including GW deployed and seeking treatment (i.e., for cognitive or health symptoms) and a control group of GW non-deployed veterans seeking neuropsychological evaluations. All veterans underwent a neuropsychological battery (**Table 3**) in addition to a structured clinical interview. In comparison to non-deployed veterans, deployed veterans had worse performance in measures of attention, visuospatial skills, and visual memory. In addition, deployed veterans endorsed worse mood symptoms. Therefore, the researchers concluded that GW deployment led to the significant neuropsychological decrements. Effect size analysis performed by Janulewicz et al. (2017) found a small effect in Trail Making Test- Part A (d = 0.43), Trail Making Test- Part B (d = 0.36), CVLT Trials 1–5 (d = −0.26), CVLT short delay (d = −0.47), CVLT long delay (d = −0.42), CVLT recognition (d = −0.33), and WMS immediate recall (d = −0.55). A medium effect was seen in WMS delayed recall (d = −0.55). A large effect or higher was seen in Digit Span backward (d = −1.00), and Block Design (d = −2.43).

Toomey et al. (2009) conducted a study examining GW veterans (deployed n = 1061, and non-deployed 1,128) on several measures of neuropsychological performance. Veterans underwent a neuropsychological battery (White et al., 2001). Results indicated that deployed veterans performed significantly worse on a measure of attention flexibility (i.e., Trails A-B) in comparison to non-deployed veterans. The meta-analysis completed by Janulewicz et al. (2017) did not return any notable effect sizes.

Overall, these studies comparing deployed GW veterans to non-deployed veterans showed some consistency in relative impairment within major cognitive domains, including simple and sustained attention, complex tracking, working memory, acquisition and retention of information when simply comparing deployment status rather than symptomatic vs. non-symptomatic groupings.

SYMPTOMATIC VS. NON-SYMPTOMATIC DEPLOYED GW VETERANS AND NEUROPSYCHOLOGICAL PERFORMANCE

Hom et al. (1997) first investigated symptomatic GW veterans (n = 26) in comparison to healthy GW veteran controls (n = 20) on neuropsychological and psychological measures (**Table 3**).

Psychological functioning was measured using validated surveys and a clinical interview. Symptomatic veterans showed significantly worse performance on measures of overall brain function or derived composite scores from neuropsychological measures (Halstead Retain Impairment Index). In addition, symptomatic veterans showed greater impairment than controls on the Halsted Category Test and Trails Making Test- Part B (d = 0.69 per Janulewicz et al., 2017), indicating poor abstract reasoning and problem solving/flexibility; measures of executive functioning. Of note, Janulewicz et al. (2017) also found a large effect size for Block Design (d = −1.57) for this study. The researchers concluded that these results supported the presence of worse neuropsychological and mood functioning in veterans with GWI as classified by Haley syndromes (Haley et al., 1997). However, these researchers hypothesized that mood complaints were secondary to the physical dysfunction consistent with GWI symptoms and did not solely account for GWI presentation. This study exhibited several limitations including a small sample size.

Anger et al. (1999) investigated mood and neuropsychological differences in GW veterans with unexplained medical symptoms. Veterans underwent a medical examination conducted by a physician blind to case/control designation; controls were determined as those not endorsing any GW related symptoms. Symptomatic and non-symptomatic veterans (N = 101) completed a series of tests assessing psychological and neuropsychological functioning (**Table 3**). Anger et al. (1999) found statistically significant differences on neuropsychological testing only for the Oregon Dual Task Procedure (ODTP) computerized test measure after controlling for multiple comparisons. Using these results, researchers divided groups based on speed as "slow cases" and "other cases." Consistent with performance on the ODTP, veterans in the "slow case" group showed slower responses than controls on Symbol Digit, Simple Reaction Time, Digit Span Forward, and Digit Span Backward. Therefore, Anger et al. (1999) reported slower neurobehavioral performance on digit recall tasks and increased psychological distress in those with GWI symptoms. However, slow performance was exhibited in a sub group of GW cases ("slow cases"). These "slow cases" also showed deficits in working memory, attention and response speed indicating a more severe subgroup. These results were also not otherwise explained by mood or PTSD and were consistent with the literature investigating deficits in those with organophosphate poisoning.

Storzbach et al. (2000) conducted a study investigating the performance of GW veterans with unexplained symptoms (n = 241) on psychosocial and neurobehavioral measures in comparison to a veteran control group (n = 113). In regard to the mood measures, there was a significant difference between groups in that symptomatic veterans were higher on nearly all mood measures with nearly all measures demonstrating a large effect size. Additionally, the case group endorsed worse physical, mental, and health-related functioning (SF-36), greater combat exposure scale measures, and PTSD symptoms. In regard to neuropsychological testing, the case group had worse performance on Symbol Digit and ODTP forced choice and forced latency scores with a small effect size (Smith, 1968; Binder, 1993). The researchers concluded that, as they found differences in psychosocial and cognitive tests, stress has a major role in GW symptoms as either a precursor or a result of the experienced symptoms. However, these conclusions are limited in that the researchers did not control for mood when investigating neuropsychological performance.

Storzbach et al. (2001) expanded upon these findings using the same measures to assess psychosocial and cognitive functioning in 239 symptomatic GW veterans and 112 control veterans. However, they identified a "slow group" using a modified cutoff as established by Anger et al. (1999). The slow group had worse performance in comparison to controls in all measures, except the Serial Digit Learning Test again indicating a more impaired subgroup.

Binder et al. (2001) investigated cognitive performance in symptomatic GW veterans (n = 94) as defined by chronic fatigue syndrome (CFS). Groups were divided based on CFS criteria (Fukuda et al., 1994) with 32 participants comprising the case group and 62 participants comprising the control group. Neuropsychological testing was conducted using the same battery described in Anger et al. (1999). Results indicated that those in the case group performed worse on reaction time ODTP latency, and ODTP number correct. Limitations of this study include the use of a computerized measure that may have been less sensitive then measures with an examiner and a shorter battery with less global neurocognitive implications and the classification of GWI as CFS.

Bunegin et al. (2001) built their hypothesis on the premise that GW symptoms are linked to CNS dysfunction. Previous research has shown that GW veterans experience cognitive issues and headaches from chemical odors (Bell et al., 1990; Miller and Prihoda, 1999) which is similar to transient ischemia. Therefore, researchers investigated cognitive performance and middle cerebral artery blood flow velocity (MCABFV) in both symptomatic (n = 8) and asymptomatic GW veterans (n = 8) when exposed to different air conditions (i.e., clean air, placebo acetone condition, and low levels of acetone). All participants were tested using NES-2 computerized assessment (Letz, 1991). The results of the study suggested that both symptomatic and asymptomatic GW veterans performed similarly in cognitive tests when comparing the performance across different air exposures. However, pooled data across conditions revealed significantly lower performances in measures of memory and executive functioning in symptomatic GW veterans. Additionally, there were statistically significant differences between asymptomatic and symptomatic GW veterans in MCABFV as symptomatic GW veterans demonstrated a depressed response across all conditions.

Lange et al. (2001) conducted a study examining symptomatic and healthy GW veterans on cognitive functioning; however, symptomatic was defined using established criteria for CFS and Multiple Chemical Sensitivity (Cullen, 1987; Fukuda et al., 1998). Additionally, Lange et al. (2001) identified and accounted for presence of PTSD and major depression in a group of 87 GW veterans (healthy controls = 39; GWI = 48). Both healthy and symptomatic GW veteran groups were administered tests sensitive to attention, concentration or information processing, verbal and visual memory, abstraction and conceptualization, visuo-perceptual and perceptual-motor functions, and fine motor functioning. Analyses found significant results in attention,

concentration, and information processing, as well as abstraction and conceptualization. Tests reflecting attention and information processing as well as tests of abstraction and concentration were significantly different with symptomatic veterans showing worse performance than non-symptomatic controls. In addition, regression analyses were conducted controlling for mood outcomes; results indicated the symptomatic group remained significant on some tests (NES simple reaction time) but were no longer significant for other tests. Mood-related diagnoses were not correlated with performance on the CPT; therefore, case status was the only predictor and remained significant in symptomatic GW veterans. Lange et al. (2001) concluded that symptomatic veterans exhibited deficits on attention, concentration, and information processing over and above the impact of mood related disorders. Limitations of the study include using GWI terminology inconsistent with the field where current case criteria is determined using Kansas or CDC criteria rather than CFS and MCS.

Wallin et al. (2009) investigated neuropsychological performance in a small sample derived from the National Health Survey of GW veterans (Case group with CDC criteria = 25, Control = 16). Veterans underwent neuropsychological testing (**Table 3**) in addition to psychological testing. Wallin et al. (2009) found no significant differences between groups on neuropsychological testing. However, there were differences in GWI cases on measures of depression, somatic complaints, and anxiety. Wallin et al. (2009) concluded a stronger influence of psychological factors over neurological factors. Several limitations were present in this study including a small sample size. Correspondingly, an effect size analysis conducted by Janulewicz et al. (2017) found meaningful effects (>0.20) in Block Design (d = −0.73), Trail Making Test, Part-A (d = 0.39), Trail Making Test, Part-B (d = 0.51), CVLT, long delay (d = −0.66), Pegboard, dominant (d = 0.37) and Pegboard, non-dominant (d = 0.31) that would have shown significant differences in a larger study sample.

These eight studies had similar findings regarding cognitive domains when investigating symptomatic vs. non-symptomatic veterans indicating the more refined criteria than deployed vs. non-deployed. In the symptomatic groups there was consistency in attention deficits as measured Digit Span Forward in several studies (**Table 3**). Additionally, psychomotor speed as measured by Symbol Digit and Simple Reaction Time was sensitive to symptomatic veterans (**Table 3**). Finally, the most consistent finding regarding attention was in a measure of sustained attention – CPT, which was observed in several studies. Executive functioning was more variable with less consistency in test measures used. Therefore, there was few similarities between studies (i.e., no more than two studies had similar findings in Category Test and Trail Making Test- Part B). In regard to memory, several studies found memory impairment as measured by the CVLT-II (**Table 1**). These findings are supported by both imaging and animal models of memory. Regarding visuospatial functioning, there was consistency of results in that the symptomatic group showed worse performance on Block Design in multiple studies (Hom et al., 1997; Proctor et al., 2006; Chao et al., 2010). Finally, there was some consistency in motor coordination as measured by a Pegboard test in several studies (Proctor et al., 2006; Chao et al., 2010, 2011). In terms of mood measures, these studies did not have more than two studies that were consistent in mood results. However, health outcomes as measured by the SF-36 were different in the case symptomatic groups (Anger et al., 1999; Storzbach et al., 2000; Wallin et al., 2009).

These studies were able to demonstrate a stronger argument for neurological dysfunction given the clear operational definitions of symptomatic vs. non-symptomatic groups. However, these studies were still very diverse in regard to the measurement style (i.e., computer versus paper and pencil) and test battery and what determined 'caseness.' These studies also highlight the importance of using more objective measures of neurological biomarkers to make a stronger argument for behavioral and neurological connections. As noted before, future research using CDEs for case criteria and neuropsychological batteries would be highly beneficial given their sensitivity to changes in veterans with GWI as well as creating more consistency across studies.

NEUROTOXICANT EXPOSURE AND NEUROPSYCHOLOGICAL PERFORMANCE

Several studies have assessed neuropsychological functioning in relation to neurotoxicant exposures during the war including sarin, pesticides and pyridostigmine bromide (PB) anti-nerve gas pills. Results of these studies are reported below and in **Table 4**.

In the Proctor et al. (1998) paper described above, when comparing those exposed or not exposed to self-reported chemical warfare agents, significant differences were found in measures of tension and confusion (POMS), long term visual memory (WMS-R Visual Reproduction), short term verbal memory (CVLT), and attention/working memory (Digit Span). However, those that reported exposure to chemical warfare agents during the war also had lower scores in comparison to the unexposed group. When controlling for mood or malingering, the results did not change, indicating performances were not fully explained by mood disorders and likely represented sequelae from toxicant exposures.

Lindem et al. (2003a) as described above assessed veterans with PTSD and chemical exposure, analyses showed worse performance in sustained attention, motor speed, and motor coordination. Furthermore, researchers concluded that severity of PTSD was a contributing factor to issues with short-term verbal memory (acquisition, retrieval, semantic clustering). Additionally, this pattern suggested difficulties with sustained attention, planning, and executive functioning that may point toward issues with hypervigilance. Finally, self-reported chemical weapons exposure showed specific deficits in sustained attention, perseverative responses, visual memory, and mood measures.

Proctor et al. (2006) examined the relationship between DOD-estimated levels of sarin and cyclosarin exposure and neuropsychological functioning. A stratified random sample of GW veterans completed a medical and history questionnaire,

TABLE 4 | Neurotoxicants and Neuropsychological Performance.

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Key Findings and Conclusions
White et al., 2001	193 GWV, 47 Germany deployed veterans	WAIS-R CPT Trail Making Test PASAT WCST Digit Span CVLT* WMS-R* Finger Tapping Purdue Pegboard POMS* TOMM	– Compared deployed and non-deployed veterans – Detailed account of toxicant exposure – Stratified Random sample	– TOMM scores evidenced possible poor effort in some participants – Multiple comparisons	– Pesticide exposure by self-report was associated with worse mood functioning on all POMS subscales. – Chemical weapons exposure by self-report was associated with worse mood functioning on the POMS subscales of tension and confusion as well as poorer – performance on attention/executive functioning, memory and mood measures.
Sullivan et al., 2003	207 treatment seeking GWV (120 referred for neuropsych evaluation), 53 treatment seeking non-deployed veterans	WAIS-R Information WAIS-R Digit Span Trail Making Test NES CPT Stroop Test Paced Auditory Serial Addition Test Wisconsin Card Sort Test* CVLT WMS-R Paired Associate Learning WMS-R Visual Reproductions Hooper Visual Organization Test WAIS-R Block Design Finger tapping Purdue Pegboard POMS TOMM	– Investigated deployment, treatment seeking, use of pyridostigmine bromide (PB) and PTSD on cognitive functioning – Matched by control group that was also treatment seeking	– Self-report of exposure – Sample size small for exposure comparisons	– PB use in GWVs showed worse performance on an executive system task. – GWVs with PTSD versus those without PTSD showed no significant differences – There were no significant interaction effects of PB and PTSD on cognitive functioning.
Vasterling et al., 2003	72 GWVs deployed and 33 non-deployed GWVs	WAIS-R Digit Span WCST AVLT CVMT Purdue Pegboard WAIS-R Information	– Selection of a non-treatment seeking group of GWVs – Comparison of deployed GWVs to a group of GWVs mobilized but not deployed – Use of olfactory and neurocognitive measures with demonstrated sensitivity to neurotoxic exposures but not to organophosphates	– Sample was regionally recruited	– No evidence that performance on olfactory or neurocognitive measures were related to self-reported exposure to GW toxicants – GWVs reporting more significant exposures reported greater severity of health symptoms and more severe cognitive symptoms, than those reporting less significant exposures – Symptoms of emotional distress were positively correlated with self-report of health and cognitive complaints
Proctor et al., 2006	140 Army GWV with modeled estimates of nerve agent exposure	CPT Trail Making Test WAIS-R Digit Span WCST Finger Tapping Purdue Pegboard* WAIS-R Block Design* CVLT WMS-R verbal paired associate learning WMS visual reproduction	– Stratified random sampling strategy – Examined performance by exposure to sarin and cyclosarin by DOD modeling – Sample was unaware of sarin and cyclosarin components, analyses were conducted prior to exposure knowledge	– Etiology undetermined given the risk of another illness between exposure and measurement (i.e., no baseline health information) – Limited objective information about exposures – Plume estimates only by unit	– Exposure associated with poor fine psychomotor dexterity (d = 0.44) and visuospatial abilities (d = 0.43) in a dose-response manner – The difference on the motor task was equivalent to the performance effect of being approximately 20 years older and for the block design task, being 15 years older. – Higher exposure was not significantly related to mood state.

(Continued)

TABLE 4 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Key Findings and Conclusions
Toomey et al., 2009	1061 deployed GWV and 1128 non-deployed GWV	WAIS-III Digit Span Trail Making Test* PASAT CPT WCST CVLT* RCFT* Finger Tapping* Purdue Pegboard* TOMM WRAT-III	– Investigated differences in deployment, toxicant exposure, and GWI status – Large sample size, stratified random sampling method – Use of factor analysis – Use of Khamisiyah exposure data	– Low study participation rates from overall larger sample – Cross-sectional design – Neuropsychology raters were not blind to condition – Self-reported PB, pesticide, oil well fire, vaccine exposure	– Those with Khamisiyah exposure modeled sarin exposure showed poor motor speed after controlling for mood – Those reporting proximity to SCUD missiles had lower motor speed – Those reporting CARC paint exposure had worse visual memory – Khamisiyah exposure was associated with poorer verbal memory, beyond emotional distress and demographic variables
Chao et al., 2010	40 GWV with a history of DOD notified sarin cyclosarin exposure risk and 40 non-exposed matched GW veteran controls	CPT Trail Making Test* WAIS-III Digit Span Short Category Test COWAT* Grooved Pegboard* WAIS-III Digit Symbol, matching WAIS-III Block Design* WAIS-III Verbal Comprehension Index* CVLT-II WMS-III Logical Memory BVMT-R TOMM	– Use of DOD modeled Khamisiyah data for sarin/cyclosarin exposure – Demographically matched groups	– Lack of information regarding the unit and rank of veterans – Lack of information regarding symptom severity (i.e., CMI, smoking status, head injuries) – Lack of cumulative exposure for all GW veterans – Plume estimates only by unit	– No differences in cognitive measures after controlling for poor effort (i.e., failure of TOMM). – No correlation between unit-level dose-estimates and neuropsychological data in the exposed veterans. – In exposed veterans, hippocampal volume correlate positively with verbal comprehension scores, while total GM volume correlated positively with performance on verbal fluency and visuospatial ability and negatively with time to complete the Trail Making Test and time to place all pegs in the pegboard with the non-dominant hand. – In exposed veterans, total WM volume correlated positively with verbal fluency, and visuospatial function.
Chao et al., 2011	64 sarin and cyclosarin exposed GWVs and 64 "matched" unexposed GWVs	CPT* WAIS-III Digit Span* Trail Making Test Short Category Test CVLT-II* Grooved Pegboard TOMM	– Used matched controls to compare structural and functional differences in veterans with suspected neurotoxicant exposure – Use of more sensitive MRI (4T) – Use of sensitive tests for neuropsychological and mood outcomes	– Lack of information regarding veteran's unit, severity of GWI symptoms, smoking status, or history of head injury – Neurotoxicant exposure measured at unit over individual level	– Reduced gray matter and white matter in exposed veterans which was linked to sarin/cyclosarin exposure, over and above confounding demographic, clinical, and psychosocial variables. – Exposed veterans made more omission errors and had slower response times on CPT; omission errors was also linked to sarin neurotoxicant exposure – Positive correlation between GM and WM volume, on CVLT performance and digit span backward in the exposed veterans.

(Continued)

TABLE 4 | Continued

References	Group (N)	Neuropsychological Tests	Strengths	Limitations	Key Findings and Conclusions
Chao et al., 2016	136 GWVs: 106 who reported hearing chemical alarms sound	WAIS III Block Design* Digit Span* CVLT		– Had to rely on self-reports of deployment-related exposures – Lack of pre-GW measurements of brain structure and function – Didn't measure experience and exposure that took place *after* the GW – Small sample size – Lack of a non-deployed GW-era veteran control group	– Self-reported frequency of hearing chemical alarms was inversely associated with and significantly predicted performance on the Block Design visuospatial task. – This effect was partially mediated by the relationship between hearing chemical alarms and lateral occipital cortex volume. – Volumes of the lateral occipital cortex, right inferior frontal cortex, and right supramarginal gyrus were positively correlated with Block design raw scores. – Volumes of lateral occipital cortex, right supramarginal gyrus and right precuneus were negatively correlated with the frequency of hearing chemical alarms. – No dose-effect relationship between Khamisiyah exposures and Block Design raw scores – Frequency of hearing chemical alarms sound was inversely correlated with Backward Digit Span raw scores but not with raw scores on CVLT learning trial 2 or with CVLT short-delay free recall.
Sullivan et al., 2018	159 GW-deployed preventative medicine personnel who had varying levels of pesticide exposure	WAIS-III information subtest Boston Naming Test Trail Making Test* CPT* WCST Finger Tapping Grooved Pegboard HVOT RCFT* Stanford-Binet Copying Test CVLT II POMS* TOMM	– Grouped veterans by exposure (low/high) to PB and pesticides – Sample had sophisticated knowledge of exposure as they were part of the medical team	– Multiple analyses – Exposures of PB and pesticide may be correlated – Classifications of groups based on self-report	– High pesticide/high PB had worse information processing speed, attention (i.e., errors), visual memory, and increased mood complaints, after controlling for either CMI, PTSD, or depression. – High pesticides/low PB group was the worst performing in terms of visual memory recall while the low pesticides/low PB and low pesticides/high PB group performed significantly better. – Dichlorvos (pest strips) exposure was the best predictor of poorer performance in the attention and psychomotor domains. Methomyl (fly bait) exposure and lindane (delouser) were the best predictors of affective complaints in the mood domain. Bendiocarb and lindane were the best predictors for the visuospatial domain.

*See original journal articles in first column for test references. *Denotes significance of $p < 0.05$.*

a semi-structured environmental interview, neuropsychological testing, and psychological testing. Veterans were grouped based on exposed vs. non-exposed status as determined by modeled plume exposure estimates obtained from the DoD (from the Khamisiyah weapons depot detonations in March 1991). Results indicated significant differences in groups on psychomotor and visuospatial abilities (e.g., Purdue Pegboard and Block Design) with higher exposure associated with worse outcomes, or a dose response effect with exposure. However, one limitation is the gap between exposure and outcome measurement (4–5 years), thereby making it impossible to determine if it was a delayed or immediate exposure effect. Importantly however, this was the only study conducted before awareness of possible sarin exposure and before DOD notification letters were sent to those exposed at Khamisiyah, Iraq thus reducing bias based on knowledge of exposures.

Toomey et al. (2009) compared 2,000 veterans as described above and additionally performed analyses within the deployed veterans with and without sarin exposure from the Khamisiyah weapons depot detonations as classified by DOD notification (Winkenwerder, 2003). Results showed toxicant exposure was associated with motor speed deficits on CPT over and above mood related effects. In addition, depressive symptoms and exposure to self-reported contaminated food and water were related to worse scores of sustained attention measures. Veterans self-reporting CARC paint exposures had worse visual memory functioning. Veterans reporting being exposed to nerve agents during the war had worse verbal memory functioning and those reporting being near SCUD missiles had lower motor speed.

Chao et al. (2010) investigated neuropsychological performance (White et al., 2001) and MRI results between 40 GW veterans with a history of DOD notified sarin/cyclosarin exposure risk from Khamisiyah and 40 non-exposed matched GW veteran controls. When comparing the controls to the group exposed to sarin/cyclosarin, there were no differences in cognitive measures after controlling for poor effort (i.e., failure on the TOMM). However, the group with sarin exposure had less total gray matter and hippocampal volume on brain imaging. Limitations included lack of information regarding the unit and rank of veterans, lack of information regarding symptom severity (i.e., CMI, smoking status, head injuries), lack of cumulative exposure for all GW veterans, and plume estimates only by unit. Per Janulewicz et al. (2017), there were meaningful effects for Block Design ($d = -0.32$), CPT, reaction time ($d = 0.42$), CVLT long delay ($d = -0.34$), and Pegboard, non-dominant ($d = 0.27$).

Chao et al. (2011) then expanded on these prior findings using a different and larger cohort of veterans (sarin exposed $n = 65$; unexposed controls = 64) and a stronger 4T magnet MRI. Group comparisons on neuropsychological tests showed that sarin exposed veterans had more omission errors and slower reaction time on the CPT. Additionally, there was reduced gray matter and white matter volume in comparison to the control group. Regression analyses also revealed that GWI status was associated with errors of omission, as well as reduced gray matter and white matter volume. Janulewicz et al. (2017) also found meaningful effects in CPT reaction time ($d = 0.38$), Trail Making Test, Part A ($d = -0.64$), Pegboard, dominant ($d = -0.28$), and pegboard non-dominant ($d = -0.036$).

Sullivan et al. (2003) used a sample of 260 veterans including GW deployed and treatment seeking and a control group of GW non-deployed veterans not seeking treatment. Veterans were also compared based on PTSD and the use of pyridostigmine bromide (PB) anti-nerve gas pill usage during the war. All veterans underwent a neuropsychological battery (**Table 1**) in addition to a structured clinical interview to determine PTSD status. Veterans exposed to PB showed worse performance on a measure of executive system functioning. However, there was no difference between those with and without PTSD on neuropsychological measures and no interaction effect of PB use and PTSD diagnosis. Therefore, the researchers concluded that GW deployment and PB exposure led to the significant neuropsychological decrements.

White et al. (2001) – compared deployed and non-deployed veterans as described above. In this study, pesticide exposure by self-report was associated with worse mood functioning on POMS mood scales. Chemical weapons exposure by self-report was also associated with worse performance on attention/executive functioning, memory and mood measures.

Sullivan et al. (2018) investigated how differing levels of pesticide exposure and PB intake contributed to neuropsychological outcomes in GW veterans. The researchers recruited veterans with functional knowledge of their exposure to neurotoxicants based on their military occupational specialties as military pesticide applicators and/or preventative medical personnel. The four veteran comparison groups were based on pesticide and PB exposures. Participants completed a full neuropsychological test battery (**Table 4**). Veterans were also assessed for psychological functioning. GWI was determined by CMI criteria (Fukuda et al., 1998). Results showed that high pesticide/high PB exposed group showed significantly slower CPT reaction time and higher POMS symptoms. These neuropsychological decrements remained significant with PTSD as a covariate, demonstrating a main effect on attention reaction time in comparison to the low pesticide/low PB group. Additionally, the high pesticide exposure/low PB group was significantly worse on a measure of visual memory compared to the low pesticide/high PB and low pesticide/low PB groups. Significant differences were found in psychomotor, mood, attention, and memory domains when controlling for covariates (i.e., age, education, gender). Researchers found that a higher rate of CMI was associated with the high pesticide/high PB group which evidenced worse cognitive performance in attention, motor, and memory domains. Overall, results showed that high pesticide/high PB exposure had worse performance on information processing reaction times, attentional errors and visual memory accompanied by increased mood complaints. Limitations of this study include multiple analysis with a smaller sample size, increasing the chance of finding significance. Additionally, it is possible that, although the sample had a sophisticated knowledge of their exposure, their exposures were correlated (i.e., exposure to PB associated with exposure to nerve agents, and pesticides). Additionally, pesticide and PB classifications were reliant on self-report exposure.

Vasterling et al. (2003) compared 72 GW deployed veterans and 33 non-deployed veterans. They compared a full neuropsychological battery and used an olfactory test as a sensitivity measure of toxicant exposure. Results showed no evidence that performance on olfactory or neurocognitive measures were related to war-zone duty or to self-reported exposure to GW toxicants. Symptoms of emotional distress were positively correlated with self-report of health and cognitive complaints. However, the olfactory test has not been shown to be sensitive to organophosphate exposures, the most commonly associated exposure with GWI.

Research on neurotoxicant exposures varied in regard to the toxicant explored (i.e., PB, pesticides or cyclosarin/sarin) and methodology used (i.e., objective or subjective measures). Research would be improved by including more objective biomarkers of past toxicant exposure when comparing deployed and non-deployed troops. Suggested objective biomarkers could be immunological, genetic, or metabolic in nature and would strengthen the link between toxicant exposure and neurological dysfunction given these variables reflect compromised functioning at the time of the study rather than retrospective measures such as self-report or military dose estimate reports. Although markers of past organophosphate exposures have previously been elusive, more recent downstream effects from these exposures have been preliminarily identified and can be utilized (Abou-Donia et al., 2017). Additionally, some studies also addressed investigating illness status or treatment seeking groups (David et al., 2002; Sullivan et al., 2003). Research would improve with more consistent grouping of individuals based on established criteria for GWI (i.e., Steele, 2000). Finally, utilizing recommended CDEs consistently across studies for assessing mood and neuropsychological performance and consistent questions about neurotoxicant exposures would benefit future research as study results would be more comparable.

MOOD AND PTSD CONTRIBUTIONS TO NEUROPSYCHOLOGICAL PERFORMANCE

The following five studies that were previously reviewed above concluded that neuropsychological performance was either attributable to mood symptoms or equivalent to controls after accounting for mood-related symptoms (Axelrod and Milner, 1997; Sillanpaa et al., 1997; Storzbach et al., 2000; David et al., 2002; Proctor et al., 2003). Axelrod and Milner (1997) found that GW veterans had elevated scores in a MMPI measure thought to represent body complaints. Sillanpaa et al. (1997), using a model of psychological factors, found that depression (as measured by the MMPI) and anxiety (as measured by the STAI) significantly predicted neuropsychological performance in attention, motor, and executive functioning while an early model of GWI failed to produce significance. Storzbach et al. (2000) found that there was a significant predominately large effect difference in the case group on scores from multiple PTSD and psychological scales. David et al. (2002) found that symptomatic individuals had higher scores on depression, PTSD and anger scales. Finally, Proctor et al. (2003) found that there was significant difference in the POMS mood scales (Fatigue and Confusion) between those reporting GWI symptoms versus controls.

Vasterling et al. (1998) examined GW veterans with (n = 19) versus without PTSD (n = 24) on measures of attention and memory dysfunction. GW veterans with PTSD performed worse on the WAIS-R Arithmetic test and made more commission errors on the CPT. The GW veterans with PTSD also had worse performance in the Auditory Verbal Learning Test (AVLT) and Continuous Visual Memory Test (CVMT). Vasterling et al. (1998) hypothesized that the presence of intrusions (i.e., inability to inhibit thoughts or experiences related to trauma) could contribute to these patterns of symptoms. Using a principal component analyses, the researchers found that cognitive intrusions symptoms, particularly re-experiencing phenomenon, was related to poorer performance on memory and attention measures (**Table 2**). Therefore, they hypothesized that PTSD may lead to problems inhibiting inaccurate answers and filtering information unrelated to the task at hand. Of note, the study was limited given that the sample was specifically chosen to have PTSD and they also had other co-morbid diagnoses (i.e., major depression, dysthymia, panic disorder, social phobia, obsessive-compulsive disorder, and somatoform disorder), and included a small sample size making it difficult to control for potential confounds.

In these five studies, there was a lack of consistent use of the same psychological measures making comparing across studies difficult. In addition, some of these psychological tests that measure body complaints and pain, can be interpreted as representing physical or psychological impairments. However, these results showed that depression and PTSD are noteworthy covariates that should be accounted for when investigating neuropsychological performance in GW veterans. Nevertheless, many studies of toxicant exposure and GWI status show neuropsychological deficits even after controlling for mood (Vasterling and Bremner, 2006; Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI], 2008). Correspondingly, mood can also be affected by toxicant exposures such as those experienced in the GW indicating another reason why mood should also be assessed in neuropsychological assessments (Sullivan et al., 2018). The recently recommended CDEs for GW research also include measures of PTSD, depression and mood (**Table 1**).

MOTIVATION AND MALINGERING EFFECTS ON NEUROPSYCHOLOGICAL FUNCTIONING

Lindem et al. (2003c) investigated motivation as a contributing factor impacting neuropsychological results in GW veterans. Using a test of malingering and motivation performance validity test (TOMM), the veterans were grouped by those with high scores ($\geq$48) and those with low scores ($\leq$47). Mood related disorders were established using the structured clinical interviews (SCID and CAPS). Results indicated a significant

difference on measures of attention, executive functioning, and memory between those with high and low scores on the TOMM. Results also showed some inconsistency across performance given expected patterns with deficits in cognition. Specifically, veterans with lower TOMM scores had lower scores on a verbal memory measure (i.e., Verbal Paired Associates on WMS-R) whilst having higher scores on another test of verbal memory (i.e., CVLT), highlighting the variability that is associated with lower motivation. Additionally, more cognitively challenging items (Trails B, WCST) were more sensitive to low motivation as they required more effort than other tests of simple attention and concentration (Trails A, Digit Span Forward and Backward). Limitations included a small sample of veterans with poor effort (n = 18) and a lack of significant clinical measures. The researchers concluded that motivation was an important factor to consider when assessing cognitive performance in GW veterans.

Barrash (2007) proposed that neuropsychological examinations of GW veterans may be unreliable given possible poor effort, invalidating neuropsychological results. A sample of 399 veterans deployed in the GW were divided into three groups: participants without impairments, participants exhibiting impairment with credible results or participants with impaired and non-credible results. Participants underwent a full neuropsychological battery with results adjusted based on age, gender, and estimated premorbid intellect. In addition, veterans completed measures assessing psychological functioning and subjective cognitive complaints. Malingering was measured using the Exaggeration Index of the AVLT (Rey, 1964), Recognition Memory Test (RMT), performance across cognitive domains, error types, and MMPI-2 validity indices. Researchers found lower levels of non-credible performance among GW veterans, and those with non-credible validity results had worse impairment on nearly all neuropsychological tests. In addition, those in the non-credible group were more likely to endorse worse subjective cognitive symptoms and emotional and social impairment. Therefore, Barrash (2007) concluded that non-credible results are relatively rare in GW populations (<1%). In addition, researchers found consistently worse performance patterns in non-credible profiles indicating that a malingering measure should be used in neuropsychological assessments. Barrash (2007) noted some limitations including a small sample of the non-credible group, decreased statistical power, and a lack of measures indicating the reason for poor effort.

Both of these studies show a lower rate than expected in GW veterans for malingering or lowered motivation performances. However, it was demonstrated that low effort can lead to worse outcomes on neuropsychological testing as well as variable test performances that makes it difficult to interpret the true cognitive profile of GW veterans with low effort. Therefore, it is recommended that all neuropsychological batteries include measures of motivation and malingering that can be used as covariates in analyses or where those performing sub-optimally on these measures can be removed from data analyses. An element of the recent CDEs for neuropsychological assessment includes a motivational measure (CVLT Forced Choice, **Table 1**).

DISCUSSION

Gulf War illness is a CMI, impacting the health of a significant amount of GW veterans; however, the etiology and treatment of GWI remains somewhat elusive, prompting the demand for more research. Research investigating the neuropsychological underpinnings of GWI is especially needed given the prevalence of cognitive symptoms in GW veterans, possibly the second most reported symptom in GWI (Smith et al., 2012).

Early studies of neuropsychological functioning and GW veterans focused more on the etiology of these symptoms with conflicting results pointing either toward a mood related or neurological cause. These studies did not use an established criterion and compared groups based on their deployment status (deployed, non-deployed) and/or symptom presence (reporting symptoms, not reporting symptoms). Therefore, early review papers such as Axelrod and Milner (2000) recommended that further research should use more testable operational definitions of GWI (see **Table 2**).

Despite the efforts to establish criteria for GWI, researchers continued to find mixed results on the etiology of GWI centering on the debate of a mood related or neurotoxicant underpinning or both. Vasterling and Bremner (2006) highlighted that the impact of mood and the discrepancy between subjective reports and objective measurements made it more difficult to determine the etiology of any deficits observed. Further studies controlled for mood effects in analyses (i.e., PTSD or depression), however, different outcomes and case criteria used continued to make clear comparisons across studies difficult to interpret. For example, David et al. (2002) found substantial evidence of mood related nature of GWI using the Fukuda et al. (1998) CDC criteria in United Kingdom veterans,. Wallin et al. (2009) expanded on these findings using the CDC criteria in United States veterans and only found differences in GWI on depression, somatic complaints, and anxiety and were underpowered to detect neuropsychological impairments. Further research using the Haley GW syndromes eluded to more physiological causes (Hom et al., 1997). Nevertheless, focusing on these criteria or the presence of GW-related symptoms did not necessarily clarify the etiology of GWI. However, no study to date has compared the IOM and Kansas case criteria when comparing neuropsychological outcomes. Because the Kansas criteria has been shown to be a more specific case criteria than other measures used in prior studies (CDC, ME/CFS, 'slow' cases), this may provide more clarity with regard to neuropsychological impairment profiles in veterans with GWI.

In addition, there is now consistent evidence across nine papers comparing neurotoxicant exposures and neuropsychological outcomes (see **Table 4**). Seven out of the nine studies found significant neuropsychological differences when comparing exposures through either sarin/cyclosarin, organophosphate pesticides or PB anti-nerve gas pills. These studies point toward a pattern of neurotoxicant exposure and neurocognitive decline given their relative similarity to other occupationally exposed groups of agricultural workers or pesticide applicators (Ismail et al., 2012; Mackenzie Ross et al., 2013). Therefore, neurotoxicant exposures may have an impact

on particular neuropsychological domains including attention, executive system, memory and motor functioning as a result of chemicals that impact acetylcholine inhibition and induce neuroinflammation (Sullivan et al., 2003, 2018; Proctor et al., 2006; Toomey et al., 2009).

Additional research focused on other pertinent topics in GWI including subjective memory and effort. Briefly, one of the three studies found a correlation between subjective memory and objective memory functioning. Future research would benefit from consistency of subjective and objective cognitive queries.

As PTSD can be a relevant comorbidity in GW veterans, this review also included research investigating PTSD and GW veterans in relation to neuropsychological performance. In two out of three smaller studies, veterans with PTSD who had served in the GW showed worse performance in verbal memory (i.e., working memory, intrusions, recognition, and interference), visual memory, general intellectual ability, sustained attention, motor speed, and visuospatial skills (Vasterling et al., 1998, Lindem et al., 2003a). However, these findings were not replicated in a larger study of treatment seeking veterans (Sullivan et al., 2003).

The GW was a short combat mission of just 4 days of actual ground war, resulting in a PTSD prevalence rate of less than 10% of GW veterans in population-based studies (Research Advisory Committee on Gulf War Veteran's Illnesses [RAC-GWVI], 2008). Although studies of convenience samples with self-referred participants generally have higher PTSD rates. Prevalence rates of comorbidity between those with GWI and PTSD need to be fully investigated. Given the significant overlap of comorbidity in convenience sample studies, treatment studies should consider comorbid therapies where both disorders can be treated at the same time.

Correspondingly, GWI and stress or PTSD symptoms as a comorbid condition is supported by neuroinflammation and HPA axis research. O'Callaghan et al. (2015) and Koo et al. (2018) found that stress, as induced via corticosterone exposure, extended the impact of neuroinflammation from diisopropyl fluorophosphate (i.e., DFP sarin surrogate), in a rat model of GWI. This suggests that the effect of the neurotoxicant is worsened by the stressor rather than the cause. Furthermore, O'Callaghan et al. (2015) identified cytokine biomarkers (i.e., TNF-alpha, interleukin 6) which were more highly elevated after corticosterone exposure. Additionally, they found that treatment with anti-inflammatory antibiotic minocycline reduced the inflammatory response. Koo et al. (2018) found that DFP exposure was associated with wide spread microstructural integrity changes on diffusion imaging in the thalamus, amygdala, piriform cortex, and ventral tegmentum area whereas the rats also treated with corticosterone had more restricted patterns within the hypothalamus and hippocampus. Ashbrook et al. (2018) supported this finding identifying epigenetic biomarkers or transcriptional histone modification and DNA methylation in genes possibility linked to neuroinflammation and cognition in a rat model of GWI with DFP and corticosterone.

In research with human participants, Golier et al. (2012) found that GW veterans with PTSD were more likely to have higher plasma adrenocorticotropic hormone after exposure to corticotropin-releasing factor. Additionally, this change was associated with higher exposure to PB. Golier et al. (2012) concluded that this reflects HPA dysfunction in GW veterans. Furthermore, Golier et al. (2016) found that treatment via mifepristone was associated with improvements in verbal learning in GW veterans with CMI which was mediated by cortisol change levels suggesting some overlap with cognitive functioning and HPA axis health.

Suggestions for future research include using measures consistent across studies. Additionally, future research should continue to utilize more objective measures of neurological dysfunction (i.e., imaging, genetic studies, immunological factors) in conjunction with recommended neuropsychological and psychological test measures. These studies show the importance of controlling for PTSD and other mood effects when comparing neuropsychological outcomes in veterans with GWI. Although research is varied on GWI and its sub components (i.e., PTSD, effort, and subjective reporting), there remains strong evidence of neuropsychological decrements. The etiology of these results remains unclear but has been further linked to neurological dysfunction.

Mood factors remain relevant given their potential to exacerbate neurological dysfunction by possibly proliferating neuroinflammation (O'Callaghan et al., 2015; Koo et al., 2018). Despite these efforts, the heterogenous nature of methodologies investigating neuropsychological deficits limits the ability to truly identify the etiology of the neuropsychological decline without instituting common data elements of core tests used in all future neuropsychological studies. However, research has been improved to support evidence of GWI leading to deficits measurable through neuropsychological batteries, particularly in areas including attention, memory, motor functioning, and executive functioning. Notable improvements include the use of established criteria and measuring toxicant exposure especially through objective biomarkers (Abou-Donia et al., 2017). However, future research would benefit from continuing to use established criteria when investigating neuropsychological performance in GWI. Linking objective biomarkers with neuropsychological outcomes could provide potential markers for treatment development. For instance, research on cytokine profiles of GWI have shown immunological homeostatic shifts, which lends credence to a neurological etiology in GWI and treatment avenues to pursue (Golier et al., 2012; Craddock et al., 2015; Abou-Donia et al., 2017). Finally, there has been a lack of research investigating GWI and PTSD comorbidity.

CONCLUSION

In conclusion, neuropsychological research in GWI has improved in methodology but continues to leave questions regarding the etiology and cognitive difficulties in veterans. Future research should utilize improved methods with the use of standardized methodology and assessment batteries, increase measurement sensitivity and increase consistency while expanding into additional realms of study

(i.e., immunological and neuroimaging biomarkers) that could further explain the underlying pathobiology of GWI. Through this research, clinicians can utilize sensitive neuropsychological instruments which in turn will more effectively inform treatment efficacy for the multitude of veterans impacted by GWI and its co-morbidities.

Therefore, the neuropsychological CDEs (Gulf War Illness Research Program [GWIRP], 2019) is an excellent resource for identifying highly recommended measures for future research to allow direct comparison of study results. It is also imperative that these measures are adapted for use in imaging studies to understand the functional and structural underpinnings of cognitive impairments and changes over time in this aging group of veterans who are at higher risk for chronic medical conditions (Zundel et al., 2019).

Our recent meta-analysis of neuropsychological studies found impairments in visuospatial, attention, executive function, and learning and memory domains which were found in three or more prior studies (Janulewicz et al., 2017). This literature review supports these findings while considering the impact of neurotoxicant and mood factors. Future research assessing treatments or investigating biomarkers of GWI should include neuropsychological outcomes in the domains of visuospatial, attention and executive function, and learning and memory. Specifically, tests to include are Block Design, Trail Making Test, Digit Span, and CVLT, as these are known sensitive measures in GW veterans. For the clinician, GW veterans are an aging population at higher risk for chronic medical conditions and therefore their subjective cognitive complaints should be documented and evaluation with the sensitive neuropsychological measures recommended in this review.

AUTHOR CONTRIBUTIONS

MJ, JK, NK, MK, KS, and TC contributed to the conception and design of the review. MJ, CZ, and TC compiled review materials. MJ wrote the first draft of the manuscript. JK, NK, MK, CZ, KS, and TC wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

This research was funded by the United States Department of Defense Congressionally Directed Medical Research Programs (CDMRP) awards (http://cdmrp.army.mil/), grant number [W81XWH-16-1-0632] (Craddock – PI), [W81XWH-13-2-0085] (Morris – PI), [W81XWH-13-2-0072] (Sullivan – PI), and grant number [W81XWH-16-1-0552] (Craddock – PI). The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the United States Department of Defense.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 14 October 2019
doi: 10.3389/fphys.2019.01229

Dysbiosis-Associated Enteric Glial Cell Immune-Activation and Redox Imbalance Modulate Tight Junction Protein Expression in Gulf War Illness Pathology

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OPEN ACCESS

Edited by:
Sarah C. Pearce, United States Army Combat Capabilities Development Command, United States

Reviewed by:
Xin Cong, Peking University, China
Gregory Weber, United States Army Combat Capabilities Development Command, United States

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Specialty section:
This article was submitted to Gastrointestinal Sciences, a section of the journal Frontiers in Physiology

Received: *17 May 2019*
Accepted: *09 September 2019*
Published: *14 October 2019*

Citation:
Kimono D, Sarkar S, Albadrani M, Seth R, Bose D, Mondal A, Li Y, Kar AN, Nagarkatti M, Nagarkatti P, Sullivan K, Janulewicz P, Lasley S, Horner R, Klimas N and Chatterjee S (2019) Dysbiosis-Associated Enteric Glial Cell Immune-Activation and Redox Imbalance Modulate Tight Junction Protein Expression in Gulf War Illness Pathology. Front. Physiol. 10:1229. doi: 10.3389/fphys.2019.01229

About 14% of veterans who suffer from Gulf war illness (GWI) complain of some form of gastrointestinal disorder but with no significant markers of clinical pathology. Our previous studies have shown that exposure to GW chemicals resulted in altered microbiome which was associated with damage associated molecular pattern (DAMP) release followed by neuro and gastrointestinal inflammation with loss of gut barrier integrity. Enteric glial cells (EGC) are emerging as important regulators of the gastrointestinal tract and have been observed to change to a reactive phenotype in several functional gastrointestinal disorders such as IBS and IBD. This study is aimed at investigating the role of dysbiosis associated EGC immune-activation and redox instability in contributing to observed gastrointestinal barrier integrity loss in GWI via altered tight junction protein expression. Using a mouse model of GWI and *in vitro* studies with cultured EGC and use of antibiotics to ensure gut decontamination we show that exposure to GW chemicals caused dysbiosis associated change in EGCs. EGCs changed to a reactive phenotype characterized by activation of TLR4-S100β/RAGE-iNOS pathway causing release of nitric oxide and activation of NOX2 since gut sterility with antibiotics prevented this change. The resulting peroxynitrite generation led to increased oxidative stress that triggered inflammation as shown by increased NLRP-3 inflammasome activation and increased cell death. Activated EGCs *in vivo* and *in vitro* were associated with decrease in tight junction protein occludin and selective water channel aquaporin-3 with a concomitant increase in Claudin-2. The tight junction protein levels were restored following a parallel treatment of GWI mice with a TLR4 inhibitor

SsnB and butyric acid that are known to decrease the immunoactivation of EGCs. Our study demonstrates that immune-redox mechanisms in EGC are important players in the pathology in GWI and may be possible therapeutic targets for improving outcomes in GWI symptom persistence.

Keywords: dysbiosis, peroxynitrite, RAGE, S100B, nitric oxide, p47 phox, SsnB, sodium butyrate

INTRODUCTION

Gastrointestinal disturbances are one of the most commonly reported chronic symptoms among veterans who returned from the Persian Gulf war of 1990–1991 (Murphy et al., 1999; Dunphy et al., 2003; Koch and Emory, 2005; White et al., 2016). About 14% of veterans who suffer from Gulf War illness (GWI) complain of some form of gastrointestinal (GI) problems such diarrhea, pain and gas etc. (Dunphy et al., 2003; Koch and Emory, 2005). According to Coker et al. (1999), the most commonly reported gastrointestinal issues reported among United States and British Gulf war (GW) veterans were diarrhea, vomiting, and stomach problems. A study by Dunphy et al. (2003) showed veterans of the Persian GW presented with diarrhea and had rectal hypersensitivity as did Zhou et al. (2018) who reported increased somatic hypersensitivity and pain among some GW veterans with GI issues.

Although the veterans report these symptoms, the prospective study by Koch and Emory (2005) did not find any significant clinical markers of disease pathology in blood or intestine tissue of deployed participants. Similarly, one of our own studies which reported metabolic reprogramming in liver as a result of leaky gut and endotoxemia did not find any biochemical markers of liver damage or altered metabolism in a mouse model of GWI. This was surprising because we had had previously shown that exposure to GW theater chemicals resulted in an alteration of gut microbiome and concomitant TLR4 mediated gastrointestinal and neuroinflammation with endotoxemia (Alhasson et al., 2017; Seth et al., 2018). This elusive nature of GWI is a strong reason for further studying underlying mechanisms of this condition in order to obtain effective therapies.

Of emerging interest in inflammatory gastroenterology are enteric glial cells (EGC) which reside in close proximity with the neurons of the enteric nervous system. These cells are similar in structure and physiology to astrocytes of the brain but are not excitable (Gershon and Rothman, 1991; Bassotti et al., 2007; Ochoa-Cortes et al., 2016). Initially, the principal function of EGC was thought to be providing mechanical support to enteric neurons. However, recent studies have shown that these cells play an important role in regulating the gastrointestinal microenvironment through several mechanisms, which have been extensively reviewed (Bassotti et al., 2007; Yu and Li, 2014; Sharkey, 2015). EGC were found to significantly modulate gut homeostasis through release of growth factors (von Boyen et al., 2011; Hansebout et al., 2012; Grubisic and Gulbransen, 2017) cytokines and prostaglandins (Hansebout et al., 2012; Steinkamp et al., 2012; Yu and Li, 2014) but may also play a pathogenic role by contributing to nitrosative stress and proinflammatory cytokines when exposed to stressful or toxic stimuli in the gut. Moreover, studies have found that EGC have the ability to "sense" a change in microbiome from probiotic to pathogenic, possibly through toll like receptors (TLRs). A study by Turco et al. (2014), found that adhesive *E. Coli* seem to activate a TLR-S100β/RAGE-iNOS signaling pathway in human EGC, while probiotic lactobacillus did not. Another study found that when EGC were treated with lipopolysacharrides (LPS), there was activation of TLRs with a release of S100B and nitric oxide (NO) (Cirillo et al., 2009; Rosenbaum et al., 2016). In this reactive state, EGC produce proinflammatory cytokines and chemokines e.g., (IL-1β, TNF-α, MCP-1) and release of inducible NO which may contribute to oxidative stress in the gut (von Boyen et al., 2011; Yu and Li, 2014; Ochoa-Cortes et al., 2016).

In irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), it is well known that an altered microbiome plays a significant role in the pathogenesis of the disease (Menees and Chey, 2018). In IBS for example, patients were found to have a decrease in abundance of Bifidobacteria and Lactobacillus but an increased prevalence of pathogenic species like *Escherichia* spp., Shigellas, and several Clostridia (Distrutti et al., 2016). Furthermore, it has been observed that metabolic diseases e.g., diabetes and obesity also present with increased ratio of Firmicutes to Bacteriodetes (Conlon and Bird, 2014; Johnson et al., 2017). Studies concerning the mechanisms of these gastrointestinal diseases have found that change of EGC phenotype from homeostatic to pathogenic is a characteristic of these diseases (Cabarrocas et al., 2003; Linan-Rico et al., 2016; Chen et al., 2018). A study by Wang et al. reported a significantly increased expression of glial fibrillary acidic protein (GFAP), Tyrosine receptor kinase B and Substance P in the colon of IBS patients with a correlated increase in intestinal inflammation (Wang et al., 2016). Other studies show that a loss in EGC resulted in poor gastrointestinal health characterized by loss of gut barrier integrity (Brown et al., 2016; Morales-Soto and Gulbransen, 2019).

Our previous research reported an altered microbiome in a murine model of GWI with increase in Firmicutes over Bacteriodetes and a decrease in several butyrogenic bacteria. This dysbiosis was accompanied by activation of TLR4, increased inflammation, a leaky gut, endotoxemia with release of damage associated molecular patterns (DAMPS) such as HMGB1 in gulf war chemical treated mice compared to controls (Alhasson et al., 2017; O'Callaghan et al., 2017; Seth et al., 2018). Interestingly, a recent study by Hernandez et al., showed that exposure to pyridostigmine bromide a known gulf war chemical exposure resulted in enteric neuronal and glial reactivity and inflammation (Hernandez et al., 2019).

This current study investigates the contribution of EGC in observed inflammatory phenotype which we and others have observed in GWI. We test the hypothesis that, the altered microbiome which results in increased pathogen associated molecular patterns (PAMPS) (e.g., LPS, flagellin and other immunostimulatory bacterial parts), leaky gut and increase in circulatory DAMPS (e.g., HMGB-1) in GW-chemical (Permethrin and pyridostigmine bromide) treated mice results in a reactive EGC phenotype compared to mice treated with vehicle control treated mice and mice co-exposed with GW chemicals and antibiotics. Through this reactive EGC phenotype intestinal cells such as enteric neurons and epithelial cells might be further affected leading to a vicious cycle of consistent proinflammatory state. This constant proinflammatory state of intestinal cells might answer the persistence of gastrointestinal, systemic and neuro inflammation in gulf war illness. The study uses a murine model of GWI and *in vitro* studies with EGCs and intestinal epithelial cells to elucidate possible mechanisms to explain this observed inflammation observed in GWI.

MATERIALS AND METHODS

Pyridostigmine bromide (PB), Permethrin (Per), Sodium Butyrate, Sparstolonin B (SsnB), Corticosterone, Neomycin trisulfate hydrate, Enrofloxacin, Apocynin (APO), Phenylboronic acid (FBA) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Lipopolysaccharides (LPS), LPS-RS (TLR4 inhibibitor) were purchased from Cayman chemical company (Ann Arbor, MI, United States), Rat High mobility group box 1 protein (HMGB-1) Rat (rec) (His) was purchased from Chimirigen, Mediatech, Inc. (Manassas, VA, United States), Anti-TLR4, anti-flotillin-1, anti-S100B, anti-GFAP, anti-ASCII and anti-Caspase 1, anti-TLR5, anti-3NT, anti-GP91, anti-P47phox, anti-NOS 2, anti-HMGB-1 and anti-aquaporin-3 primary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, United States), anti-claudin 2, anti-TLR2 and anti-occludin were purchased from Abclonal Technology (Woburn, MA, United States) was while anti-NLRP-3, anti-RAGE were purchased from Abcam (Cambridge, MA, United States). Fluorescence-conjugated (Alexa Fluor) secondary antibodies, ProLong Gold antifade mounting media with DAPI were purchased from Thermofisher Scientific (Grand Island, NY, United States), Apoptosis ApopTag® Fluorecein *in situ* detection kit from Millipore (Billerica, MA, United States), The Pierce LAL Chromogenic Endotoxin Quantification Kit from Thermo Scientific (Waltham, MA, United States) and Griess reagent system from Promega corporation (Madison, WI). All other chemicals which were used in this study were purchased from Sigma unless otherwise specified. Paraffin-embedding of tissue sections on slides were done by AML laboratories (Baltimore, MD, United States).

Animals

Adult wild-type male (C57BL/6J mice) were purchased from the Jackson Laboratories (Bar Harbor, ME, United States). 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, United States. Mice were housed individually on 7090 Sani-Chip bedding from Teklad (Madison, WI, United States) and fed with 8904 irradiated chow diet from Teklad (Madison, WI, United States) at 22–24°C with a 12-h light/12-h dark cycle. All mice were sacrificed after animal experiments had been completed. Immediately after terminal anesthesia, mice's small intestine was collected and dissected for further experiments, while fecal pellets were collected from the colon and immediately stored in sterile Eppendorf tubes for microbiome analysis. The tissues were fixed using 10% neutral buffered formalin. Distal segments of small intestines were used for the staining and visualizations.

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 (Zakirova et al., 2015; O'Callaghan et al., 2017). The treated wild-type mice group (GW) were dosed tri-weekly for 1 week with PB (2mg/Kg) and Permethrin (200 mg/kg) by oral gavage. After completion of PB and Permethrin dosages, mice were administered corticosterone intraperitoneally (i.p.) with a dose of 100μg/mice/day for 5 days of the week for 1 week to represent war stress. 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 (CONT) of mice received saline injections and vehicle for oral gavage (6% DMSO) in the same paradigm. Similarly, another group of mice (GW + AB) were exposed to PB/Permethrin and corticosterone as in above mentioned dosages along with antibiotics (Neomycin 45 mg/kg and Enrofloxacin 1mg/Kg) thrice per week for 2 weeks for intestinal decontamination and obtaining gut sterility, while another group (AB) were exposed to antibiotics (Neomycin, 45 mg/kg and Enrofloxacin 1 mg/Kg) for 2 weeks. A fifth group of mice was treated with PB/Permethrin and corticosterone, but with Sodium butyrate (10 mg/Kg) and Sparstolonin B (SsnB) 3 mg/Kg (GW + SsnB + BT). We have shown before that SSnB is a potent TLR4 antagonist and Butyrate decreases intestinal inflammation in GWI.

Cell Culture

Enteric Glial Cell Culture

Immortalized rat EGC were obtained from ATCC® (ATCCCRL-2690). Plated EGC were maintained in DMEM media supplemented with 10% FBS until treated. Cells were serum starved in DMEM supplemented with 1% FBS for 12 h and then exposed to vehicle control and chemicals. Cells were then treated with vehicle control-PBS (VEH), LPS (1 μg/mL), HMGB-1 (100 ng/mL), SsnB (10 μg/mL), Sodium butyrate (5 mM)and inhibitors FBA (100 μM) and Apocynin (100 μM) with either HMGB-1, LPS or antibiotics (neomycin and enrofloxacin cocktail) at different dilutions ranging from (1X to 1000X)

TABLE 1 | Rat primer sequence.

Rat_IL-1β	Sense: CCTCGGCCAAGACAGGTCGC
	Antisense: TGCCCATCAGAGGCAAGGAGGA
Rat_NLRP-3	Sense: TGCATGCCGTATCTGGTTGT
	Antisense: ATGTCCTGAGCCATGGAAGC
Rat_TNF-α	Sense: CAACGCCCTCCTGGCCAACG
	Antisense: TCGGGGCAGCCTTGTCCCTT
Rat_ASCII	Sense: GGACAGTACCAGGCAGTTCG
	Antisense: GTCACCAAGTAGGGCTGTGT
Rat_Caspase 1	Sense: GACAGGTCCTGAGGCCAAAG
	Antisense: AAAAGTTCATCCAGCAATCCATTT
Rat_MCP 1	Sense: TAGCATCCACGTGCTGTCTC
	Antisense: CAGCCGACTCATTGGGATC
Rat_18S	Sense: GGATCCATTGGAGGGCAAGT
	Antisense: ACGAGCTTTTTAACTGCAGCAA
Rat_NOS2	Sense: AGCAGAGTTGGTGCAGAAGC
	Antisense: GGGAATAGCACCTGGGTTTT
Rat_Claudin1	Sense: AGGTCTGGCGACATTAGTGG
	Antisense: CGTGGTGTTGGGTAAGAGGT
Rat-ZO-1	Sense: GGAAATGTGTAAATCACCTGGAAGA
	Antisense: CCAAAGAACAGAAGACCACCAAC
mm_18S	Sense: TTCGAACGAACGTCTGCCCTATCAA
	Antisense: ATGGTAGGCACGGCGATA
mm_Claudin1	Sense: TTTCGCAAA GCACCGGGCATACA
	Antisense: GCCACTAATGTCGCCAGACCTGAAA
mm_ZO-1	Sense: CCACCTCTGTCCAGCTCTTC
	Antisense: CACCGGAGTGATGGTTTTCT
mm_TLR2	Sense: ACCAAGATCCAGAAGAGCCA
	Antisense: CATCACCCGGTCAGAAAACAA
mm-TLR4	Sense: GGAGTGCCCGCTTTCACCTC
	Antisense: ACCTTCCGGCTCTTGTGGAAGC
mm-TLR5	Sense: TGTAAAGTACTGGTGCCCGTGTGT
	Antisense: ACTGCGCAAACATTCTGCTGGC
mm-NOS-2	Sense: CGCTGGCTACCAGATGCCCG
	Antisense: GCCATAGCGGGCTTCCAGC

The primer sequences are given as 5′-3′ orientation; Sense: Forward primer. Antisense: Reverse primer.

(see **Supplementary Figures S2–S7**) for 24 h. After which the experiment was terminated and cells were harvested for mRNA extraction, gene expression analysis and protein expression studies. Nitric oxide production was estimated from culture fluids by measuring nitrite formation using the Griess assay.

Intestinal Epithelial Cell Culture

Immortalized rat intestinal epithelial cells (IEC-6) ATCC© CRL-1592, were obtained from ATCC. The cells were maintained in DMEM media supplemented with 10% FBS and 1x ITS until treated. Cells were serum starved in DMEM supplemented with 1% FBS for 12 h and then primed with LPS (100 ng/mL) for 12 h. Cells were then treated with culture fluids from EGC (above) for 24 h, then harvested for further analyses.

Microbiome Analysis

Microbiome was analyzed from fecal pellets and luminal contents collected from animals after sacrifice and sent to Second Genome for 16S rRNA sequencing. Microbial analyses were performed from isolated nucleic acids using the MoBio PowerMag Microbiome kit (Carlsbad, CA, United States), according to manufacturer's instructions. The microbiome data is in NCBI EBI under the accession number PRJEB19474.

Laboratory Methods

Immunofluorescence Staining

Paraffin-embedded distal part of the small intestine sections were deparaffinized using a standard protocol. Epitope retrieval solution and steamer were used for epitope retrieval of sections. Primary antibodies such as anti- GFAP, anti-S100β, anti-NOS2, anti-NLRP-3, anti-ASCII, anti-GP91, anti-P47phox anti-TLR4, anti-Flotillin, anti-aquaporin3 were used at the recommended dilution (1:200). Species-specific secondary antibodies conjugated with Alexa Fluor (633-red and 488-green) were used at advised dilution (1:300). Finally, the stained sections were mounted using Prolong gold anti-fade reagent with DAPI. Sections were observed under–Olympus fluorescence microscope using 20X, 40X or 60X objective lenses, or under confocal microscopy using Leica SP-8 with LasX-10 software at magnification of 63X.

Western Blot

Serum HMGB-1 levels were estimated from 35 μg of denatured mouse serum protein, while TLR2, 4 and 5 were estimated from 25 μg of denatured small intestine tissue by a Western Blot analysis following standard protocols. Briefly, serum was concentrated and then diluted 1:5. Tissue protein or serum protein was then separated on a Novex 4–12% bis-tris gradient gel and subjected to standard SDS-PAGE. The separated proteins were then transferred to a nitrocellulose membrane by a Trans-Blot Turbo transfer system. The membrane was then stained with Ponceau S, and then blocked with 5% BSA solution for 1 h, then incubated with primary antibody overnight at 4°C. Species-specific anti-IgG secondary antibody conjugated with HRP was used to tag primary antibody. ECL western blotting substrate was used to develop the blot The blot was then imaged using G:Box Chemi XX6 and subjected to densitometry analysis using Image J software.

Real-Time Quantitative PCR

Messenger RNA expression in small intestine and rat EGC was examined by quantitative real-time PCR analysis. Total RNA was isolated from each 15 mg small intestine tissue or 1×10^6 EGC by homogenization in Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions and purified with the use of RNeasy mini kit columns (Qiagen, Valencia, CA, United States). cDNA was synthesized from purified RNA (1 μg) using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, United States) 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, United States). Threshold Cycle (Ct) values for

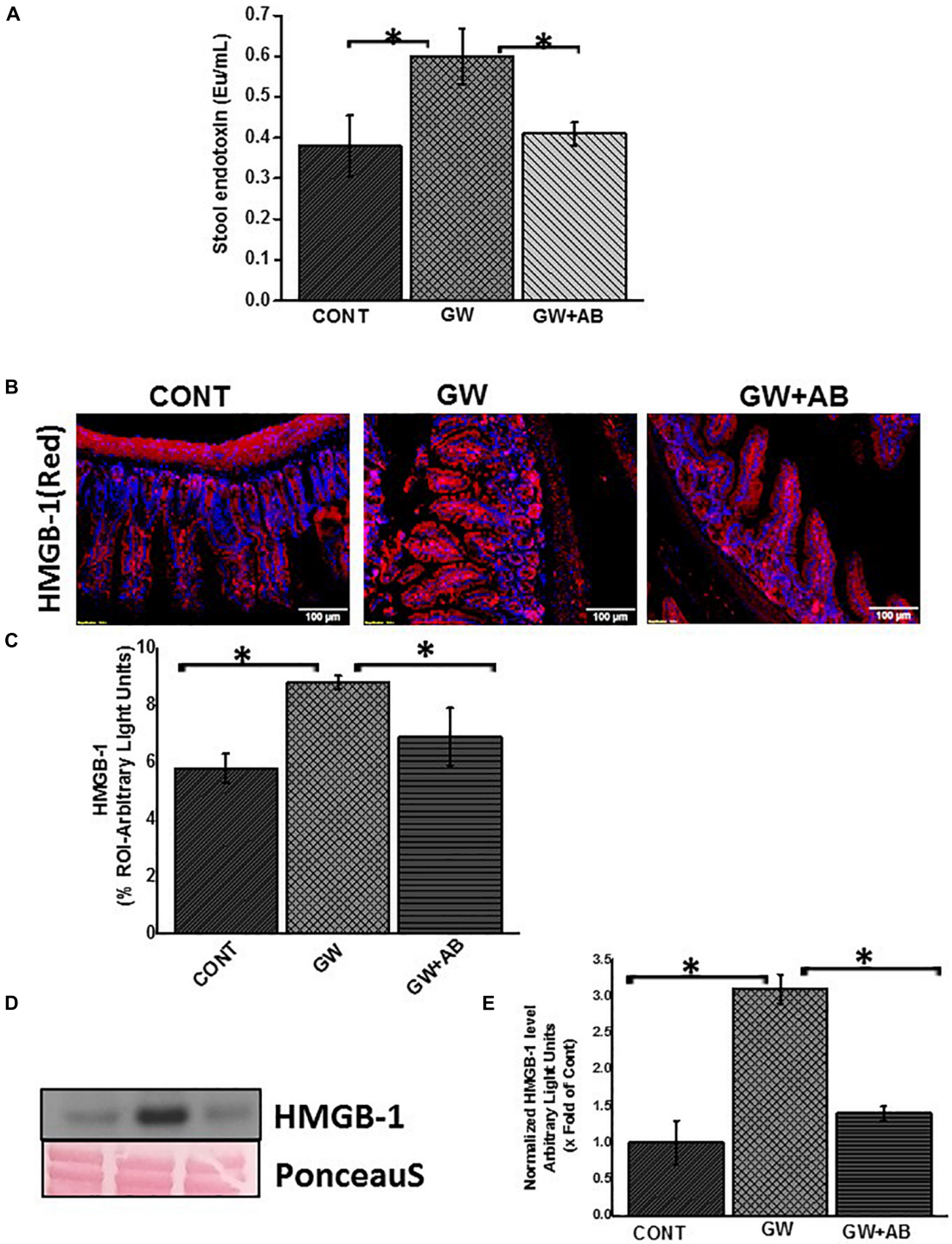

FIGURE 1 | Altered microbiome associated increase PAMPS and DAMPS. **(A)** Stool endotoxin levels. Endotoxin levels in stool samples were determined by the LAL assay. Graph **(A)** show the levels of endotoxin in Endotoxin Units (Eu) in vehicle control (CONT, $n = 9$) treated mice, gulf war chemical exposed mice (GW, $n = 9$) and mice co-exposed with antibiotics (GW + AB, $n = 9$) (*$P < 0.05$). **(B)** Expression of HMGB-1 in small intestine tissues. Expression of HMGB1 was assessed by immunofluorescence microscopy at (total Magnification 200X; scale bar 100 µm). Images show immunoreactivity in the distal part of the small intestine for vehicle control treated mice (CONT, $n = 9$), GW chemical treated mice (GW, $n = 9$) and mice co-exposed with gulf war chemicals and antibiotics (GW, $n = 9$). **(C)** Quantitative morphometric analysis of HMGB-1 immunoreactivity represented as arbitrary light units in the region of interest (% ROI) *$P < 0.05$. **(D)** Serum High mobility group box 1 (HMGB1) levels. Serum HMGB-1 levels were estimated by western blot analysis for mice treated with control (CONT, $n = 3$), Gulf war chemical exposed mice (GW, $n = 5$) and mice co-exposed to antibiotics and GW chemicals (GW + AB, $n = 3$). Ponceau red staining was used for normalization of protein. **(E)** Quantitative morphometric analysis of western blot bands normalized against total Ponceau. The *Y* axis shows HMGB-1/Ponceau S ratio (*$P < 0.05$).

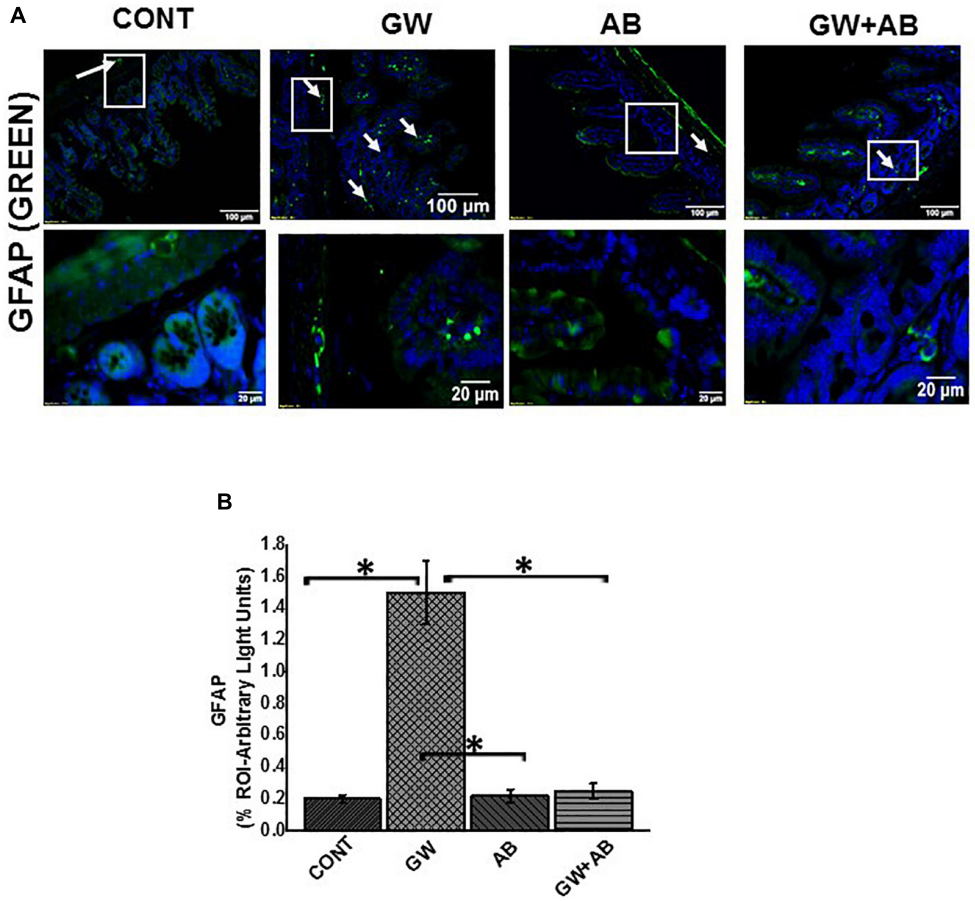

FIGURE 2 | Altered microbiome induced change in EGC phenotype to a reactive phenotype. **(A)** Expression GFAP. Expression of GFAP was assessed by immunofluorescence microscopy at (top panel magnification 200X; scale 100 μm and bottom panel magnification 600X; scale 20 μm). Images show immunoreactivity of the distal part of the small intestine for vehicle control treated (CONT, $n = 9$), gulf war chemical treated mice (GW, $n = 9$), mice treated with only antibiotics (AB, $n = 4$) and mice co-exposed with gulf war chemicals and antibiotics (GW + AB, $n = 9$). **(B)** Quantitative morphometric analysis of GFAP immunoreactivity represented as arbitrary light units as observed in the region of interest (% ROI) (*$P < 0.05$).

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 below in **Table 1**.

Endotoxin Level Detection by Litmus Amebocyte Lysate Assay

Bacterial endotoxins (EU/mL) were detecte din mouse stool samples for mice which were treated with vehicle control, gulf war chemicals, and mice co-exposed with gulf war chemicals and antibiotics using the Pierce LAL Chromogenic Endotoxin Quantification Kit according to the manufacturer's instructions. Briefly, stool samples were obtained from mice and equal weights were homogenized in endotoxin free water. The supernatant was then collected, and heat inactivated at 70°C. This was then diluted 1:300 and endotoxins quantified.

Nitrite Estimation by Griess Assay

Nitric oxide release was estimated from the cell culture fluids by measuring nitrite formation immediately after the experiment was terminated. Nitrite was measured using the Griess reagent system from Promega corporation (Madison, WI, United States) and experiments were performed according to manufacturer's protocols.

Tunel Assay

DNA fragmentation was detected using the ApopTag® Fluorescein *in situ* detection kit from Millipore (Billerica, MA, United States) by following the manufacturer's standard protocol.

Statistical Analysis

All *in vivo* experiments were repeated three times ($N = 3$) with at least 3 mice per group ($n = 9$; data from each group of three mice were pooled). All *in vitro* and laboratory analysis experiments were repeated at least three or four times. The statistical analysis was carried out by analysis of variance (ANOVA)

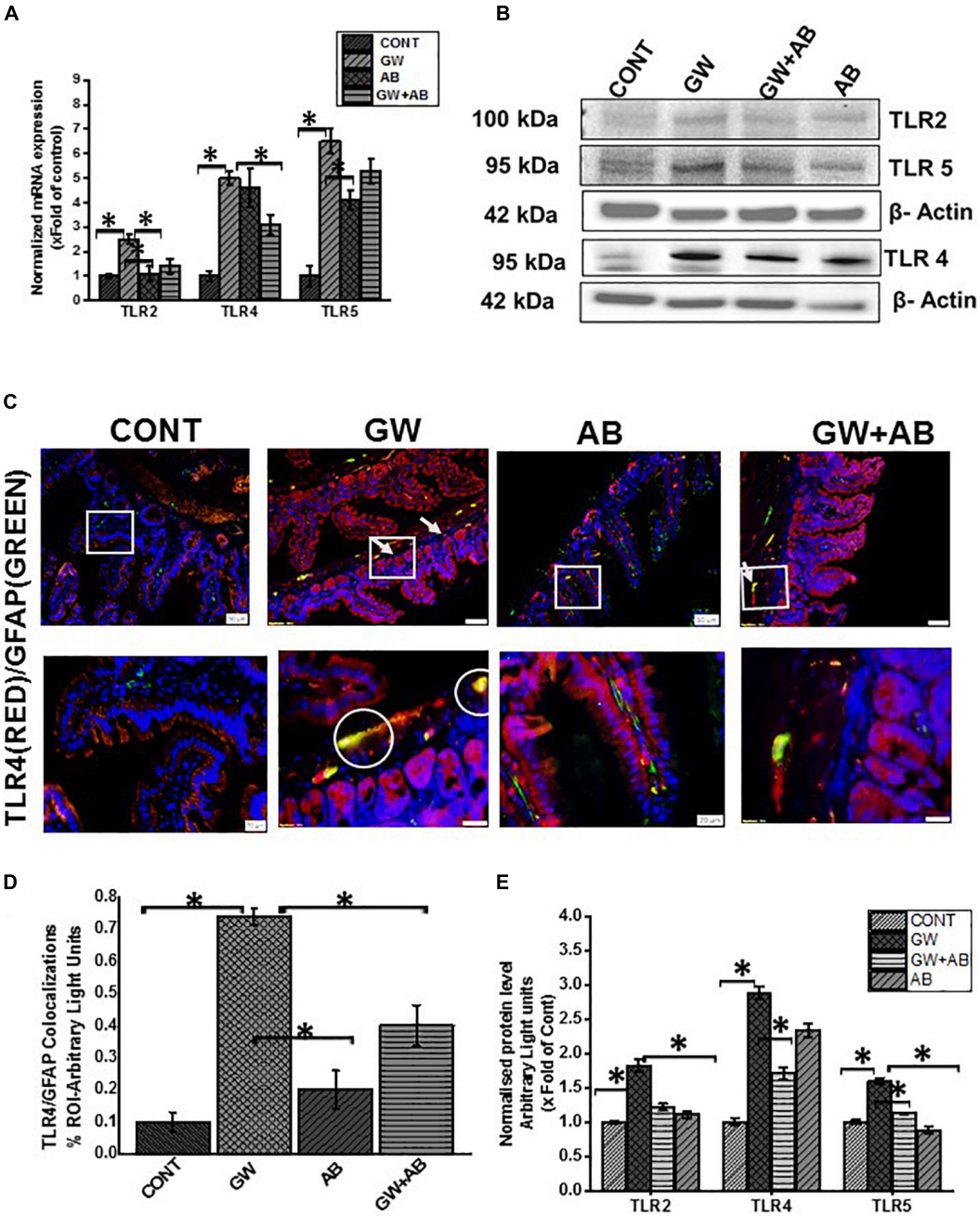

FIGURE 3 | Expression of Toll-like receptors in small intestine and EGC. **(A,B)** General mRNA and protein expression levels of toll-like receptors TLR2, TLR4, and TLR5 in small intestine of mice treated with vehicle control (CONT, $n = 9$), gulf war chemical treated mice (GW, $n = 9$) and mice treated with antibiotics only (AB, $n = 4$) and mice co-exposed with GW chemicals and antibiotics (GW + AB, $n = 9$). mRNA expression was determined by RTqPCR, while protein expression was determined by western blot analysis. **(E)** Quantitative morphometric analysis of western blot bands normalized against β-actin The *Y* axis shows protein/β-actin ratio Results are expressed as mean ± SEM for $n = 9$ ($^*P < 0.05$). **(C)** Tissue level expression of TLR4 in EGC in small intestine. Expression of TLR4 in EGC was observed by in dual labeling of TLR4 and EGC cells marker GFAP via immunofluorescent microscopy visualized at (top panel magnification 200X; scale 100 μm and bottom panel magnification 600X; scale 20 μm) in small intestine tissues obtained from mice treated with vehicle control (CONT, $n = 9$); mice treated with GW chemicals (GW, $n = 9$) mice, mice treated with antibiotics only ($n = 4$) and co-exposed with GW chemicals and antibiotics (GW + AB, $n = 3$). **(D)** Quantitative morphometric analysis of immunoreactivity of GFAP/TLR4 (yellow) is represented as colocalizations events per field from randomly chosen microscopic fields ($^*P < 0.05$).

(see **Supplementary Table S1** for F-statistics) and a Turkey's HSD test to determine specific group differences. Further we performed an unpaired student *t*-test, using Graph pad prism software (GraphPad Software Inc., La Jolla, CA, United States). For all analyses $^*P < 0.05$ was considered statistically significant and are marked as (*).

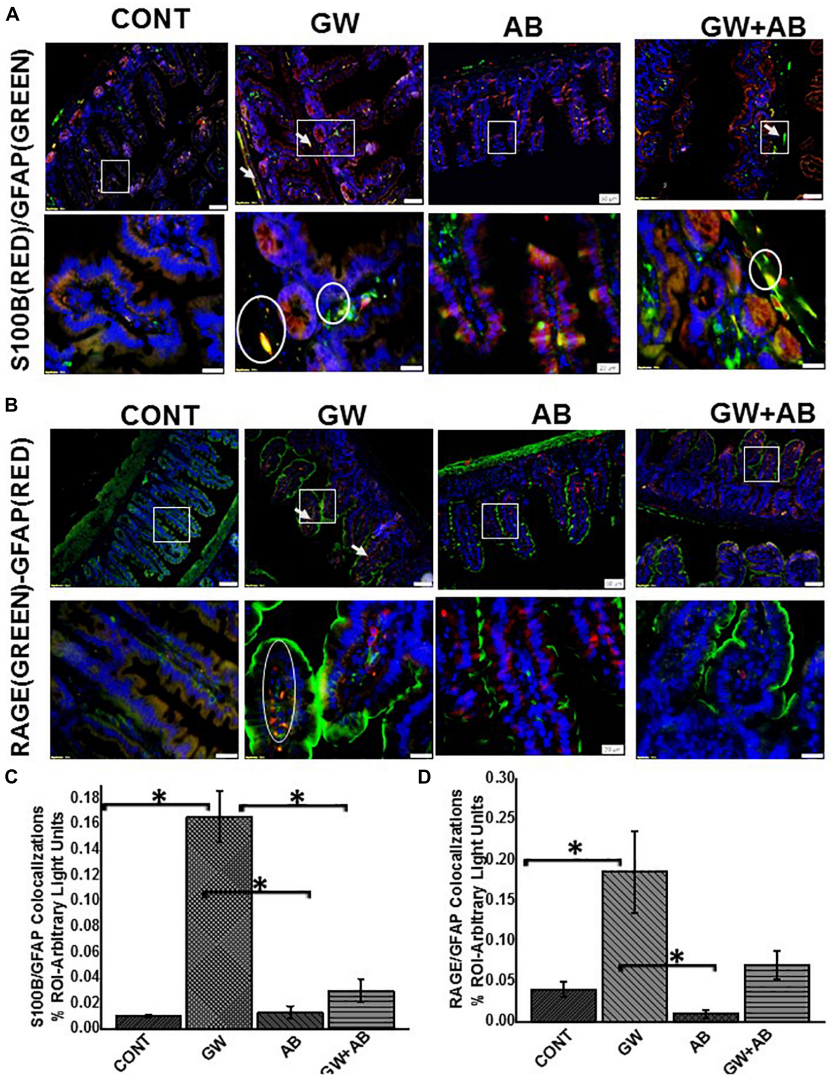

FIGURE 4 | (A) Expression levels of S100B in EGC. Protein expression levels of S100B in EGC was determined by co-staining S100B with GFAP and assessed by immunofluorescence microscopy at (top panel magnification 200X; scale 100 μm and bottom panel magnification 600X; scale 20 μm). Images show immunoreactivity in distal part of the small intestine for vehicle control treated mice (CONT, $n = 9$), gulf war chemical treated mice (GW, $n = 9$) and gulf war chemical treated mice, mice treated with antibiotics only (AB, $n = 4$) and mice co-exposed with antibiotics (GW + AB, $n = 9$). **(C)** Quantitative morphometric analysis of immunoreactivity of GFAP/S100B (yellow) is represented as colocalizations events per field from randomly chosen microscopic fields (% ROI) (*$P < 0.05$). **(B)** Expression of RAGE in EGC Protein expression levels of RAGE in EGC was determined by co-staining RAGE with GFAP and assessed by immunofluorescence microscopy at (top panel magnification 200X; scale 50 μm and bottom panel magnification 600X; scale 20 μm). Images show immunoreactivity in distal part of the small intestine for vehicle control treated mice (CONT, $n = 9$), gulf war chemical treated mice (GW, $n = 9$) mice treated with antibiotics only (AB, $n = 4$) and gulf war chemical treated mice co-exposed with antibiotics (GW + AB, $n = 3$). **(D)** Corresponding quantitative morphometric analysis of immunoreactivity of GFAP/RAGE (yellow) is represented as colocalizations events per field from randomly chosen microscopic fields (% ROI) (*$P < 0.05$).

RESULTS

Altered Microbiome Is Associated With Increase in PAMPs and DAMPs in Gulf War Chemical Exposed Mice

Studies have shown an association between altered microbiome and increase in endotoxin levels in serum or feces (Palone et al., 2016; Palone et al., 2018; Seth et al., 2018). In this study, using the LAL assay, we estimated the endotoxin levels (PAMPS e.g., LPS) in the stools of mice which were treated with GW chemicals in comparison with the controls and found that there was a significant increase in endotoxin levels of mice treated with GW chemicals compared to the controls (**Figure 1A**; $P < 0.05$). We further assessed the amount of HMGB-1 which was released in the small intestine (**Figures 1B,C**) using immunofluorescence microscopy and in the blood circulation (**Figures 1D,E**) using a western blot analysis for serum HMGB-1 levels in the circulation. These high amounts of DAMPS and

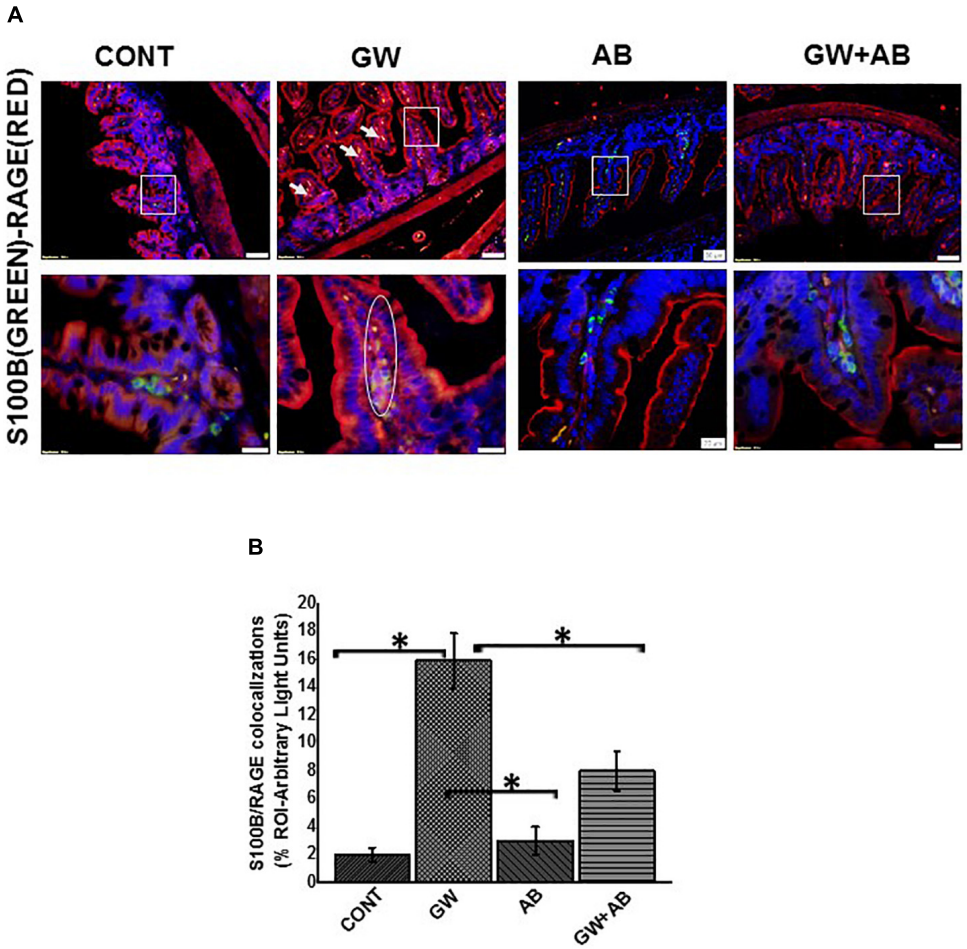

FIGURE 5 | Formation of S100B/RAGE complex in small intestine. **(A)** S100B(Green)/RAGE(Red) complex formation expression in EGC in small intestine tissues. Protein expression levels were assessed by immunofluorescence microscopy of tissues at (top panel magnification 200X; scale 50 μm and bottom panel magnification 600X; scale 20 μm). Images show immunoreactivity the distal part of the small intestine for gulf war chemical treated mice (GW, $n = 9$), vehicle control (CONT, $n = 9$), mice treated with antibiotics only ($n = 4$) and mice co-exposed GW chemicals and antibiotics (GW + AB, $n = 9$). **(B)** Quantitative morphometric analysis of immunoreactivity for S100B/RAGE is represented as colocalization events per field for randomly chosen fields (% ROI) (*$P < 0.05$).

PAMPS in the body will reach the EGC and cause persistent glial reactivity.

Altered Microbiome (Dysbiosis) Correlates With an Increased Expression of GFAP While Gut Decontamination With Antibiotics Decreases GFAP in Intestinal Enteric Glial Cells

Enteric glial cells which are found in close proximity with enteric neurons are very abundant in the lamina propria, mucosa and sub mucosal regions of the small intestine (Bassotti et al., 2007). Using immunofluorescence microscopy, we found that there was a significant increase ($P < 0.05$) in GFAP expression in the small intestine of mice treated with GW chemicals (PB + BER) compared to the control group, and mice co-exposed to GW chemicals and antibiotics (**Figures 2A,B**). The increased expression of this protein has been associated with a reactive EGC phenotype in IBS and IBD (Akimoto, 2000; von Boyen et al., 2011).

Altered Microbiome Correlates With a Reactive EGC Phenotype Through Activation of Toll-Like Receptors While Gut Decontamination via Antibiotic Usage Reversed Activation

Our previous studies showed that the altered microbiome was associated with an activation of Toll like receptors such as TLR4 and TLR5 in GW chemical treated mice (Dyer and Walker, 1993; Seth et al., 2018). In this study we show that there was a significant increase mRNA (**Figure 3A**) and protein expression (**Figures 3B,E**) levels of TLR 2, 4, and 5 in mice which were exposed to gulf war chemicals (Permethrin and pyridostigmine bromide) compared to mice treated with only vehicle control and mice co exposed with GW chemicals and antibiotics ($P < 0.05$).

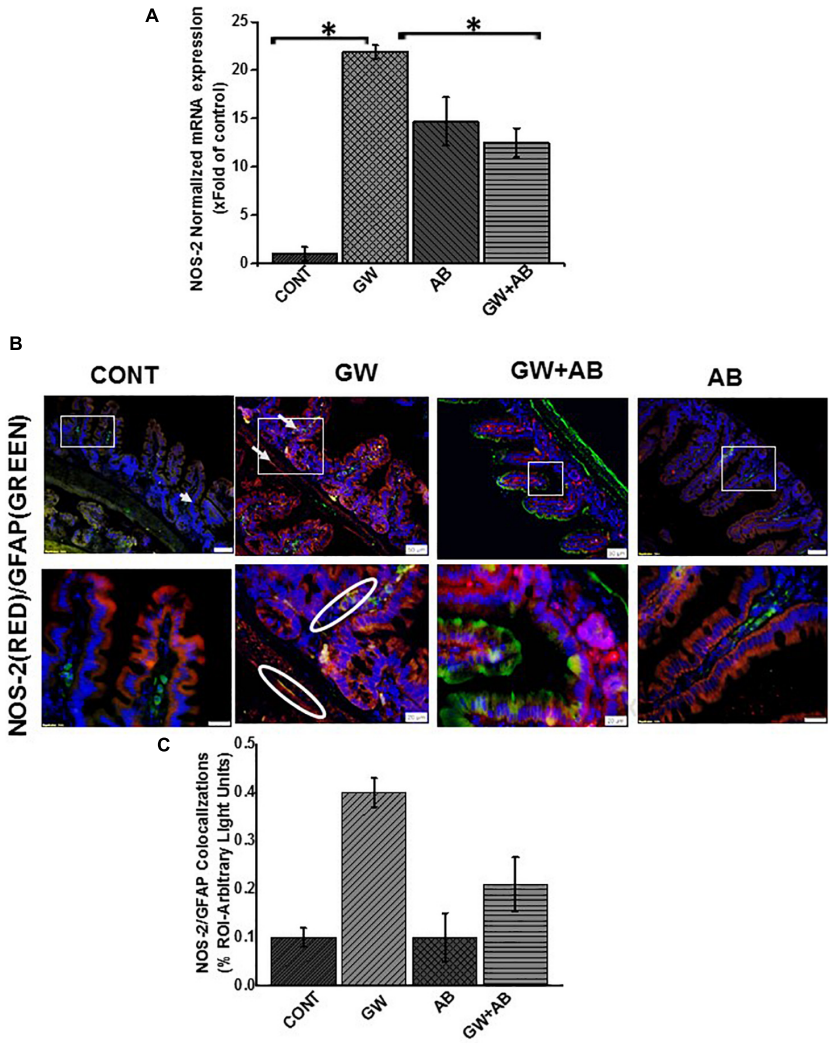

FIGURE 6 | Activation of NOS-2 in small intestine. **(A)** NOS-2 mRNA expression in the small intestine of intestine of mice treated with vehicle control (CONT, $n = 9$), gulf war chemical treated mice (GW, $n = 9$), mice treated with antibiotics only (AB, $n = 4$) and mice co-exposed with GW chemicals and antibiotics (GW + AB, $n = 9$. $^*P < 0.05$) was determined by RTqPCR. **(B)** Protein expression levels of NOS- 2 in enteric glial cells was determined by immunofluorescence microscopy of tissues and imaged at (top panel magnification 200X; scale 50 μm and bottom panel magnification 600X; scale 20 μm). **(C)** Quantitative morphometric analysis of immunoreactivity for GFAP/NOS-2 represented as colocalization events per field for randomly chosen fields (% ROI).

We further detected a significant increased expression of TLR4 on EGC (TLR4/GFAP colocalizations) in GW chemical treated (GW) mice compared to Vehicle (CONT) and mice co-exposed with GW chemicals and antibiotics (GW + AB) ($P < 0.05$) (**Figures 3C,D**) by immunofluorescence microscopy.

Altered Microbiome Associated Increased Expression of S100B in Reactive EGC Resulting in NOS-2 Expression While Antibiotic Usage for Depletion of Bacteria Reversed Such Activation

Using immunofluorescence microscopy, we found that there was a significant increase in the expression of S100B in GW chemical treated mice compared to mice treated with vehicle control and mice which were co exposed with GW chemicals and antibiotics ($P < 0.05$) (**Figures 4A,C**). We also found that there was a significant increase in RAGE expression in GW chemical treated mice in EGC by co-staining RAGE and GFAP ($P < 0.05$) compared to vehicle control treated mice. However, this increase was not significant for mice treated with both GW chemicals and antibiotics (**Figures 4B,D**).

We then studied the interaction between RAGE and S100B using immunofluorescence microscopy assuming that a co-localization of these two proteins would suggest complex formation and aid interaction. We showed that there was significant increase ($P < 0.05$) in S100β/RAGE complex formation in GW chemical exposed mice (GW) and mice treated with vehicle control (CONT) or mice co-exposed with

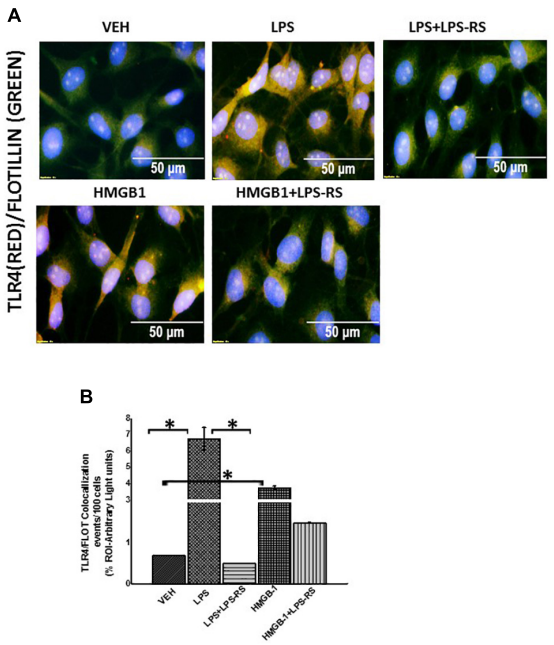

FIGURE 7 | Exposure to DAMPS and PAMPS cause activation of TLR4 in EGC. **(A)** TLR4 activation in rat Enteric Glial Cells (EGC) (n = 6). Colocalization of TLR4 and Flotillin in EGC treated with either vehicle control (VEH) or LPS, LPS + LPS-RS, HMGB-1 or HMGB1 + LPS-RS determined by immunofluorescence microscopy at (magnification 400X; scale 50 µm). **(B)** Quantitative morphometric analysis of TLR4/Flotillin colocalizations between red and green immunoreactivity were determined for every 100 cells per field. Fields were chosen randomly, and colocalizations represented as immunoreactivity in the region of interest (% ROI) (*$P < 0.05$).

GW chemicals and antibiotics (GW + AB) (**Figures 5A,B**). In **Figure 6A** using RTqPCR we found that there was a significant increase in mRNA expression of inducible nitric oxide synthase in the small intestine of GW chemical treated mice and mice treated with vehicle control and mice co exposed with antibiotics and gulf war chemicals (n = 9, $p < 0.05$). Further, we showed that there was a marked increase in inducible nitric oxide synthase (NOS-2) expression in the intestine tissues of mice treated with GW chemicals (GW) compared to mice treated with vehicle control (CONT) and mice co-exposed with GW chemicals and antibiotics (GW + AB), although this increase was not significant (P = 0.075, and 0.11 respectively) (**Figures 6B,C**).

These results are evidence of activation of a TLR-S100β/RAGE-iNOS pathway in association to an altered microbiome *in vivo* as suggested by a decrease of activation following the use of antibiotics to ensure gut decontamination.

Exposure to PAMPS (e.g., Lipopolysaccharides) and DAMPS (e.g., HMGB-1) Causes the Activation of TLR4-s100β/RAGE-NO Pathway in EGC

EGC can respond to an over balance in gut microorganisms by detecting PAMPS on/from the pathogen these bacteria such as cell wall, nucleic acid, flagella etc and mount an effective immune response through toll like receptors or NOD-like receptors (Turco et al., 2014).

Using immunofluorescence microscopy, we found that there was significant increase in TLR4 expression when we treated rat EGC with LPS or HMGB-1 (**Figures 7A,B**, $P < 0.05$). We also found an increase in S100β/RAGE complex formation in LPS and HMGB1 treated cells compared to cells treated with vehicle control ($P < 0.05$) (**Figures 8A–C**). However, the difference between expression of these receptors was not significantly different between the cells treated with HMGB1 alone compared to those treated with HMGB1 + LPS-RS to block the TLR4 receptor. This indicates that possibly, DAMPS like HMGB1 can trigger inflammatory pathways in EGCs via several other receptors apart from TLR4.

We further evaluated the activation of inducible nitric oxide synthase and release of nitric oxide in the rat EGC treated with LPS or HMGB1 (**Figures 9A–D**). We used RT q PCR to evaluate the expression of nitric oxide synthase in rat EGC (**Figure 9A**). Our results showed a significant increase in the expression of iNOS in cells treated with LPS or HMGB1 compared to vehicle control ($P < 0.01$). We also found that there was a significant increase in the protein expression of NOS-2 in LPS and HMGB-1 treated cells compared to cells treated with vehicle control only as evaluated by immunofluorescence microscopy (**Figures 9B,C**) (n = 3, $P < 0.05$). Finally, we investigated whether there was a release of nitric oxide by the cells (**Figure 9D**). We found that NO release was significantly increased LPS (2.6 fold) (*$P < 0.05$), but only a marked increase in cells treated with HMGB1 (P = 0.07) compared to vehicle control treated cells. Together, these results indicate the activation of a TLR-S100B/RAGE pathway that subsequently led to the increased production of nitric oxide, especially in response to microbial PAMPS.

Activation of NADPH Oxidase 2 (NOX-2) and Increased Peroxynitrite Formation in Small Intestine Following Dysbiosis and Its Reversal by Gut Sterility

NADPH oxidase-2 with its subunits P47 phox, P67 phox, P22 align with GP91 in the membrane to form the NOX-2 membrane assembly. We detected the activation of NOX-2 by immunofluorescence dual labeling of GP91phox and P47phox subunits of the enzyme. We found a significant increase ($P < 0.05$) GP91phox-P47phox co-localization events in GW chemical treated mice (GW) compared to mice treated with vehicle (CONT, n = 9), mice exposed to antibiotics only (AB, n = 4) and mice co-exposed with GW chemicals and antibiotics (GW + AB, n = 9) (**Figures 10A,B**). Furthermore, we found a marked increase in peroxynitrite, an indicator of redox sensitive tyrosyl radicals in EGC of mice treated with GW chemicals compared to mice treated with either vehicle control or GW chemicals and antibiotics (**Figures 10C,D**) (*$P < 0.05$).

Activation of NOX-2 and Increased Peroxynitrite Formation in Rat EGC

Studies have showed that NADPH oxidases are activated in response to pathogenic stimuli in human EGC

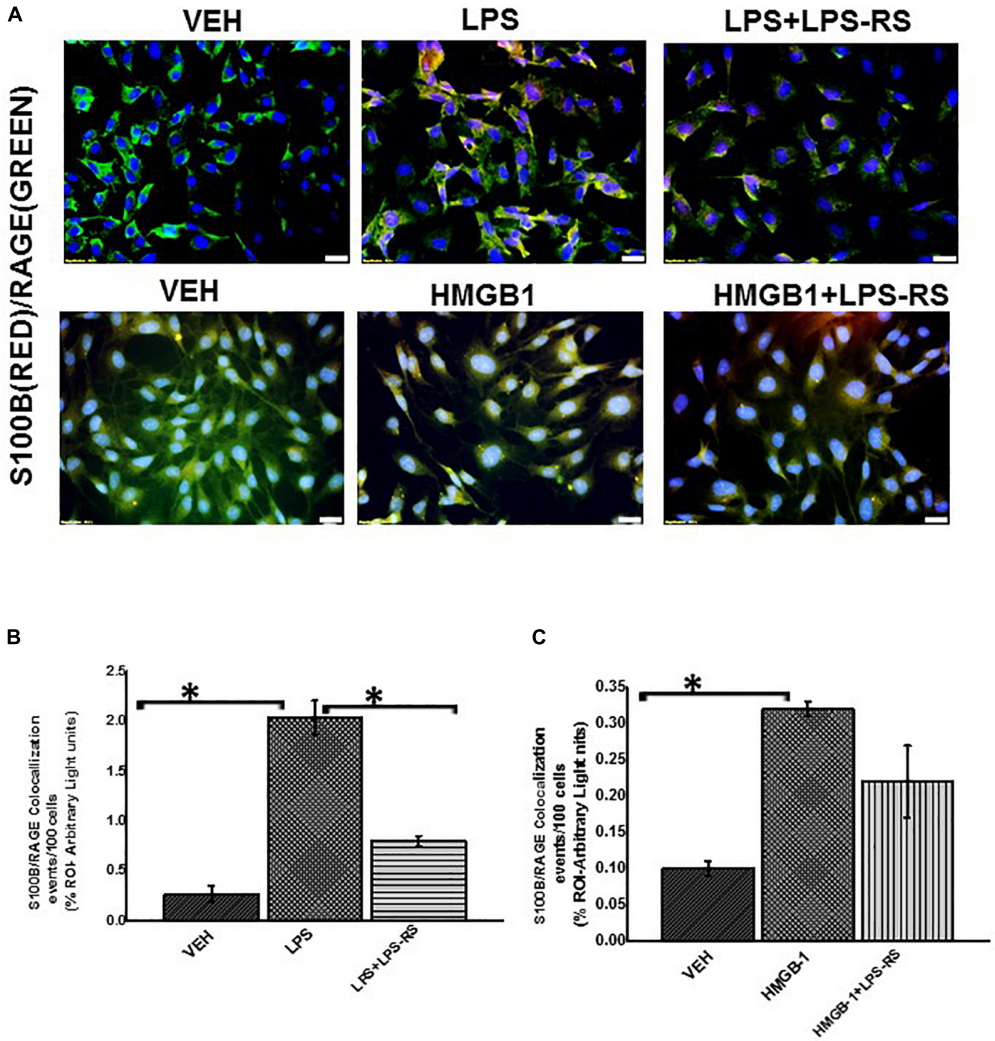

FIGURE 8 | EGC exposed to LPS or HMGB-1 change to a reactive phenotype. **(A)** S100B/RAGE complex formation in rat Enteric Glial Cells (EGC) ($n = 6$). Colocalization of S100β and RAGE in EGC treated with either vehicle control (VEH) or LPS, LPS + LPS-RS, HMGB1 or HMGB-1 + HMGB-1 + LPS-RS was determined by immunofluorescence microscopy at (total magnification 400X and scale 20 μm). **(B,C)** Quantitative morphometric analysis of S100B/RAGE complex formation. Colocalizations between red and green immunoreactivity were determined for every 100 cells per field for randomly chosen fields and represented as immunoreactivity in the region of interest (% ROI) (*$P < 0.05$).

(Macchioni et al., 2017). We found that treatment of EGC with LPS or HMGB1 significantly increased their expression of NOX-2 (**Figures 11A–D**) ($P < 0.05$). This activation was observed by immunofluorescence microscopy by detecting co-localization events (per 100 cells) between two key subunits of the NOX-2 enzyme complex. One in the lipid membrane GP91phox (labeled with green secondary antibody) and P47phox (labeled with red antibody). We found a significant increase in these co-localizations in LPS or HMGB1 treated cells compared to vehicle control treated cells (VEH) and cells treated with LPS/HMGB1 and Apocynin (LPS + APO) a NADPH inhibitor (Apocynin blocks the transport of p47 phox to the membrane) (*$P < 0.05$).

NOX-2 induced superoxide and nitric oxide react rapidly to form peroxynitrite, an indicator of redox related formation of tyrosyl radical and subsequent formation of tyrosine nitration We also observed that there was a significant increase in formation of peroxynitrite (shown by increased 3- nitrotyrosine formation) in LPS or HMGB1 treated EGC compared to Vehicle control (VEH) treated and LPS or HMGB1 and apocynin (LPS + APO) or (HMGB1 + APO) (**Figures 12A–D**) treated EGC ($P < 0.05$, $n = 6$).

Oxidative Stress Triggers Activation of NLRP-3 Inflammasome Which Results in Increased Inflammation

Reactive oxygen species (ROS) can trigger activation of inflammasomes resulting in caspase 1 mediated cleavage of Il-1β and IL-18 proinflamatory cytokines (Abais et al.,

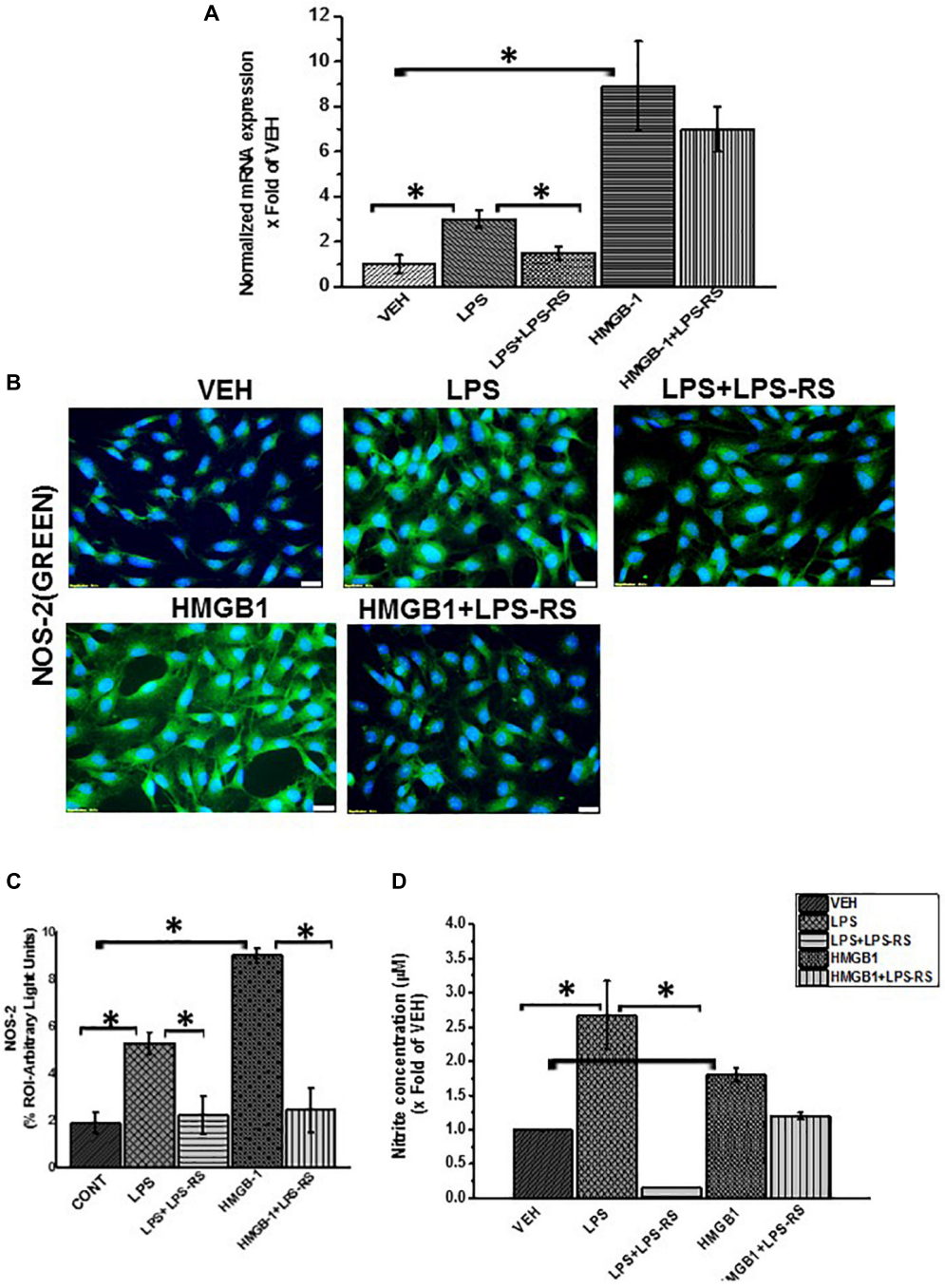

FIGURE 9 | Activation of inducible nitric oxide synthase by LPS or HMGB-1. **(A)** mRNA expression of NOS-2 in EGC exposed to vehicle control (VEH), LPS, LPS + LPS-RS or HMGB-1 and HMGB-1 + LPS-RS ($n = 6$) expressed as x fold of the vehicle control. mRNA expression was determined by qRTPCR. **(B)** NOS-2 protein expression in the cells was detected by staining with green fluorescent antibody and counterstained with DAPI (blue) and viewed at (total magnification 400 µm and scale 20 µm). **(C)** Quantitative morphometric analysis of NOS-2 in rat EGC per 100 cells in different fields and represented as immunoreactivity in the region of interest (% ROI). **(D)** Nitrite concentration in EGC ($n = 8$). Nitric oxide production in EGC supernatants was estimated by Griess assay from freshly harvested supernatants. Nitrite concentration is reported as X fold increase over the vehicle control (VEH) (*$P < 0.05$).

2015). Our results (**Figures 13A,B**) showed significant increase in mRNA expression of NLRP-3, Caspase-1, IL-1β and TNF-α in LPS treated EGC but not HMGB1 treated cells which only showed an increase in TNF-α expression compared to the vehicle control ($P > 0.05$). Treatment of EGC with LPS and apocynin (LPS + APO) and FBA (LPS + FBA) significantly decreased the observed mRNA expression ($P < 0.05$).

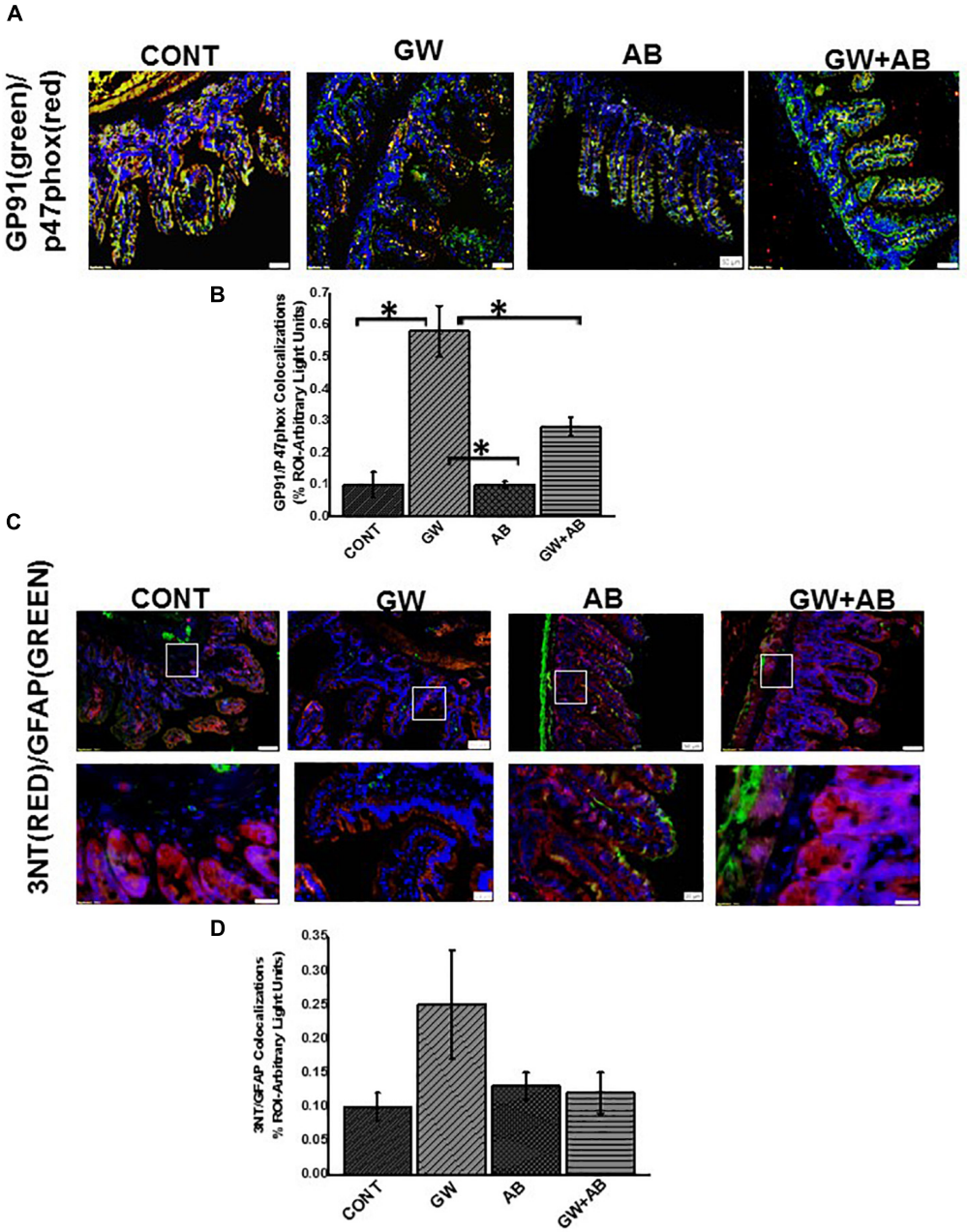

FIGURE 10 | NADPH oxidase 2 and peroxynitrite mediated oxidative stress *in vivo*. **(A)** NOX-2 activation in small intestine assessed by immunofluorescence microscopy (n = 9) (at total magnification 200X and scale 50 μm). Activation of NOX 2 was studied through observing colocalizations between GP91phox (labeled with green fluorescent antibody) and P47 phox (labeled with red fluorescent antibody) subunits of the NADPH 2 oxidase complex resulting in a yellow region. Colocalizations were determined in small intestine tissues of CONT, GW, AB, and GW + AB chemical exposed mice. **(B)** Graphical representation of morphometric analysis of colocalization events of GP91phox and P47phox in the region of interest. **(C)** Immunoreactivity of 3-nitrotyrosine (3NT) in EGC assessed through observing colocalizations between GFAP (labeled with green fluorescent antibody) and 3NT phox (labeled with red fluorescent antibody) at top panel magnification 200X, scale 50 μm and bottom panel magnification 600X and scale 20 μm) Colocalizations were determined in small intestine tissues of CONT, GW, AB, and GW + AB chemical exposed mice. **(D)** Graphical representation of morphometric analysis of colocalization events of GFAP (green) and 3NT (red).

We then investigated the protein expression of NLRP-3 and ASCII and adaptor protein of NLRP-3 in rat EGC using immunofluorescence microscopy. We found that cells treated with LPS but not HMGB1 treated cells showed a significant increase in NLRP-3 and ASCII complex formation compared to Vehicle control treated cells (VEH) indicating activation of the NLRP-3 inflammasome, when EGC encounter PAMPS. We further found that treatment of cells with LPS and FBA (LPS + FBA) showed a significant decrease in NLRP-3

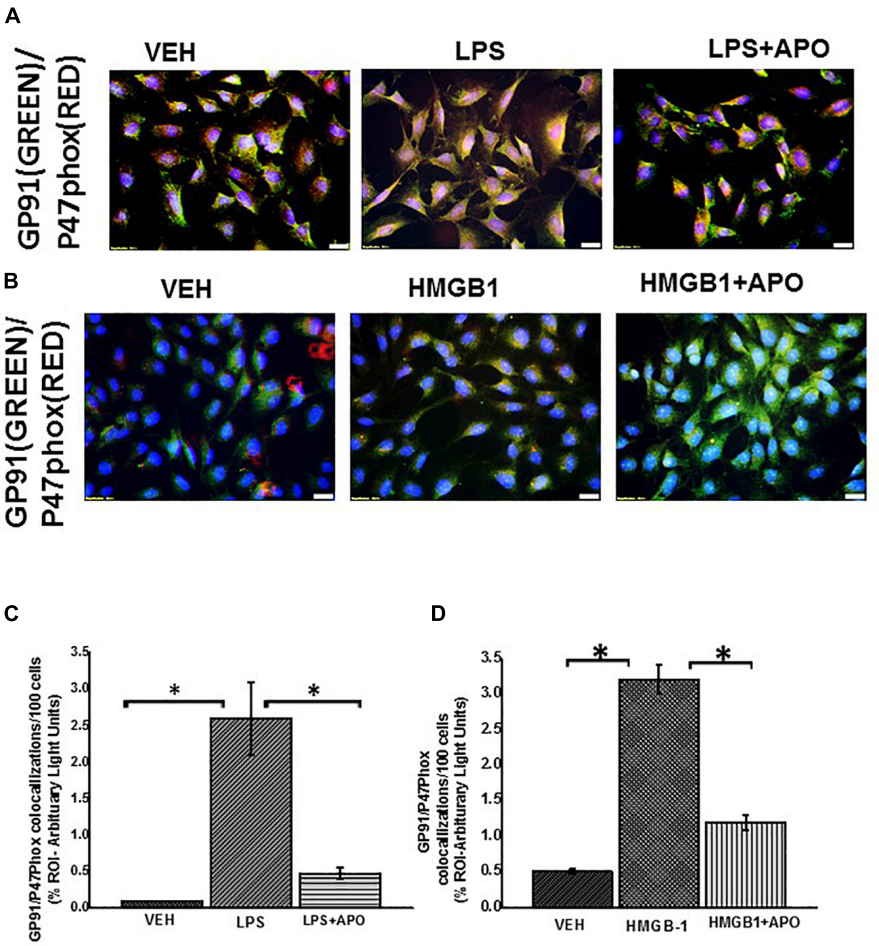

FIGURE 11 | NADPH oxidase 2 activation in rat EGC. **(A,B)** Activation of NOX-2 in rat EGC (n = 6). Activation of NOX 2 was studied through observing colocalizations between GP91phox (labeled with green fluorescent antibody) and P47 phox (labeled with red fluorescent antibody) subunits of the NADPH 2 oxidase complex resulting in a yellow region. Colocalization (yellow) of GP91phox and P47phox was detected in vehicle VEH, LPS, LPS + apocynin (LPS + APO), HMGB-1, HMGB-1 + APO treated cells at total magnification 400X; scale 20 μm). **(C,D)** Morphometric analysis of GP91/p47phox colocalization events in rat EGC per 100 cells in different fields (*$P < 0.05$).

protein activation ($P < 0.05$), (**Figures 13C–E**) suggesting the role of NOX-2 derived peroxynitrite as a candidate for the inflammasome formation.

Increased DNA Fragmentation in Reactive Rat EGC Following Stimulation With LPS and Its Dependence on NOX-2-Induced Oxidative Stress

Increased pathogenic stimuli were found to initiate apoptosis in EGC (Macchioni et al., 2017). We also investigated the fate of these reactive EGCs when continually exposed to PAMPs or DAMPs though a tunel assay to detect fragmented DNA.

We found that LPS or HMGB1 treated cells showed a significant increase in co-localization events per 100 cells compared to cells treated with only vehicle control ($P = 0.043$) (**Figures 14A–D**). There was a significant decrease in tunel events when cells were treated with LPS and Apocynin but not FBA. And when cells were treated with HMGB1 and apocynin or FBA, there where was no significant decrease in tunel events.

Reactive EGC Contribute to Inflammation and Intestinal Barrier Integrity in Small Intestine: Gut Decontamination by Antibiotics and Blocking EGC Immune Activation Restores Gut Barrier Protein Levels in GWI Mice

Cytokines, ROS and growth factors etc affect tight junction proteins, water channels and processes such as differentiation, apoptosis etc. (Bush et al., 1998; Bush, 2002; Yu and Li, 2014).

In this study, we showed that when LPS primed intestinal epithelial cells were treated with culture fluid from EGC, there was a significant increase in mRNA expression of proinflammatory cytokines in IEC-6 cells (**Figure 14E**)

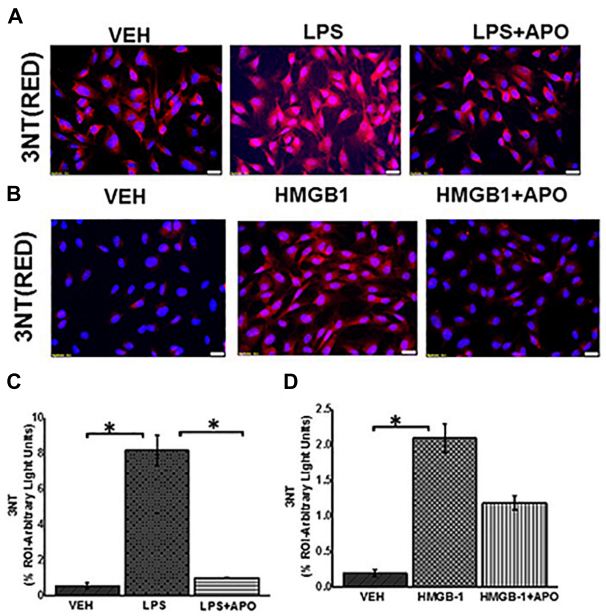

FIGURE 12 | Peroxynitrite formation in EGC. **(A,B)** Expression of 3NT in rat EGC ($n = 6$). Immunoreactivity of 3NT was detected in vehicle (VEH), LPS, LPS + APO, HMGB-1 and HMGB-1 + APO treated cells by staining with red fluorescent antibody and counterstained with DAPI (blue) viewed at total magnification 400X; scale 20 μm). **(C,D)** Morphometric analysis of 3NT in rat EGC per 100 cells in different fields (*$P < 0.05$).

($P < 0.05$). LPS primed IEC-6 cells which were treated with culture fluids from EGC treated with LPS (LPS-SN) showed a significant increase in mRNA expression of IL-1β, MCP1 and TNF-α when compared to the vehicle control (VEH) ($P < 0.05$). LPS primed cells treated with culture fluids from Vehicle control treated EGC showed a significant decrease in MCP-1 expression ($P < 0.05$) and a marked decrease in TNF-α but no significant decrease in IL-1β expression compared to the LPS primed IEC-6 cells treated with culture fluids from LPS treated EGC.

To ensure that EGC immune activation via an altered microbiome plays a significant role in gut barrier protein expression in the intestine, we studied the GW chemical treated mice for their protein levels of aquaporin, a selective water channel, occludin and claudin-2. Results showed that administration of antibiotics was associated with significantly restored the levels of aquaporin 3 in the intestine of GWI-treated mice when compared to GW-treatment (**Figures 15A–C**). Levels of occludin were also restored when compared to controls but were significantly elevated when compared to GW-mice only (**Figures 15D–F**). Claudin-2 levels have been found to be increased in association with gut integrity loss. Our results showed that use of antibiotics significantly decreased the levels of claudin-2 in antibiotic treated mice when compared to GWI-mice alone (**Figures 15G–I**).

To show that EGC immune activation as a result of TLR4 and specific inflammation was responsible in part in causing differential expression of tight junction proteins that may play a significant role in gut barrier protein integrity loss, we chose to use two significant compounds that has been studied for their TLR-antagonism (SSnB) and anti-inflammatory properties (Sodium Butyrate-BT) specifically in the intestine. Results showed that a combined use of TLR4 antagonist and butyrate markedly increased aquaporin levels (**Figure 15A**) in the small intestine when compared to GW-mice while levels of Claudin-2 were significantly decreased in the small intestine following SSnB + BT administration when compared to the same group (**Figure 15D**). Occludin which is decreased in GW mice and plays a significant role in maintaining gut barrier integrity was also restored to normal levels in the diseased mice following administration of SSnB + BT (**Figure 15G**). The results suggested that blocking TLR4 and subsequent immunoactivation that resulted in a reactive EGC phenotype in mice due to dysbiosis can be reversed by the use of these antagonists. Also, the results show that reactive EGCs might have a significant role in causing gut barrier dysfunction following activation via release of PAMPs and DAMPs and can be a cause of symptom persistence in GWI.

Reactive EGC Modulate Tight Junction Proteins and Aquaporins in Intestinal Epithelial Cells

We investigated the hypothesis that EGC which are exposed to DAMPS (e.g., HMGB-1) and PAMPS (e.g., LPS) modulate intestinal tight junction proteins and selective water channels by treating LPS primed IEC-6 cells with culture fluids freshly collected from EGC which have been treated with LPS (LPS-SN), HMGB1 (HMGB1-SN), vehicle (VEH-SN), LPS + SsnB + Butyrate (LPS + SsnB + BT) or HMGB1 + SsnB + Butryate (HMGB1 + SsnB + BT). Protein expression was studied by immunofluorescence microscopy observed at a total magnification of 400X; scale 10 μm. We found that expression of aquaporin-3 was significantly increased when IEC-6 cells were treated with LPS-SN, while when they were treated withHMGB1-SN the expression was significantly decreased compared to IEC 6 cells only treated with vehicle control (VEH) ($n = 3$, $p < 0.05$). IEC-6 cells treated which were treated with culture fluids from vehicle control treated EGC (VEH-SN) showed only a slight increase in aquaporin-3 protein expression, while IEC-6 cells treated with inhibitors SsnB and Butyrate together with LPS or HMGB1 restored expression of aquaporin 3 almost back to similar levels as IEC-6 cells treated with vehicle control (VEH) (**Figures 16A,B**). Claudin 2 expression increased significantly when IEC-6 cells were treated with culture fluids from EGC treated with LPS-SN ($n = 3$; $p < 0.05$), but only markedly, when with HMGB1 treated culture fluids (HMGB1-SN). And when IEC-6 cells were treated with culture fluids for EGC treated with SsnB and butyrate together with HMGB1 or LPS, claudin 2 levels were decreased similar to vehicle control treated cells (VEH) (**Figures 16C,D**). Higher magnification (630X and scale bar 20 μm) images taken under confocal microscopy are included to show the localization of this protein in the membrane (**Figure 17A**). IEC-6 cells that were treated with culture fluids from EGC

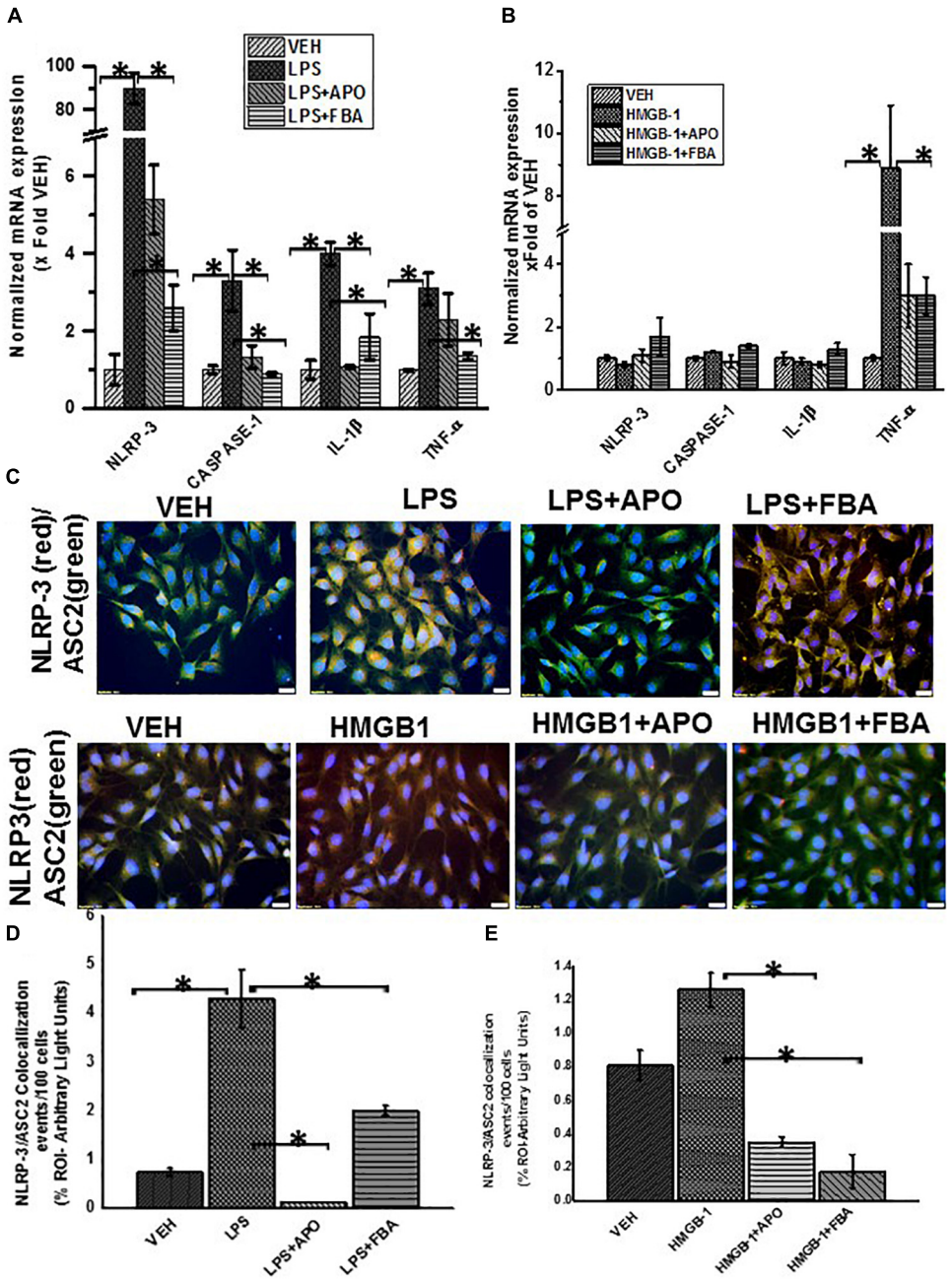

FIGURE 13 | ROS mediated activation of NLRP-3 inflammasome and inflammation in EGC. **(A,B)** Quantitative real time PCR (qRT-PCR) analysis of inflammasome and inflammation ($n = 6$). mRNA expression of NLRP-3, caspase-1, IL-1β and TNF-α in rat EGC which were treated with vehicle (VEH), LPS + apocynin (LPS + APO), LPS + phenylboronic acid (LPS + FBA), HMGB-1, HMGB-1 + APO. mRNA expression is represented as a fold change of the vehicle control. Data points are represented as mean ± SEM ($n = 3$) (*$P > 0.05$). **(C)** NLRP-3/ASC2 protein expression in rat EGC assessed by immunofluorescence microscopy and viewed at total magnification 400X; scale 20 μm). Colocalization events were determined for every 100 cells per field in cells treated with LPS, LPS + APO, LPS + FBA, HMGB-1, HMGB-1 + APO, HMGB1 + FBA. **(D,E)** Quantitative morphometric analysis of fluorescence intensity of NLRP-3/ASC2. Fields for morphometric analysis were randomly selected from different fields per slide and represented as% region of interest (% ROI) (*$P < 0.05$).

treated with LPS (LPS-SN) or HMGB1(HMGB1-SN) showed a significant decrease in occludin protein expression compared to vehicle control treated IEC-6 cells (VEH) (**Figures 16E,F**). Treatment with culture fluids from EGC which had been treated with LPS or HMGB1 together with inhibitors (SsnB and butyrate) showed a restoration in occludin levels compared to the control (VEH), although the effect was stronger in IEC 6 cells treated with LPS + SsnB + BT compared to HMGB1 + SsnB + BT treated EGC culture fluids. Higher magnification (630X and scale;20 μm) images taken under

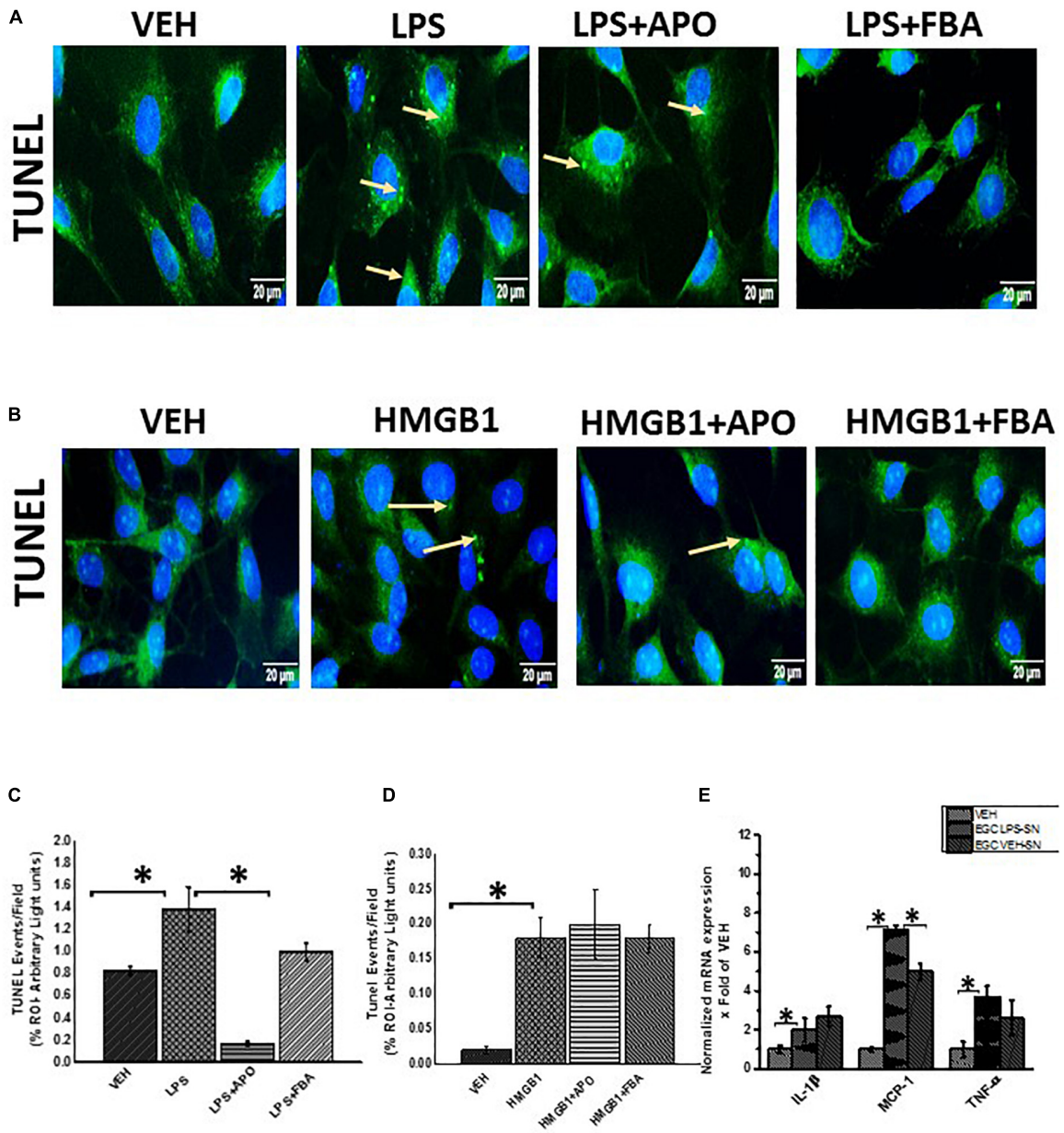

FIGURE 14 | DNA fragmentation of rat EGC. **(A,B)** Tunel assay showing DNA fragmentation ($n = 6$). DNA fragmentation was determined by the Tunel assay in Vehicle (VEH), LPS, LPS + APO, LPS + FBA, HMGB-1, HMGB-1 + APO, HMGB-1 + FBA treated cells viewed at total magnification 400X; scale 10 µm. **(C,D)** Quantitative morphometric analysis of fluorescence expression of Tunel positive cells represented as Tunel events per field (*$P < 0.05$). **(E)** Effect of EGC culture fluids on LPS primed intestinal epithelial cells. mRNA expression of IL-1β, MCP-1 and TNF-α in IEC-6 cells which have been primed with LPS and treated with culture fluids from EGC treated with LPS and vehicle control ($n = 6$) represented as x Fold of the vehicle control.

confocal microscopy were used to show the localization of this protein in the membrane (**Figures 17A,B**). The tubulin staining used in occludin images to show the extent of occludin traversing the tubulin outline thus signifying their apical membrane localization as widely perceived (Shown by white arrows).

These results indicate that reactive EGC are strong players in modulating tight junction protein expression through production of factors that may influence gut barrier integrity.

DISCUSSION

Our results propose a possible molecular mechanism to explain the altered microbiome associated inflammation in a cellular level and poor gastrointestinal health which we observed in our studies on GWI (Alhasson et al., 2017; Seth et al., 2018). The results reported in this study are an advancement to our previous reported work in Gulf War illness pathology. Since gut sterility by antibiotics reversed immunopathology in GWI

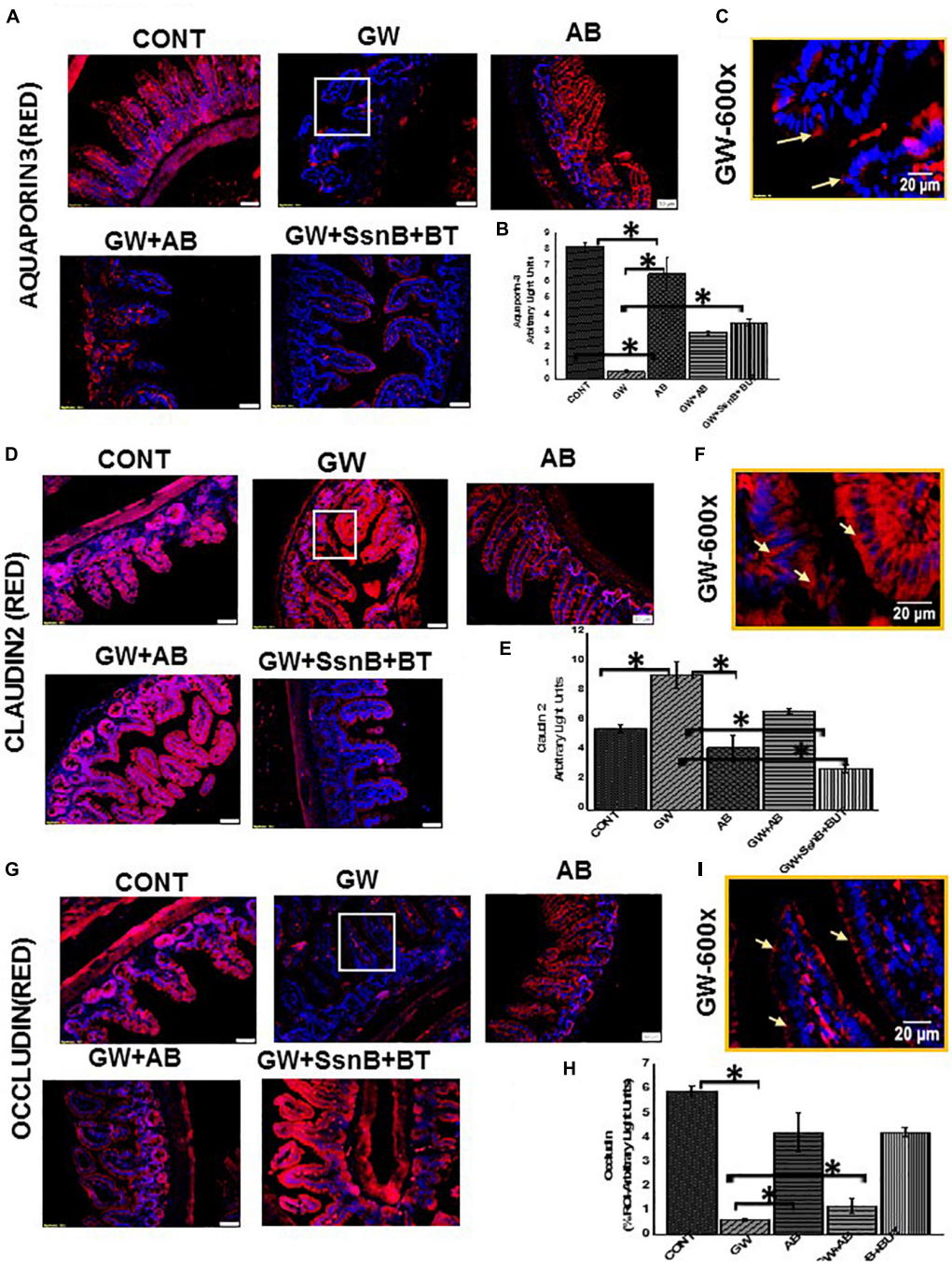

FIGURE 15 | Expression of claudin 2, occludin and aquaporin 3 in mouse small intestine. **(A,D,G)** Protein expression of occludin, claudin 2 and aquaporin 3 in mouse small intestine was determined by immunofluorescence microscopy and visualized at total 200X; scale 50 μm in tissues obtained from mice treated with vehicle control (CONT, $n = 6$); mice treated with GW chemicals (GW, $n = 9$) mice co-exposed with GW chemicals and antibiotics (GW + AB, $n = 9$) and mice treated with GW chemicals, Sparstolonin B (SsnB) and Sodium butyrate (GW + SsnB + BT) ($n = 6$). **(C,F,I)** Higher magnification images for GW chemical treated group (GW) focusing on the apical or apical-lateral membranes total magnification 600X; scale 20 μm). **(B,E,H)** Quantitative morphometric analysis of immunoreactivity of occludin or aquaporin 3 is represented as (% ROI) (*$P < 0.05$).

we used the same approach to correlate the observed gut dysbiosis with EGC immunoactivity. We found a novel role of altered microbiome in causing a reactive EGC phenotype characterized by activation of toll-like receptors, RAGE/S100B and increased expression of nitric oxide synthase. This pathway contributes to NADPH oxidase mediated generation of ROS which trigger inflammation. The mechanism of NOX2 mediated inflammation is well accepted as we and others have shown the

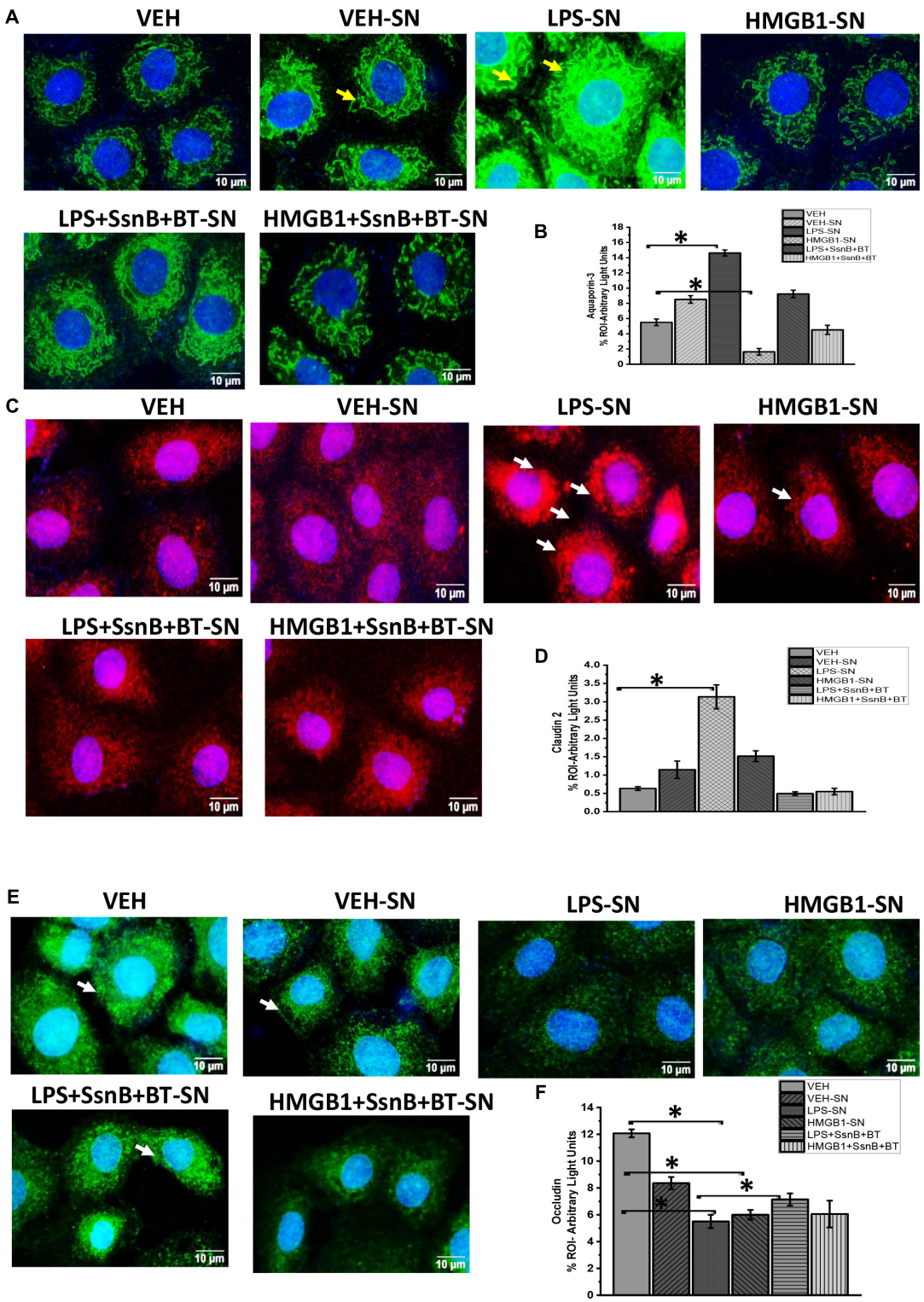

FIGURE 16 | Protein expression of aquaporin 3, claudin 2 and occludin in intestinal epithelial cells. **(A,C,E)** Protein expression of aquaporin-3, claudin-2, and occludin in IEC 6 cells treated with culture fluids from Vehicle (VEH-SN), LPS (LPS-SN), HMGB1 (HMGB1-SN) and inhibitors SsnB and butyrate (LPS + SsnB + BT and HMGB1 + SsnB + BT) treated EGC. The expression of these proteins was studied by immunofluorescence microscopy and viewed at 400X total magnification; scale 10 µm. Yellow arrows indicate the localization of the proteins in the cell membrane. **(B,D,F)** Quantitative morphometric analysis of immunoreactivity of aquaporin, claudin 2, and Occludin represented as (% ROI) ($n = 3$, $^*P < 0.05$).

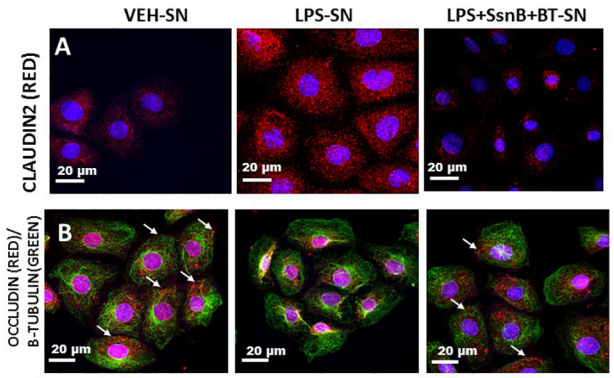

FIGURE 17 | Cellular localization of Claudin 2 **(A)** and Occludin **(B)** protein expression in intestinal epithelial cells via confocal microscopy. Protein expression of Claudin 2 **(A)** or Occludin **(B)** in the cell membrane of intestinal epithelial cells treated with culture fluids from EGC treated with Vehicle (VEH-SN), LPS (LPS-SN) and LPS with SsnB and butyrate (LPS + SsnB + BT). The images were viewed under a confocal microscope at a total magnification of 630X and scale 20 μm. Occludin membrane localization was based on the tubulin staining that visualizes the outline of the cell.

role of peroxynitrite mediated inflammation in liver, kidney and gastrointestinal disturbances (Das et al., 2015; Alhasson et al., 2016) Further, we propose that this increased inflammation and ROS may result in enteric gliopathy and a later episodes of enteric neuropathy although this needs to be investigated further and is a speculation at this point. With continued production of proinflammatory cytokines and other destructive factors (e.g., ROS) by reactive EGC, the entire or part of the epithelial barrier in the gut might lose its integrity. This hypothesis is further strengthened by our results from the supposed blockage of TLR4 and inflammation by using SSnB and Butyrate (thus blocking EGC immune-activation), further exacerbating the observed gastrointestinal pathology in gulf war illness. This explanation not only helps us understand the acute phase of gulf war associated gastrointestinal inflammatory disorders, but also could explain why these symptoms may persist for long since a vicious cycle might exist following a continuous assault on the intestinal epithelial cells.

Enteric glial cells are important regulators of the gastrointestinal tract health. They can influence the gut microenvironment both positively or negatively depending on surrounding conditions (Capoccia et al., 2015; Ochoa-Cortes et al., 2016; Grubisic et al., 2018). Remarkably they can respond to the presence of bacteria pathogens through this TLR-RAGE/S100β-iNOS pathway. This is through the recognition of bacterial parts such as cell wall, high bacterial populations, DNA etc via toll like receptors. With an altered microbiome, there is proliferation of certain bacterial species at the expense of others and this change upsets the natural healthy balance in microbiome. This disruption happens due to several stressful stimuli e.g., infection, diet or exposure to chemicals such as in the case of gulf war illness.

Our previous studies have clearly shown that exposure to GW chemicals indeed results in altered microbiome (Alhasson et al., 2017; Seth et al., 2018) (**Supplementary Figure S1**). We found an increase in the Firmicutes/Bacteriodetes ratio with significant increases in several Firmicutes genera in gulf war chemical treated mice compared to the vehicle controls. Further we found an associated loss in bacteria populations such as Bacteroides, Oscillibacter and Ruminiclostridia. Increased abundance of Bacteroides for example are associated with healthy gastrointestinal states (Johnson et al., 2017), while Oscillibacter and Ruminiclostridia have also been shown to be abundant in healthy controls in studies of Crohn's disease (Svolos et al., 2019). The decline in beneficial microbiota may have allowed for the proliferation of several bacteria populations at genus level, which usually exist in low percentages. There was a rise in several Coriobacteria, Bacilli and Verrucomicrobia bacteria. These have all been associated to increase in IBS and IBD (Distrutti et al., 2016). This upset balance of bacteria population dynamic results in normally benign bacterial populations to become pathogenic and could cause EGC to change to a reactive phenotype through toll-like receptor signaling (Zhang et al., 2015).

Both the altered microbiome and reactive EGC phenotypes have been linked to several diseased states of the gut such as IBS, IBD, gut hypersensitivity etc. (Conlon and Bird, 2014; Wang et al., 2019). However, there is scanty information concerning the true mechanism of how they contribute to these diseases. Our current study showed a correlation between altered microbiome and a reactive EGC phenotype in small intestine. Mice treated with GW chemicals (GW) had a higher expression of GFAP a protein whose increased expression has been associated with IBS, S100β/RAGE complex formation and finally an increase in nitric oxide synthase activity in EGC. These proteins were not increased in vehicle control treated mice (CONT) and their expression was significantly less in mice treated with GW chemicals and antibiotics (GW + AB). This emphasizes the role of microbiome in contributing to EGC reactive phenotype. Though we have used antibiotics to ensure gut contamination or sterility, the use of such approach may not be ensuring complete gut sterility in mice. Often the use of such antibiotics can selectively lead to bacteriostatic effects in healthy fauna while elevating the abundance of harmful bacteria in the gut. The use of germ free mice is the best approach for conducting studies where the endpoint is to assess the role gut bacteria in the pathology. Though it has to be admitted that antibiotic use for ensuring gut sterility is a standard approach where use of germ-free mice is a constraint. The use of antibiotics in our study is thus a limitation and needs further corroborative studies in future using the germ free model.

Mechanistically, we showed that the increased activation of NOX-2 following an altered microbiome and associated activation of the EGCs in GW chemical exposed mice plays a significant role in contributing to the observed intestinal inflammatory phenotype. NOX-2 in EGC and in adjacent intestinal cells, participates in oxidative stress which results from the increase in nitric oxide production. We found that the generated ROS triggered activation of the NLRP-3 inflammasome which further caused increase in inflammation and programmed cell death as showed by increased DNA fragmentation (tunel assay) in rat EGCs stimulated with LPS and/or HMGB1.

This increased inflammation and direct loss in enteric glia has been reported as in Chron's disease (Cornet et al., 2001; Ochoa-Cortes et al., 2016). The reactive inflammatory glial phenotype is detrimental to the health of the gastrointestinal tract because it produces destructive factors which interact with surrounding cells in the intestine e.g., intestinal epithelial cells, enteric neurons etc. In our study we showed that when EGC conditioned media was applied to primed epithelial cells, there was an increase in proinflammatory cytokine expression such as IL1β, MCP-1 and TNF-α which can be conducive to a leaky gut microenvironment. Furthermore, we observed that a reactive EGC phenotype can also have detrimental effects on the EGCs as shown by increased DNA fragmentation and cell death through the increased inflammation and ROS generated. This ultimately may result in programmed cell death in glia by pyroptosis or apoptosis as shown elsewhere (Macchioni et al., 2017). The general loss in enteric glia could lead to suboptimal functioning of enteric neurons and even enteric neuropathy (Bassotti et al., 2018). This mechanism could be a possible explanation for the symptoms of GWI which continue to persist for 25 years though the present report does not study the role of an activated EGC on intestinal neurons. However, the effect of the reactive EGCs on intestinal epithelial cell barrier integrity can be profound as a blockade of the EGC activation mechanisms by SSnB and butyrate prevented protein alterations in the tight junctions. The results are also interesting since we observe a cyclical pattern of epithelial cell damage-activation of EGCs and a link to altered expression of tight junction proteins such as claudin-1,2, occludin or ZO-1 that may contribute to gut-leakiness, that eventually might fuel a continuous persistence of inflammation in the local intestinal microenvironment.

CONCLUSION

We report that EGC are important players in GWI gastrointestinal disease pathology and respond to the altered microbiome in the host gut by converting to a reactive phenotype which greatly affects the healthy functioning of the gastrointestinal tract. This reactive phenotype significantly contributes to oxidative stress which further triggers inflammation, loss of gut barrier integrity and possibly death of enteric glia and enteric neurons, although further investigations need to be carried out to confirm these neuronal effects. Further, these findings provide insights into how a possible altered microbiome may be contributing to the observed GWI intestinal epithelial cell inflammatory phenotype by destabilizing the redox status of glial cells and adjacent epithelial cells via NOX-2 mediated peroxynitrite generation, inflammasome activation and release of pro inflammatory cytokines. It has to be further realized that more concrete evidence will be needed that involves germ free mice to conclude with certainty that microbiome alterations definitely dictate the observed EGC effects and therefore remains a limitation in this study. Antibiotic use for gut sterility should be overcome with more stringent experimental designs such as germ-free mouse models and gnotobiotic mice. Nevertheless, the present evidence will therefore be valuable to consider EGC nitric oxide production, formation of peroxynitrite, a redox signaling intermediate and inflammation pathways as therapeutic targets in gulf war illness.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the USC Institutional Research Board (USC IACUC).

AUTHOR CONTRIBUTIONS

SC and DK designed and performed the experiments. DK, SS, DB, RS, YL, AM, MA, and AK performed the experiments. KS, PJ, and SL analyzed and interpreted the data. PN and MN analyzed the data and edited the manuscript. NK, KS, and RH edited the manuscript. SC and DK wrote the manuscript, edited and analyzed the data.

FUNDING

Funding for this project was provided by DoD-IIRFA grant: W81XWH1810374 and VA Merit CX001923-01 awarded to SC; W81XWH-16-1-0556 to SL; W81XWH-13-2-0072 to KS; P01AT003961, P20GM103641, R01AT006888, R01ES019313, R01MH094755, and VA Merit Award BX001357 to MN and PN. This material is based upon work supported (or supported in part) by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development vide grant no. CX001923-01 to SC. SC is a 5/8th employee with the US Department of Veterans Affairs.

ACKNOWLEDGMENTS

We are deeply grateful to Benny Davidson (Instrument Resource Facility, University of South Carolina School of Medicine) and AML Labs (Baltimore, MD, United States) for providing technical and consultation services. We acknowledge the technical expertise of Dr. Avik Mukherjee (Hunt Optics and Imaging Inc.) for his help with imaging studies. We also highly appreciate Second Genome and the department staff at the Pathology, Microbiology and Immunology, School of Medicine for their excellent analysis of the microbiome.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01229/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GW and handling Editor declared their shared affiliation at the time of the review.

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International Journal of
Environmental Research and Public Health

MDPI

Article

The Gut-Microbiome in Gulf War Veterans: A Preliminary Report

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Received: 6 September 2019; Accepted: 1 October 2019; Published: 4 October 2019

Abstract: Gulf War Illness (GWI) is a chronic multi-symptom disorder affecting the central nervous system (CNS), immune and gastrointestinal (GI) systems of Gulf War veterans (GWV). We assessed the relationships between GWI, GI symptoms, gut microbiome and inflammatory markers in GWV from the Boston Gulf War Illness Consortium (GWIC). Three groups of GWIC veterans were recruited in this pilot study; GWV without GWI and no gastrointestinal symptoms (controls), GWV with GWI and no gastrointestinal symptoms (GWI-GI), GWV with GWI who reported gastrointestinal symptoms (GW+GI). Here we report on a subset of the first thirteen stool samples analyzed. Results showed significantly different gut microbiome patterns among the three groups and within the GWI +/−GI groups. Specifically, GW controls had a greater abundance of firmicutes and the GWI+GI group had a greater abundance of the phyla bacteroidetes, actinobacteria, euryarchaeota, and proteobacteria as well as higher abundances of the families Bacteroidaceae, Erysipelotrichaceae, and Bifidobacteriaceae. The GWI+GI group also showed greater plasma levels of the inflammatory cytokine TNF-RI and they endorsed significantly more chemical weapons exposure during the war and reported significantly greater chronic pain, fatigue and sleep difficulties than the other groups. Studies with larger samples sizes are needed to confirm these initial findings.

Keywords: Gulf War illness; microbiome; Gulf War; veterans; inflammation; cytokines; exposure

1. Introduction

Gulf War Illness (GWI), a debilitating multi-symptom illness, has affected and altered the quality of life for thousands of US Gulf War veterans (GWV). Approximately 700,000 troops from the US were deployed to the Persian Gulf and the estimates are that this illness afflicts a third of those who were deployed [1]. Shortly following the end of the Gulf War in 1991 veterans began reporting a constellation of health symptoms from multiple body systems [1,2]. Researchers began what has now become a multi-decade long quest to discover the causes of this illness. Over the years, substantial

evidence has accumulated supporting a link between deployment to the Persian Gulf during Operation Desert Shield/Operation Desert Storm, environmental exposures from the war and the development of GWI-related symptoms [1–5]. More specifically, researchers have identified a link between specific toxicant exposures including pesticides, anti-nerve gas pills (pyridostigmine bromide, PB) and nerve agent chemical weapons (sarin/cyclosarin), during deployment and development of GWI-related health symptoms [1–5]. In addition, it has recently been shown that GW veterans have not gotten better over time and may be getting worse as well as developing more chronic health conditions at a younger age than the general population [6].

GWI is a chronic health disorder that involves multiple body systems and includes multiple health symptoms. The hallmark symptoms of GWI according to the widely used Kansas criteria are fatigue, musculoskeletal pain, cognitive difficulties, gastrointestinal issues (GI), respiratory problems and skin rash [2,7,8]. A growing body of evidence indicates that GWI is associated with diverse central nervous system (CNS) and immune alterations suggesting a neuroinflammatory component to the disorder, but the specific pathobiological processes driving the diverse GWI symptoms have not been clearly elucidated [1,9–11]. Animal studies indicate that a chronic CNS inflammatory state can develop in response to an insult—chemical injury, infection or physical trauma—that mobilizes the CNS defense systems via activation of glia, the brain's primary immune response cells, and release of chemical messengers (i.e., cytokines) that precipitate a complex set of symptoms. These symptoms have been identified as impaired memory and learning, increased pain sensitivity and persistent fatigue, a symptom complex similar to that of GWI [10–15]. Recent studies have also demonstrated CNS inflammatory effects of GW-related exposures (PB, pesticides, depleted uranium, nerve agents) and additional innate immune processes (oxidative stress markers) that potentially explain the mechanism contributing to the full spectrum of GWI symptoms including those of the gastrointestinal (GI) system. Specifically, it is postulated that GWI can occur through activation of the innate immune system through toll like receptors (TLR4) present in the CNS and the GI tracts [10–12,16–18]. Another not mutually exclusive hypothesis for GWI centers on mitochondrial impairment as the impetus for the disorder [19–21].

Much of our research done to date on specific symptomology of GWI has focused on the CNS effects including cognitive and mood difficulties experienced by GW veterans. Only more recently has attention started to turn toward understanding the relationship between CNS effects and other debilitating symptoms including GI disturbances and chronic pain experienced by GW veterans. Veterans with GWI report experiencing numerous chronic GI symptoms including; abdominal pain/discomfort, bloating, nausea, vomiting, diarrhea as well as a clinical diagnosis of Irritable Bowel Syndrome (IBS) in many cases [22–24]. Our group has recently demonstrated the role of the gut microbiome in a mouse model of GWI on the gut microbiome-brain axis and the enteric nervous system [16,25,26]. This work revealed a potential link between the altered gut microbiota to gut leaching, GI inflammation, systemic endotoxemia, and neuroinflammation through activation of toll-like receptor 4 (TLR4) located on glial cells in the brain and the GI system [16]. TLR4 activation leads to production and signaling of proinflammatory cytokines. To date, the gut microbiome and its relationship with innate immune system activation and neuroinflammatory cytokine blood markers has not been examined in GW veterans.

In order to more fully assess the relationships between GWI, GI symptoms and potential innate immune activation and neuroinflammation in GW veterans, we translated our prior GW-relevant animal studies of microbiome and proinflammatory cytokines (i.e., gut microbiome-brain axis) into a pilot study with GW veteran participants from the Boston Gulf War Illness Consortium (GWIC) study. The Boston GWIC was designed to have both preclinical and clinical epidemiologic studies working in tandem to translate results as expediently as possible to the clinic. The goal of this GWIC funded microbiome call-back pilot study was to determine (1) whether the gut microbiome diversity of GW veterans with GWI and GI symptoms (GWI+GI) is different than the gut microbiome of GW veterans with GWI and no GI symptoms (GWI-GI) and/or different than GW veterans without GWI or GI symptoms (controls) and (2) whether these three groups differ with respect to innate immune

system activation as shown by proinflammatory cytokine levels in circulating plasma and (3) whether these markers correlate with self-reported toxicant exposures from the war.

2. Materials and Methods

2.1. Human Subjects

The GWIC includes a series of clinical studies with the primary objective of providing a cohesive understanding of the pathobiological mechanisms responsible for the varied symptoms of GWI in order to provide a rational and efficient basis for identifying beneficial treatments and diagnostic markers. Inclusion criteria for GWIC required deployment to the Gulf War between August 1990 and July 1991. Exclusion criteria included chronic illness diagnoses that could otherwise account for the symptoms endorsed by veterans. These included autoimmune, central nervous system, or major psychiatric disorders that could 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 either at least moderate severity or multiple mild symptoms within the domain [7]. GWIC participants not meeting Kansas GWI or exclusionary criteria were considered controls.

To date, the GWIC has recruited and examined over 230 GW veterans. Each of these veterans has undergone an extensive assessment, including numerous health-related surveys, a neuropsychological test battery, brain imaging and collection of blood and saliva samples [27]. Specifically related to the gastrointestinal tract, subjects were asked to report whether they experienced the following symptoms in the past 6 months: abdominal pain or cramping, diarrhea, nausea or upset stomach, other non-specified GI disorders. In addition, they were asked to report whether they had received a diagnosis of IBS from a doctor as part of the medical conditions questionnaire. The GWIC study was awarded exploratory funds to perform promising pilot studies to be used as preliminary data for future larger studies. One of the approved pilot studies was the current GWIC call back study of microbiome patterns in GW veterans. Thirty GW veterans who had participated in the GWIC study were targeted for further participation in this microbiome pilot study.

For this pilot study three groups of veterans from the GWIC cohort were recruited including: 1. GW veterans without GWI who reported no gastrointestinal symptoms/disorder (GW controls), 2. Gulf War veterans with GWI who reported no gastrointestinal symptoms/disorders (GWI-GI) and 3. Gulf War veterans with GWI who reported gastrointestinal symptoms/disorders (GW+GI).

Participants for this pilot call back study were recruited by telephone after they completed the GWIC study protocol and asked to fill out a brief subject screening questionnaire and provide a stool sample by mail to the study investigators. Once the subjects received the stool collection kit they were asked to collect the sample at their homes at their own convenience and mail back to Boston University GWIC investigators.

2.2. Demographics, Deployment Exposures and Health Symptom 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) [7,28,29]. Self-reported exposures were obtained from the Kansas GW Experiences and Exposure Questionnaire and the SNAC [7,28]. Health outcomes were measured by the medical conditions questionnaire in which the participant endorsed whether or not they had a confirmed diagnosis of queried health outcomes including memory loss, chronic fatigue syndrome, fibromyalgia and IBS [28]. Additional validated health symptom surveys were completed by study participants including the Multi-dimensional Fatigue Inventory (MFI-20), McGill Pain Inventory and the Pittsburgh Sleep Quality Index where higher scores indicated more symptoms [30–32].

2.3. Plasma Cytokine Analyses

Plasma was separated and stored at −80 °C until assayed. An 18 cytokine Quansys multiplex panel was used to measure cytokines in plasma samples in the GWIC study. The proinflammatory cytokines including IL1α and IL1β, and soluble receptors for TNF Receptor I (TNF-RI), TNF Receptor II (TNF-RII) as well as Th1, Th2, Th17 and anti-inflammatory cytokines were measured with a multiplex chemiluminescent assay using Quansys instrument and reagents in methods previously reported [33]. To determine if circulating proinflammatory cytokines levels were different across the three groups in this pilot study, plasma samples were examined by symptom group. In this study, chemiluminscent imaging concentrations of IL-1a, 1b, 2, 4, 5, 6, 8, 10, 12 (p70), 13, 15, 17 and 23, IFNγ, TNFα, TNFβ, TNF-RI and TNF-RII in plasma samples were examined.

2.4. Stool Sample Collection

Subjects were mailed a stool collection kit, a Second Genome (Second Genome, San Fransisco, CA, USA) stool collection vial, a pre-paid envelope to return the collection vial and a questionnaire pertaining to antibiotic use over the past 6 months. The Second Genome's stool collection vial is a self-collect, high-quality stool sample at home kit. Each collection vial was barcoded and added with nucleic acid stabilizing solution. This aids in the rapid homogenization and stabilization of DNA/RNA at the time of collection and protects high-quality samples during transport and long-term storage. Veterans were asked to collect their stool and then transfer a small amount of stool into the vial. This vial was then placed in a zipper pouch with absorbent material in case of spills. The zipper pouch was then placed into a plastic bubble wrap shipping envelope and mailed to investigators at Boston University School of Public Health. Once received the stool sample was stored at −20 °C and then shipped to collaborators at the University of South Carolina (USC) for analysis. Samples were stored at −20 °C at the USC facility until a significant number of samples were received from each of three study groups. Further, samples were shipped to Second Genome facility (Second Genome, South San Francisco, CA, USA) for further processing and analysis of gut microbiome.

2.5. Gut Microbiome Analysis

2.5.1. Sample Isolation

Second Genome performed nucleic acid isolation with the Qiagen MagAttract PowerMicrobiome(Qiagen, Germantown, MD, USA) DNA/RNA Kit according to the manufacturer's guidelines and optimized for high-throughput processing. All samples were quantified via the Qubit® Quant-iT dsDNA High Sensitivity Kit (Invitrogen, Life Technologies, Grand Island, NY, USA) to ensure that they met minimum concentration and mass of DNA.

2.5.2. Library Preparation

To enrich the sample for bacterial 16S V4 rDNA region, DNA was amplified utilizing fusion primers designed against the surrounding conserved regions which are tailed with sequences to incorporate Illumina (Illumina, Inc, San Diego, CA, USA) adapters and indexing barcodes. Each sample was PCR amplified with two differently barcoded V4 fusion primers and PCR products were quantified by fluorometric method (Qubit or PicoGreen from Invitrogen, Life Technologies, Grand Island, NY, USA). Samples that met the post-PCR quantification minimum were pooled equimolar and advanced for sequencing.

2.5.3. Profiling Method

A pool containing 16S V4 enriched, amplified, barcoded samples were loaded into a MiSeq® (Illumina, Inc, San Diego, CA, USA) reagent cartridge, and then onto the instrument along with the flow cell. After cluster formation on the MiSeq instrument, the amplicons were sequenced for 250 cycles

with custom primers designed for paired-end sequencing. QC and QA metrics are maintained for all sample handling, processing, and storage procedures.

2.5.4. Data Analysis

The microbiome sequence and data analysis were carried out at several separate stages: pre-processing, summarization, normalization, alpha diversity metrics (within-sample diversity), beta diversity metrics (sample-to-sample similarity), ordination/clustering, sample classification, and significance testing. For all analyses, $p < 0.05$ was considered statistically significant. Second Genome's analysis software package was used for statistical analysis of microbiome diversity. For descriptive purposes, one-way ANOVA tests and Chi-square were used as appropriate, to compare symptom groups. For these analyses, SAS 9.4 (Statistical Analysis Systems, SAS Institute, Cary, NC, USA) was used.

This study was approved by the Boston University Medical Campus Institutional Review Board and well as the USAMRDC: Human Research Protection Office (HRPO).

3. Results

3.1. Human Subjects

3.1.1. GWIC Full Study Cohort

Of the 230 GW veterans recruited into GWIC, 196 met Kansas criteria for GWI and 34 did not meet Kansas GWI criteria or Kansas exclusionary criteria and were considered healthy controls. Gastrointestinal symptoms are a prominently reported problem in veterans with GWI, however, they are not seen in all cases. Sixty-two percent of veterans with GWI reported abdominal pain or cramping, sixty-six percent reported diarrhea, sixty-two percent reported nausea or upset stomach and forty-eight percent reported being diagnosed with IBS. In comparison, in our control group 9% percent of veterans reported abdominal pain or cramping, 12% percent reported diarrhea, 18% reported nausea or upset stomach and 12% reported being diagnosed with IBS.

3.1.2. GWIC Microbiome Pilot Call Back Study Cohort

Of the 30 participants targeted, a total of 27 GWV agreed to participate to date in the GWIC pilot call back study and provided a stool sample for analysis. In Group 1 (GW controls) there were 7 subjects, in Group 2 (GWV with GWI and no GI symptoms; GWI-GI) there were a total of 5 subjects, Group 3 (GWV with GWI and GI symptoms; GWI+GI) there were 14 subjects. Of those, only a subset has had their stool samples analyzed to determine their gut microbiota. In Group 1, there were 5 subjects, Group 2 included 3 subjects and Group 3 included 5 subjects. There were no statistically significant differences seen between the groups on demographic characteristics, p-values ranged from 0.23–0.95 (Table 1). There were also no statistically significant differences seen between the groups on Body Mass Index ($p = 0.664$) or self-reported high sugar or diabetes ($p = 0.420$) (Table 1). We examined self-reported exposures in theater and found a statistically significant difference in the groups for self-reported exposure to chemical/biological weapons (Table 1). The majority (80%) of cases with GWI+GI experienced chemical weapons exposure, 66.7% of those with GWI–GI and none of the controls experienced chemical weapons exposure. There were no statistically significant differences among the groups with respect to exposure to PB anti-nerve gas pills, pesticide treated uniforms or being present in an area that had been fogged or sprayed with pesticides (Table 1). There were significant differences among the groups with regard to pain, fatigue and sleep problems with the GWI+GI group reporting far more symptoms on the McGill Pain score, Multi-dimensional Fatigue Inventory (MFI-20) and the Pittsburgh Sleep Quality Index (PSQI) than the GWI-GI or the control group (Table 1).

Table 1. Demographic and Exposure Characteristics of Participants.

Characteristic	GW Control $n = 5$	GWI-GI $n = 3$	GWI+GI $n = 5$	p-Value
Age, years [Mean (SD [1])]	52.8 (6.7)	63.2 (15.5)	53.3 (7.2)	0.296
Height, in [Mean (SD [1])]	69.8 (1.0)	67.5 (4.4)	66.8 (2.7)	0.238
Weight, lbs [Mean (SD [1])]	198.5 (18.3)	207.0 (24.3)	217.0 (99.7)	0.904
Gender [N (%)]				0.420
Male	5 (100)	2 (66.7)	4 (80)	
Female	0 (0)	1 (33.3)	1 (20)	
Race/Ethnicity [N (%)]				0.420
Black	0 (0)	0 (0)	1 (20)	
White	5 (100)	3 (100)	4 (80)	
Education [N (%)]				0.482
High School plus other training (technical/trade)	1 (20)	0 (0)	0 (0)	
Associates Degree or 2 years of College	0 (0)	0 (0)	2 (40)	
Some College	0 (0)	0 (0)	1 (20)	
Bachelor's Degree	2 (40)	1 (33.3)	0 (0)	
Advanced or Professional Degree	1 (20)	2 (66.7)	2 (40)	
Highest Grade [Mean (SD [1])]	15 (2)	15 (1)	16 (3)	0.957
McGill Pain Score [Mean (SD)]	12 (8)	32 (7)	39 (16)	0.021
Multi-dimensional Fatigue Inventory (MFI-20) [Mean (SD)]	40 (8)	53 (7)	72 (10)	0.0005
Pittsburgh Sleep Quality Index (PSQI) [Mean (SD)]	8 (3)	7 (2)	14 (2)	0.003
Body Mass Index (BMI) [Mean (SD)]	28.6 (2.5)	31.9 (0.7)	34.0 (14.2)	0.664
Self-reported high sugar or diabetes [N (%)] No Yes	5 (100.00)0 (0.0)	2 (66.7)1 (33.3)	4 (80.0)1 (20.0)	0.420
Exposed to chemical or biological warfare agents during military service [N (%)]				0.049
No	2 (40.0)	1 (33.3)	0 (0.0)	
Yes	0 (0.0)	0 (0.0)	4 (80.0)	
Not Sure	3 (60.0)	2 (66.7)	1 (20.0)	
Taken pyridostigmine bromide (anti-nerve agent) pills [N (%)]				0.420
No	1 (20.0)	1 (33.3)	0 (0.0)	
Yes	4 (80.0)	2 (66.7)	5 (100.0)	
Wore a uniform treated with pesticides [N (%)]				0.120
No	5 (100.0)	2 (66.7)	2 (40.0)	
Yes	0 (0.0)	1 (33.3)	3 (60.0)	
Saw the area in which you lived fogged or sprayed with pesticides [N (%)]				0.228
No	3 (60.0)	3 (100.0)	2 (40.0)	
Yes	2 (40.0)	0 (0.0)	1 (20.0)	
Not Sure	0 (0.0)	0 (0.0)	2 (40.0)	

[1] SD standard deviation.

3.2. Cytokine Results

To determine if circulating proinflammatory cytokines levels were different across the three pilot study groups, plasma samples were collected and examined. Using chemiluminscent imaging concentrations of IL-1a, 1b, 2, 4, 5, 6, 8, 10, 12 (p 70), 13, 15, 17 and 23, IFNγ, TNFα, TNFβ, TNF-RI and TNF-RII in plasma samples were examined. No statistically significant differences were found among the groups for the following plasma cytokine concentrations: IL-1a, 1b, 2, 4, 5, 6, 8, 10, 12 (p 70), 13, 15, 17 and 23, IFNγ, TNFα, TNFβ, and TNF-RII (p-values ranging from 0.06–0.69). However, we found a statistically significant difference among the groups in circulating levels of TNF RI which plays a

role in regulating inflammation ($p = 0.04$), such that GW controls had lower levels (452.6 ± 183.1) than GWI-GI (819.2 ± 250.3) and GWI+GI groups (852.7 ± 244.0) (Table 2).

Table 2. TNF-RI and RII cytokine comparisons by GI symptom groups.

	GW Controls		GWI–GI		GWI+GI	
Cytokine	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
TNF-RI *	452.6	183.1	819.2	250.3	852.7	244.0
TNF-RII	721.2	79.6	876.3	62.7	541.6	122.3

* groups significantly different at $p < 0.05$.

3.3. Gut Microbiome Results

3.3.1. Sample Diversity

To assess differences in the number of different species across the three groups, species richness was calculated using OTU richness (number of operational taxonomic units). Species richness was significantly lower in the GW controls (Mean = 415 ± 83.1) than in the GWI-GI (Mean = 576 ± 12.9) ($p = 0.03$), and GWI+GI (Mean = 501 ± 56.4). Shannon index was used to determine the evenness or balance of distribution of microbiome species across groups. GW controls had lower Shannon diversity scores (3.79 ± 0.226) than GWI-GI (4.03 ± 0.147) and GWI+GI groups (3.94 ± 0.163) (Table 3).

Table 3. Sample Diversity Results by Groups.

	GW Controls		GWI–GI		GWI+GI	
Family	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
OTU Richness	415	83.1	576	12.9	501	56.4
Shannon Index	3.79	0.23	4.03	0.15	3.94	0.16

3.3.2. Phyla Distribution

Firmicutes was the most abundant phylum across all groups (Table 4, Figure 1). A significant increase was observed in the relative abundance of Firmicutes in the GW controls (80.4 ± 4.91) and GWV with GWI-GI symptoms (79.3 ± 5.19; $p = 0.03$) compared to GWV with GWI+GI symptoms (69.4 ± 4.72; $p = 0.01$). We also observed higher abundance of Bacteroidetes, Actinobacteria, and Euryarchaeota in GWI+GI (13.7 ± 6.33; 10.9 ± 5.49; and 2.09 ± 3.22) than in GW controls (9.08 ± 3.84; 9.47 ± 7.67; and 0.17 ± 0.38) or GWI- GI (8.06 ± 5.77; 7.76 ±4.21; and 0.748 ± 0.391).

Table 4. Percent relative abundance at the Phyla Level by Groups.

	GW Controls		GWI–GI		GWI+GI	
Phylum	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Firmicutes	80.40	4.91	79.30	5.19	69.40	4.72
Bacteroidetes	9.08	3.84	8.06	5.77	13.70	6.33
Actinobacteria	9.47	7.67	7.76	4.21	10.90	5.49
Verrucomicrobia	0.05	0.09	3.35	3.26	2.86	2.06
Euryarchaeota	0.17	0.38	0.49	0.63	2.09	3.22
Proteobacteria	0.68	0.61	0.75	0.39	0.91	0.72
unclassified	0.08	0.02	0.10	0.02	0.09	0.02
Tenericutes	0.00	0.00	0.13	0.22	0.02	0.06
Others	0.06	0.10	0.04	0.03	0.02	0.02

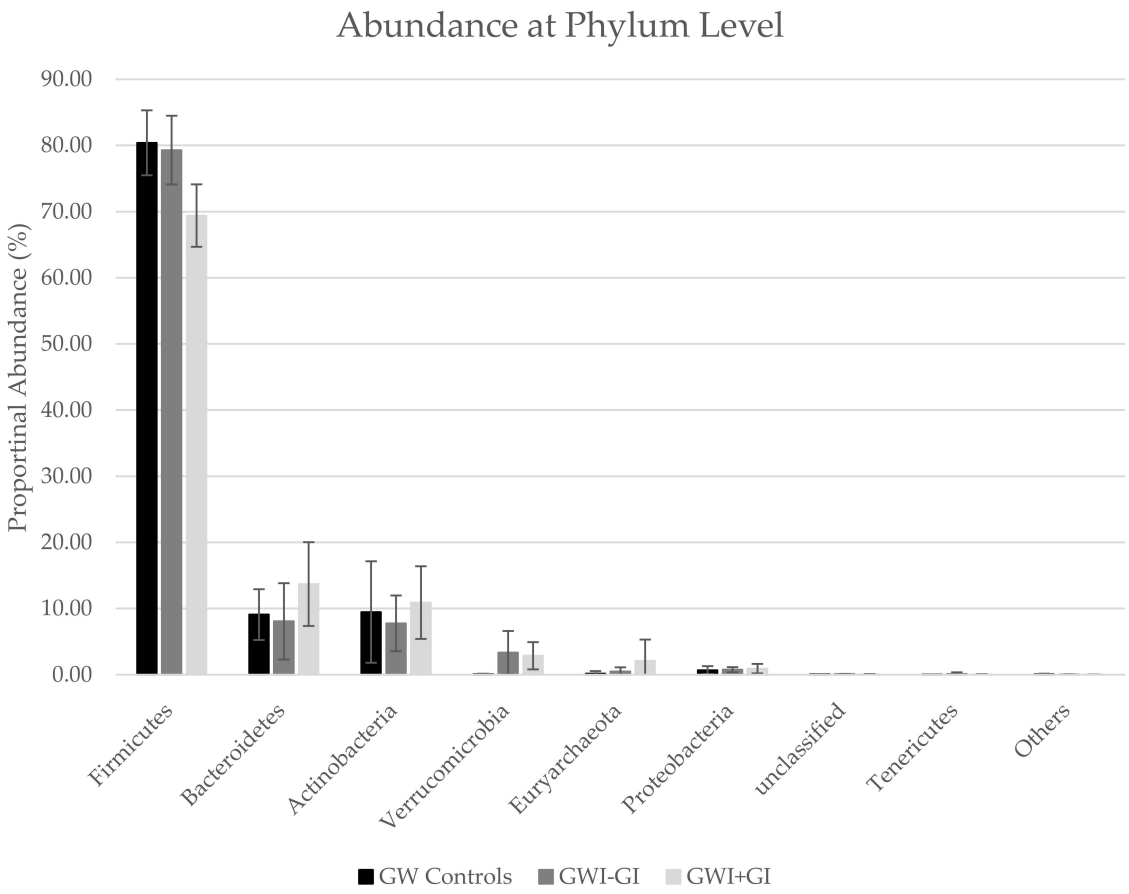

Figure 1. Proportional Abundance at the Phylum and Family Level by Group.

3.3.3. Family Distribution

At the family level, Lachnispirache was the most abundant family in the GW controls but only the second most abundant in GWI-GI and GWI+GI, where Ruminococcaceae was the most abundant (Table 5, Figure 2). A significant increase was observed in the relative abundance of Lachnispiracae in GW controls (48.1 ± 10.6) compared to GWI-GI (24.6 ± 1.79; $p = 0.03$) and GWI+GI (23.5 ± 10.3; $p = 0.03$). GWI+GI had statistically significantly higher abundance of Ruminococcaceae (27.4 ± 5.4) than GW controls (14.2 ± 4.57) and approximately the same abundance as GWI-GI (27.7 ± 12). Bacteriodaceae and Bifidobacteriaceae were also more abundant in GWI+GI (9.39 ± 6.18 and 4.01 ± 2.04) than GW controls (6.9 ± 4.06 and 3.36 ± 2.76).

Table 5. Percent relative abundance at the Family Level by Groups.

	GW Controls		GWI–GI		GWI+GI	
Family	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Lachnospiraceae	48.10	10.60	24.60	1.79	23.50	10.30
Ruminococcaceae	14.20	4.57	27.70	12.00	27.40	5.40
Erysipelotrichaceae	6.24	4.24	6.41	2.42	6.63	4.39
Streptococcaceae	1.37	1.67	7.16	11.50	3.17	4.87
Bacteroidaceae	6.90	4.06	5.78	5.57	9.39	6.18
Coriobacteriaceae	6.03	5.73	6.63	4.72	6.75	4.96
Bifidobacteriaceae	3.36	2.76	0.95	0.83	4.01	3.74
Verrucomicrobiaceae	0.05	0.09	3.35	3.26	2.83	2.04
Other	13.70	2.52	17.40	5.78	16.40	6.17

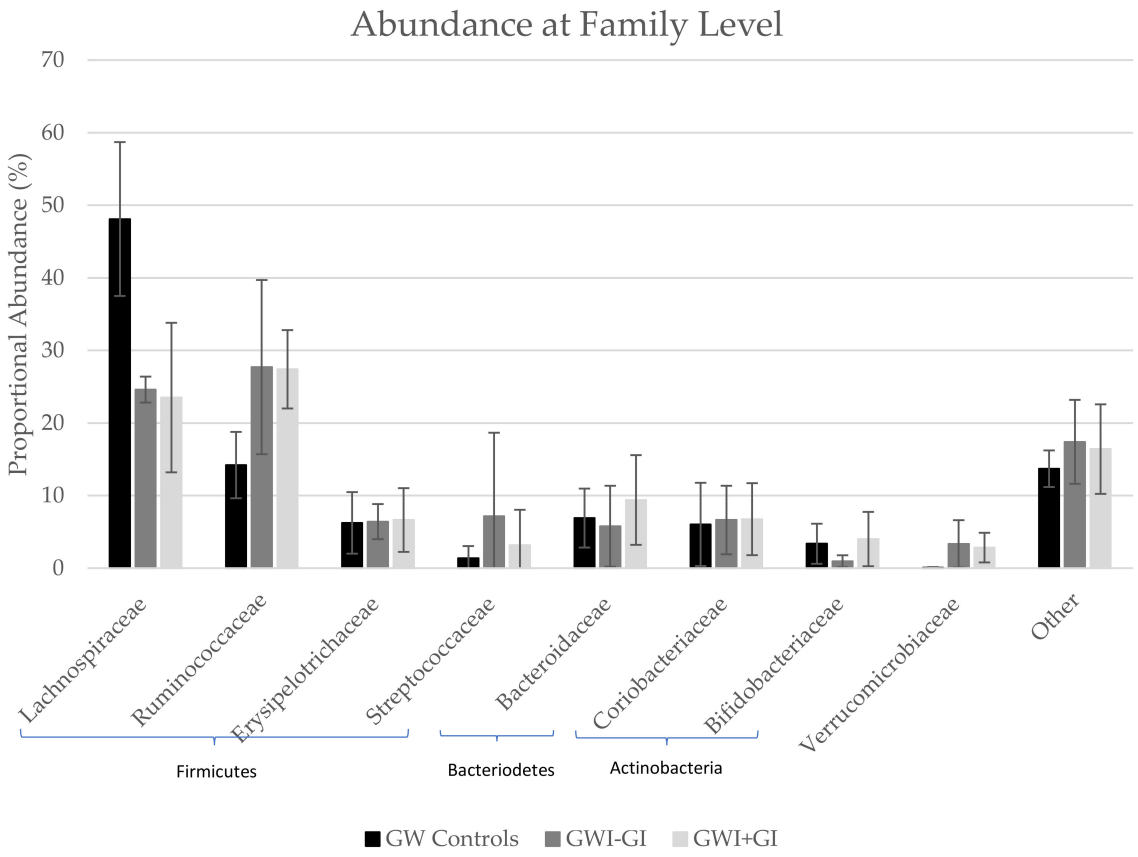

Figure 2. Proportional Abundance at the Phylum and Family Level by Group.

3.3.4. Genus Level

A number of genus were more abundant in GW controls than GWI+GI and GWI-GI. Dialister was statistically significantly enriched in GW controls compared to GWI-GI ($p = 0.008$). Roseburia was statistically significantly enriched in GW controls compared to GWI+GI ($p = 0.001$). Ruminococcus was statistically significantly more abundant in GWI-GI and GWI+GI compared to GW controls ($p = 0.038$ and $p = 0.007$). Dialister was statistically significantly less abundant in GWI-GI compared to GW controls and GWI+GI ($p = 0.008$ and $p = 0.003$).

4. Discussion

The findings from this small pilot study suggest that the gut microbiome is significantly different in veterans with GWI+/-GI and GW veteran controls. There also appears to be a significant difference between the GWI+GI group and GWI-GI group with regard to microbiome diversity. Specifically, we found that GW controls had a greater abundance of firmicutes, including the family lachnospiraceae and the genus Dialister and Roseburia compared to GWI+GI and GWI-GI. In addition, compared to GW controls and veterans with GWI+GI, veterans with GWI-GI had a higher abundance of verrucomicrobia, and verrucomicrobiaceae as well as higher abundances of the families ruminococcaceae and streptococcaceae. The GWI+GI group had a greater abundance of the phyla bacteroidetes, actinobacteria, euryarchaeota, and proteobacteria as well as higher abundances of the families Bacteroidaceae, Erysipelotrichaceae, and Bifidobacteriaceae when compared to the groups of GW control veterans and GWI-GI veterans.

A study by Rizzatti et al. [34] found an association between proteobacteria and gut inflammation and Inflammatory Bowel Disease (IBD) such as Crohn's disease and ulcerative colitis. In our study, we found a higher abundance of proteobacteria in our GWI+GI and GWI-GI compared to our GW controls. In this pilot study, we found a higher abundance of genus Roseburia in the GW controls

compared to those with GWI, both with and without GI symptoms. Researchers have found that individuals with IBS have less abundance of Roseburia when compared to controls [35]. Roseburia has been shown to help digest complex carbohydrates [36] and also aid in the production of butyrate ([37]. Butyrate (butyric acid) is a short-chain fatty acid and its beneficial effect has been recently discussed in a preclinical study of colitis and IBD [38]. In our recent investigation, sodium butyrate priming improved gut microbial dysbiosis, intestinal epithelial membrane integrity, and intestinal inflammation in a PB and permethrin exposed mouse model of GWI [26]. A separate PB-exposed animal model of GWI by another research group also recently reported intestinal glial inflammation and chronic brain glial activation [17]. Although PB pill usage and permethrin treated uniforms were reported more commonly in GWI+GI group, only self-reported exposure to chemical warfare agents (sarin) was significantly different among the groups with over three-quarters of the GWI+GI group reporting this exposure during deployment and none of the GWI-GI or controls. This suggests an additional exposure target for preclinical etiological and treatment studies of GWI+GI symptoms.

In this pilot study, a decrease in the abundance of Roseburia in GWI+GI and GWI-GI, might be a novel cause of gastrointestinal inflammation in GWI. Correspondingly, our study also found significantly increased circulating levels of the proinflammatory cytokine TNF-RI in plasma from our GWI+GI group. The levels of TNF-RI were nearly double that of controls in the GWI+GI and GWI-GI groups suggesting significantly more inflammatory signaling in the GWI groups. These results correspond with recent therapeutic targets for IBD which focus on anti-TNF therapies to reduce gut inflammation associated with the disorder including those targeted on TNF-RI specifically [39].

Additional important correlations with findings in the literature and our findings include a less abundance of Dialister and the association with depression [40] as well as arthritis [41] and the positive association between Ruminococcus and IBS [42] and lupus [43]. In our pilot study, we found that the GWI+GI group reported much higher symptom burden for pain (McGill Pain Scale), fatigue (MFI-20) and sleep (PSQI) on validated measures compared with both the GWI-GI and control groups. These finding also correspond with prior studies reporting lower pain thresholds in GW veterans with GI in other study cohorts suggesting that a targeted approach of treating inflammatory mediators in GWI+GI could potentially reduce other chronic health symptoms [22–24].

As with all studies, this study had both strengths and weaknesses. To our knowledge, this is the first study conducted in a population of Gulf War veterans aimed at examining their gut microbiome and innate immune markers. As a follow-on study to the larger Gulf War Illness Consortium, we were able to recruit a number of veterans in a short amount of time who agreed to provide us with stool samples. In addition, we were able to separate our veterans into three clearly distinct groups based on GWI and GI symptoms. A major limitation of this study is the small sample size of a pilot study resulting in low statistical power to detect differences among the groups. Additionally, we were limited in how far down the taxonomic level we were able to analyze using the current methods employed. We also may not have had the power in this small sample to detect smaller cytokine effects among the groups (IL1, TNF-alpha, IL8) that have also been previously associated with IBD. Correspondingly, due to small sample size it was not possible to assess the impact of multiple exposures (i.e., PB and chemical weapons or depleted uranium) on gut microbiome and inflammatory markers. In addition, statistical analysis was limited in this pilot study with multiple testing and no post-hoc correction which could result in Type I errors. Some veterans with GWI have been reported to experience increased infections and could have increased exposure to antibiotics which could alter their gut microbiome. Participants in our study did not use antibiotics during the two weeks prior to stool collection but future studies should get a detailed history of antibiotic use in the veterans for a longer period of time.

Recent studies on human gut microbiome emphasize its association with a variety of diseases including obesity, diabetes type 2 (metabolic disorder), inflammatory bowel disease, liver disease, cancer, and neurological disorders [44–47]. The findings from this pilot study and our recent mouse model of GWI showed both similarities and discrepancy in the microbiome composition [16]. Interestingly, in both

studies' Firmicutes and Bacteroidetes remain two major phyla of the gut microbiota. Firmicutes was decreased and Bacteroidetes was increased in human microbiota of the GWI+GI group, however, Firmicutes was increased and Bacteroidetes was decreased in mouse microbiota of GWI. A closer look at both microbiome compositions suggest that the family Ruminococcaceae has been significantly increased in both the human and mouse microbiome. Other research by Ley et al. [48] suggest that though the two dominating phyla are Firmicutes and Bacteroidetes, at lower taxonomic classification the microbiome composition differ by 85% in healthy human and mouse [49]. The variation between human and mouse microbial composition may be due to the limitations in 1) diet control (mouse model has a similar diet composition; however, human diet composition varies), sample collection (in the mouse model mainly luminol or fecal content are collected, however, in the human fecal matter are collected) and analysis methods (mouse microbiome are limited to 16s rDNA sequencing, however, human microbiome analyzed by 16s rDNA or metagenomics). As designed, our GWIC study infrastructure provided the opportunity for the initial translation of our GWI animal microbiome study results to clinical relevance in this pilot study that will lead the way for further larger, more definitive studies.

Future directions of this work should include studies that build upon these initial provocative findings and increase the sample size to further validate these initial findings. In addition to increasing the overall sample size, it would be informative to add an additional IBS or IBD non-GWV control group and additional mixed exposure GWI groups. Additional work should be done to examine the peripheral markers of inflammation to examine the link between the altered gut microbiome and blood markers of inflammation, including additional TLR4 circulating markers such as HMGB1 recently found to be associated with animal models of GWI [12]. The gut-microbiome-brain axis could also be examined further by studying the association between gut microbiome changes, neuroinflammatory signaling and cognitive and neuroimaging findings in GW veterans. With larger sample sizes, it will also be possible to assess the contribution of combination exposures (PB, pesticides, chemical weapons and depleted uranium). Finally, it is possible to alter the gut microbiome through treatments, both prescription and over-the-counter, and the next direction for treatment possibilities for ailing GW veterans could be to examine the efficacy and safety of treatment approaches that target the gut microbiome as a novel treatment approach for the varied symptoms of GWI.

5. Conclusions

The findings from this small pilot study suggest that the gut microbiome is significantly different in veterans who have GWI and those who do not have GWI. There also appears to be a significant difference between the GW veterans with GWI and GI disturbance and the GW veterans with GWI but no GI disturbance with regard to microbiome diversity. The GWI+GI group also showed greater plasma levels of the inflammatory cytokine TNF-RI and they endorsed significantly more chemical weapons exposure during the war and reported significantly greater chronic pain, fatigue and sleep difficulties than the other groups. Studies with larger samples sizes are needed to confirm these initial findings.

Author Contributions: Each author listed made substantial edits in different areas that contributed to this research project and manuscript preparation. The following lists the areas in which each author contributed; Conceptualization, P.A.J., K.S. and S.C.; Methodology, P.A.J., S.C., R.K.S., K.S., and N.K.; Formal analysis, E.Q., T.H.; Investigation, P.A.J., R.K.S., J.M.C., J.A., E.Q., T.H., R.D.H., S.M.L., N.K., K.S. and S.C.; Resources, K.S., N.K., P.A.J. and S.C.; Data curation, P.A.J., J.A., K.S., J.M.C., R.K.S. and S.C.; Writing—Original draft preparation, P.A.J., J.C. and R.K.S.; Writing—Review and editing, P.A.J., R.K.S., J.M.C., E.Q., R.D.H., K.S. and S.C.; Visualization, P.A.J., S.R., J.M.C.; Supervision, P.A.J.; Project Administration, P.A.J., R.K.S., J.A., K.S.; Funding Acquisition, P.A.J., K.K.S., S.C.

Funding: This research was funded by the Department of Defense, grant numbers GW120037 and GW170068.

Acknowledgments: We would like to thank all of the GW veterans who participated in this pilot study.

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

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