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14. ABSTRACT 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 include Boston, Miami, Bronx and San Francisco. The BBRAIN structure provides centralized cataloguing and coordination of retrospective biorepository samples from 10 collaborating institutions who are sharing 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 are included in the BBRAIN network datasets to allow for the comparison of biomarkers with behavioral outcomes. To date, data usage and material transfer agreements have been put in place with all sites and sharing has begun with retrospective data and outcomes.					
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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The primary objective of the Boston Biorepository and Integrative Network for Gulf War Illness (BBRAIN) is to establish a retrospective and prospective biorepository network for Gulf War Illness (GWI) research by data mining from existing BBRAIN collaborator specimens and by recruiting 500 additional participants' repository samples. The four prospective recruitment resource sites include Boston University, Miami VA, Bronx VA and San Francisco VA. The BBRAIN structure currently provides 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 from participants previously consented to share their samples for future research. 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.

2. **KEYWORDS:**

Gulf War Illness, Biorepository Network, GW veterans, biomarkers

3. **ACCOMPLISHMENTS:**

Major goals of the project.

The approved statement of work lists the major goals of the project and the timeline for achieving each goal:

STATEMENT OF WORK

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

Tasks	Timeline
Major Task 1: Obtain IRB/HRPO approval	Months
1a. Obtain necessary IRB approvals or Exempt status	1-3
1b. Obtain DOD Human Research Protections Office (HRPO) approvals or Exempt Status for use of de-identified samples	4-6
Milestone(s) Achieved: Regulatory reviews completed, final approvals obtained	6
Major Task 2: Obtain Biorepository Data for Analysis and Catalogue Available Biospecimens for sharing at Repository Resource Sites	Months
2a. Obtain MRI, MRS, DTI, PET imaging data from retrospective resource (RR) sites and send to BUMC for cataloguing, post-processing and machine learning analyses	6-12
2b. Obtain demographic, health symptom and cognitive outcome data from RR Sites and send to BUMC for cataloguing, post-processing and analysis	6-12
2c. Catalogue and track available tissue, blood, saliva and CSF samples from RR sites	6-12
Milestone(s) Achieved: Biorepository data mining/cataloguing complete for 10 sites	12
Major Task 3: Perform Post-Processing of Imaging Data and Machine Learning Analysis with Combined Common Data Elements	Months
3a: Perform Brain image post-processing and cataloguing of images for repository.	1-24

3b: Perform data analysis of brain imaging and machine-learning analyses including cognitive data and health symptom data in cases and controls.	1-24
Milestone(s) Achieved: Brain imaging data mining analysis studies complete and imaging, demographic, cognitive outcomes repository created.	24
Major Task 4: Preparation and Training for Clinical Study Procedures	Months
4a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire repository network	1-6
4b. Develop manuals for the neuropsychological testing protocol, specimen collection protocols and recruitment screening procedures	1-9
4c. Train researchers and staff on protocols and quality control measures for the clinical study and repository collection	1-9
Milestone(s) Achieved: Staff trained and all databases and websites ready for subject recruitment and specimen tracking.	9
Major Task 5: Screening, Recruit and assessment of GW Veterans From Four Resource Sites	Months
5a: Obtain informed consent from potentially eligible GW veterans	9-36

5b: Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, Fitbit tracker, blood draw and saliva samples from 500 study participants.	9-36
5c. Obtain participants urine and stool sample kits back by mail to BUSPH for biorepository storage.	9-36
5d. Upload Fitbit sleep, heart rate variability and blood pressure data to BUSPH DCC to merge with other datasets.	9-36
5e. Score neuropsychological tests and upload summary data to DCC for entry, cleaning and analyses.	9-36
5f. Send blood and saliva samples to Nova University for analysis of cytokine and chemokine panels and cortisol measurements and biorepository banking.	9-36
Milestone(s) Achieved: Obtained blood, saliva, urine, stool, autonomic, demographic and cognitive data from 500 participants for biorepository and analysis.	36
Major Task 6: Process, Store and Manage samples for a total of 500 new participants.	Months
6a. Process and store blood, saliva, urine and stool samples received from 4 recruitment sites at two biorepository sites	6-33
6b. Perform local laboratory analyses (CBC) for 500 participants	6-33
6c. Extract DNA and RNA from 500 saliva samples and store in repository	6-33
6d. Run batched assays for cortisol and cytokine results (18 plex cytokine assay)	6-33
6e. Provide Sample request forms and track information regarding the use of samples	6-36+
6f. Ship samples to requesting investigators	6-36+
Milestone(s) Achieved: Processed all newly collected samples into biorepository storage, ran cytokine and cortisol measurements, extracted DNA/RNA for repository.	36
Major Task 7: Merge Data and Perform interim Data Analyses	Months
7a. Merge Clinical datasets data from prior studies for brain imaging, cognitive, demographic and health symptom report data	6-18
7b. Perform machine learning and/or meta-analytic techniques on common data elements from prior study epidemiological, cognitive and brain imaging data	6-18

7c. Discussion of results and preparation of abstracts for meeting presentations and initial manuscript for publication	18-24
7d. Annual reports of progress will be written	12-24
Milestone(s) Achieved: Preliminary analysis of results and presentation of initial results at scientific meetings and potential publication. Possible diagnostic markers from machine learning and meta-analyses.	6-24
Major Task 8. Perform Final Analysis, Prepare Manuscripts for Preparation and Finalize Biorepository for Sharing of Samples	Months
8a. Merge clinical datasets for prospective and retrospective data sets for GWI cases and GW controls and other symptomatic controls (fibromyalgia, IBS, CFS)	24-30
8b. Perform data analysis comparing cytokine, cortisol, cognitive outcomes, Fitbit and self-reported health symptom outcomes within GWI cases and controls from prospective data collection	25-26
8c. Coordinate interim and final collaborative publications and/or meta-analyses and Final Study Report	24-36
8d. Finalize websites for BBRAIN biorepository with data dictionaries and a searchable indexed site	24-36
Milestone(s) Achieved: Final analyses performed, publications prepared and biorepository dataset and indexed repository website prepared	24-36

The statement of work for year 4 is inclusive of Tasks 1-7 above. The statement of work for year 4 primarily describes the completion of the start-up phase of the biorepository network including obtaining local and funder institutional review approvals for clinical studies as well as establishing protocols for sharing samples with retrospective clinical sites and finalizing clinical protocols for neuropsychological assessments, blood, saliva and Fitbit sleep and other metrics. In addition, in year 4, the plan was to recruit 400 study participants for the study protocol including cognitive evaluations and specimen collection. Progress toward completing each task is listed below.

Summary of Clinical Assessments and Tests Collected from 4 Clinical Study Sites

	Year 1				Year 2				Year 3			
Target Enrollment (per quarter)	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4
Boston University			20	20	30	30	30	30	20	10	10	
San Francisco VAMC			10	10	15	15	15	15	10	5	5	
Bronx VAMC			10	10	15	15	15	15	10	5	5	
Miami VAMC			10	10	15	15	15	15	10	5	5	
Target Enrollment (cumulative)			50	100	175	250	325	400	450	475	500	

Accomplishment under these goals.

TASK 1. OBTAIN IRB & HRPO APPROVAL (MONTHS 1-6)

Task 1a. Obtain necessary IRB approvals or Exempt status

Institutional Review Board (IRB) approvals for the prospective sites have been obtained at Boston University, Miami VA, NOVA Southeastern University, Bronx VA, and the San Francisco VA/UCSF.

The DUA and MTA documents have been finalized and approved between BU and SF VAMC, NOVA Southeastern University, Bronx VA, Mass General Hospital, Georgetown University, Beth Israel Deaconess Medical Center, Roskamp Institute and University of Alabama.

All retrospective sites/samples have received IRB approvals (this includes, BUSPH, SF VAMC, NOVA Southeastern University, Drexel University, Beth Israel/Deaconess/Harvard, Mass General Hospital, Georgetown University, and the University of Alabama).

Task 1b. Obtain final DOD Human subjects Research Protections Office (HRPO) approvals or Exempt Status for Use of de-identified samples.

All participating sites (BUSPH, SF VAMC, NOVA Southeastern University, Miami VA, and the Bronx VA) have received HRPO approvals. A no-cost extension for the next year was approved for all study site with the exception of the Bronx VA where Dr. Golier has retired from research at that VA.

TASK 2. OBTAIN BIOREPOSITORY DATA FOR ANALYSIS AND CATALOG AVAILABLE BIOSPECIMENS FOR SHARING AT REPOSITORY RESOURCE SITES (MONTHS 6-12)

The consortium coordinating center and Administrative Core at Boston University has led many monthly web and in-person meetings to maintain engagement from investigators in the consortium and update the group on the prospective and retrospective BBRAIN studies. During each of these monthly meetings, an investigator of the BBRAIN consortium was invited to speak in depth about their research topic. For example, Dr. Bang-Bon Koo spoke to the group about his MRI machine learning tool and Dr. Ian Robey discussed the status of the VA Brain Bank. These meetings were also used to update the larger consortium on any new publications and prospective study recruitment updates. Time was allotted for initiating collaboration and conversation of novel scientific questions. Table 3 lists these planning meetings. In addition, smaller working groups met throughout the year to discuss further planning and table 4 lists the working groups. Several planning meetings were held with the Data Management Service Group to develop the data sharing web software for potential collaborating investigators to request for samples and also obtain additional information on the study. Variable names of specimens from retrospective resource (RR) sites were obtained and a common variable names and data dictionary for catalogued samples have been created. We now have obtained and cataloged samples to be shared with the consortium for analysis from the 10 RR sites. Table 5 details the status of RR data sharing and processing. To date, fifteen requests have been reviewed and approved for sharing of samples through our online request forms. The details of these approved requests are outlined in table 6.

Table 2. BBRAIN Monthly Planning and EAB Meetings

Date	Type of Meeting	Discussion Items
10-27-2021	Monthly Web-meeting	Prospective and Retrospective site updates
11-17-2021	Monthly Web-meeting	Prospective and Retrospective site updates
02-23-2022	Monthly Web-meeting	Prospective and Retrospective site updates
03-30-2022	Monthly Web-meeting	Prospective and Retrospective site updates
04-27-2022	Monthly Web-meeting	Prospective and Retrospective site updates
06-29-2022	Monthly Web-meeting	Prospective and Retrospective site updates
07-27-2022	Monthly Web-meeting	Prospective and Retrospective site updates
08-31-2022	Monthly Web-meeting	Prospective and Retrospective site updates
09-28-22	Monthly Web-meeting	Prospective and Retrospective site updates

Table 3. BBRAIN Network Working Groups

Working Group	Tasks	Members
Data Management Service Group	Assist with QC issues, data cleaning, data management and sharing, website management.	Emily Sisson, 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, Emily Sisson, 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 results can inform each other.	Michael Pratt – BU Tech Transfer office Consortium PI, co-PIs Research site PIs
Behavioral Studies Working Group	Plan imaging protocols and provide quality control for multiple imaging sites. Plan behavioral testing protocols and coordinate clinical studies for comparability.	Drs. Sullivan, Killiany, Koo Krengel, Toomey, Golier, Chao, Klimas
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. Klimas, Sullivan
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

Table 4. Retrospective Resource Site Data Sharing Status

Retrospective Resource Site	Shared Data	Shared (Y/N)	Processed (Y/N)
<i>Boston University</i>	<u>GWIC samples</u> -Serum, plasma, cytokines, genetic SNP panels, saliva, cortisol -Health data, symptoms, exposure data; medical conditions -Cognitive assessments, brain imaging, Cerebral Spinal Fluid	Y	Y
<i>Harvard University: Beth Israel Deconess Medical Center</i>	<u>GWI</u> -blood samples and demographic data	Y	Y
<i>VA Boston</i>	<u>VA Biorepository Brain Bank (VABBB)</u> -Brain tissue database	Y	Maintained by VA: https://www.research.va.gov/programs/tissue_banking/als/
<i>University of Alabama</i>	<u>GWI, Healthy controls, Chronic Fatigue syndrome, Fibromyalgia cohorts</u> -plasma, serum -brain imaging scans	Y	Y
<i>Georgetown University</i>	<u>GWI, CFS, healthy control</u> -blood and brain imaging	Y	Y
<i>Harvard University: Mass General Hospital</i>	<u>GWI</u> -PET scans, blood, demographic and cognitive data	Y	Y
<i>San Francisco VA</i>	<u>GWI and healthy controls</u> -Brain imaging -saliva samples, demographic, exposure assessment and survey	Y	Y
<i>Drexel University</i>	<u>Stem Cell Biorepository</u> -hiPSCs stem cells (GWI/Controls)	Y	Y
<i>Roskamp Institute</i>	-Omics (proteomics, lipidomics, and metabolomics)	Y	Y
<i>CDC/NIOSH</i>	-mouse brain samples from GWI studies	Y	Y

Task 2a. Obtain MRI, MRS, DTI, PET Imaging data from retrospective resource (RR) sites and send to BUMC for cataloguing, post processing and machine learning analyses

Imaging data obtained from retrospective sites including the San Francisco VA and Mass General Hospital were sent to Drs. Killiany and Koo at Boston University for cataloguing, post-processing and analysis. The MRI scans were transferred electronically in either extended DICOM or par/rec format to the Center for Biomedical Imaging at Boston University School of Medicine.

The San Francisco VA site has contributed the following de-identified neuroimaging data types to the BBRAIN Imaging repository from prior studies on GW veterans: 1.5T data (T1, T2, and proton density scans) from 257 GW veterans, 4T data (T1, T2, and FLAIR scans) from 207 GW veterans, and 3T data (T1, T2 and FLAIR scans) from 230 GW veterans. These images will be categorized and stored for query for researchers interested in

using them for analyses.

Data from the Harvard University/Mass General Hospital PET and diffusion tensor imaging studies were also shared from 40 scans with Dr. Koo at Boston University as part of the planned BBRAIN imaging studies. These machine learning and multi-modal analyses are currently being conducted. Imaging data from Georgetown University and University of Alabama was also shared. Georgetown University shared T1, resting state fMRI, diffusion (DTI), and pCASL 3T data from 151 GW veterans. The University of Alabama shared T1 and ASL 3T data from 92 GW veterans. This data was added to the imaging post-processing pipeline and machine learning analyses.

Additional hard drives have been purchased to store the vast amount of imaging data that will be shared. As the images were obtained, each scan underwent quality checking that consisted 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 failed the quality check were rejected by the study and remediation discussed with the appropriate retrospective site investigator. Scans that passed the quality check were entered the post-processing pipeline for data mining and sharing.

Task 2b. Obtain demographic, health symptom and cognitive outcome data from RR sites and send to BUMC for cataloguing, post-processing and analysis

HRPO approvals and DUAs have now been obtained from all retrospective study sites and the demographic, health symptom and cognitive outcome data have now been obtained from all RR sites. Data variables names, REDCap shells, and data dictionary were obtained from retrospective sites to create common variable names for specimens that were cataloged for sharing.

To date, GWIC demographic, health symptom and cognitive data from 263 study participants has been added to the BBRAIN repository. In addition, the San Francisco VA has also shared demographic information, health symptom and cognitive outcomes from 224 GW veterans and the Roskamp Institute shared demographic information, health symptom and cognitive outcomes from 63 GW veterans.

Additional demographic, case status and health symptom data has been obtained from Georgetown University of 55 Gulf War veterans with GWI and 12 GW veterans with CFS. Similar data has been obtained from the University of Alabama on 105 GW vets without GWI, 26 GW vets with CFS, 15 GW vets with TBI and 18 GW vets with RA.

Data from RR sites have been combined with prospective BBRAIN data and analyzed. These results have been presented at conferences and monthly meetings. Ongoing analysis for cognitive outcomes and health symptom outcomes are being prepared for publication.

Task 2c. Catalogue and track available tissue, blood, saliva and CSF samples from RR sites

We have obtained and catalogued specimen data information from all retrospective resource sites (RRS) to add to the list of available samples for sharing. This has included human induced pluripotent stem cell (hiPSC) lines, serum samples, plasma samples, DNA and RNA samples and cerebrospinal fluid.

Specifically for the BBRAIN repository of hiPSC cells, we have:

- Expanded capabilities to provide partially differentiated neurospheres
- Expanded technical know how into organoid technology

TASK 3. PERFORM POST-PROCESSING OF IMAGING DATA AND MACHINE LEARNING ANALYSIS WITH COMBINED COMMON DATA ELEMENTS (MONTHS 1 - 24)

Task 3a. Perform brain image post-processing and cataloguing of images for repository

Images shared from all sites have been catalogued and inventoried. Post-processing and harmonization of images across sites has begun. Once data harmonization is complete, analysis with combined data elements will be conducted.

Task 3b. Perform data analysis of brain imaging and machine-learning analyses including cognitive data and health symptom data in cases and controls.

Machine learning multi-modal approach to brain connectomics analysis will be conducted once brain scans are post-processed and added to the machine learning data analysis computational pipeline. This work will be used to validate Dr. Koo et al.'s recent paper using GWIC data analyses (Cheng et al., 2020).

TASK 4. PREPARATION AND TRAINING FOR CLINICAL STUDY PROCEDURE (MONTHS 1 - 9)

This task has been completed. We are well underway in subject recruitment and enrollment. Additional training of the study procedure is conducted when needed for new study personnel. As previously mentioned, study coordinators meet monthly to discuss any outstanding questions and ensure consistency in study methods across sites.

Task 4a. BUSPH Data Coordinating Center (DCC) will create website, data collection forms, specimen tracking system and databases for the entire repository network

The Data Coordinating Center (DCC) now renamed 'BEDAC' at BUSPH has developed a website to assist in the management and conduct of the repository that contains the following components: a data entry application, participant and data tracking applications, reports for investigators to monitor status and research staff to manage day to day operations; public-facing web application for repository information, data requests, and data access. The website now contains publicly available informational pages, including descriptions of RS, projects, investigator profiles, and publications with links to PubMed; as well as informational areas to be used for dissemination of project results. This information is updated regularly to accurately inform veterans and other investigators. All project-specific applications (e.g., tracking, data entry and upload) are accessible via the website and restricted to project personnel through secure logins. The site also includes areas for NCC, RS and project level communications including news (announcements, meetings); manuals, forms and protocols; up-to-date personnel contact information; an area

for secure transfer of data; reports for enrollment, follow up and data management. The sites are listed at the links below:

<http://sites.bu.edu/bbrain/> and https://wwwapp.bumc.bu.edu/BEDAC_BBBrainRetro

Progress to date includes BEDAC creating needed reports from the electronic data capture tool REDCap. These reports help to track study status and materials from a large volume of participants. Weekly, a generated report of outstanding stool, urine, survey and fitbit data is sent to each site. This report allows for easy access to follow up with participants and remind them to complete data from the study. BEDAC has generated multiple analytic datasets from cleaned data to date. An updated analysis of the population's demographics is periodically sent to investigators of the consortium. Other data is requested by investigators through the sample request electronic form. The sample request electronic form and approval process is shown below:



BOSTON UNIVERSITY

BBRAIN
Boston Biorepository, Recruitment
and Integrative Network for GMI

Welcome to the BBRAIN Data Repository Request Form.
What would you like to do?

Create a New Request

OR to view/edit an existing request, please enter the email address entered
for the primary investigator

Go

Task 4b. Develop manuals for the neuropsychological testing protocol, specimen collection protocols and recruitment screening procedures

Manuals for the neuropsychological testing protocol, blood, saliva, urine and microbiome specimen collection and recruitment protocols have been compiled and finalized. The table below describes the full study protocol for the four study sites in Boston, Miami, Bronx and San Francisco.

Table 5. BBRAIN study protocol

Study Protocol	Boston		Miami		Bronx		San Francisco		Total
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	
Questionnaires (demographics, general health and symptoms, pain, fatigue, sleep, medical conditions, deployment and exposure history)	150*	50*	50	50	50	50	50	50	n = 500 (300 cases/200 controls)
Clinical evaluation and autonomic measures (medical history, height, weight, supine/standing BP and pulse; FitBit BP, HRV, sleep quality)	150	50	50	50	50	50	50	50	n = 500 (300 cases/200 controls)
Clinical lab tests (CBC, metabolic profile, lipid panel, TSH, ANA, RF)	150	50	50	50	50	50	50	50	n = 500 (300 cases/200 controls)
Biorepository Samples and Research assays (plasma cytokines /chemokines, salivary cortisol; plasma, serum, whole blood, DNA, RNA, Urine, fecal sample)	150	50	50	50	50	50	50	50	n = 500 (300 cases/200 controls)
Neuropsychological assessment (executive function, attention, memory, psychomotor function, motivation, mood)	150	50	50	50	50	50	50	50	n = 500 (300 cases/200 controls)
Longitudinal assessment (clinical evaluation, clinical lab tests, plasma cytokines, neuropsychological assessment; biorepository samples)	100	50	25	25	-	-	-	-	n = 125 (cases/ 75 controls)

*100 participants will be recruited on site and 100 participants remotely.

Task 4c. Train researchers and staff on protocols and quality control measures for the clinical study and repository collection

New study personnel who are working directly with study participants were extensively trained upon hiring via Zoom by the Boston University Coordinating Center to ensure adequate quality control of test administration and interview procedures among the study sites. Quality control measures will continue to be instituted and monitored by experienced Administrative Core investigators including Dr. Toomey as the clinical studies proceed to ensure good inter-rater reliability and to reduce tester drift among the study sites. The RAs continue to meet biweekly with the study coordinator at the Boston University Coordinating Center to ensure all study sites are adhering to the study protocol.

TASK 5. SCREENING, RECRUITMENT AND ASSESSMENT OF GW VETERANS FROM FOUR RESOURCE SITES (MONTHS 9 - 36)

Due to the ongoing COVID-19 pandemic, recruitment restrictions have varied at the different study sites. Boston University has experienced no restriction and have successfully been recruiting participants throughout the year. The San Francisco VA recruitment was placed on hiatus for human subject research visits from January 2022 to March 2022. The prospective data team at the San Francisco VA has been actively enrolling participants since March 2022. The Bronx VA and Miami VA have been enrolling participants for the duration of the year. The Bronx VA has now completed subject recruitment for their site because Dr. Golier is no doing research and is focusing only on clinical responsibilities. In the recent months, country wide inflation and more expensive travel costs have slightly hindered participant enrollment. However, this has improved most recently.

In total, 500 study participants (300 GWI cases, 200 controls) will be recruited from four RS sites; Boston, MA, Miami, FL, Bronx, NY and San Francisco, CA. At the Miami, Bronx and San Francisco sites, 100 study participants each will be recruited. At the Boston site, 100 study participants will be recruited to participate on site and an additional 100 participants not residing close to the any of the four recruitment sites will be given offered travel costs to participate at the Boston site.

To date, 480 participants have been screened. Of the 487 screened, 338 are eligible (272 GWI Cases and 49 healthy controls). Please see recruitment data tables below.

BBRAIN: Screening and Enrollment Summary
October 13, 2022

	Total	Boston	Bronx	Miami	San Francisco
Number of Subjects Contacted	487	249	55	41	142
Number of Subjects Screened	472 (96.9%)	248 (99.6%)	55 (100.0%)	39 (95.1%)	130 (91.5%)
Number of Subjects Eligible	338 (71.6%)	202 (81.5%)	32 (58.2%)	26 (66.7%)	78 (60.0%)
Number of Subjects with Appointments Made	201 (59.5%)	124 (61.4%)	18 (56.3%)	21 (80.8%)	38 (48.7%)
Number of Subjects Assessed	190 (94.5%)	113 (91.1%)	18 (100.0%)	21 (100.0%)	38 (100.0%)

	Total		Boston		Bronx		Miami		San Francisco	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	392	56	215	31	34	2	36	1	107	22
Number of Subjects Eligible	288	50	172	30	30	2	25	1	61	17
Number of Subjects Assessed	158	32	93	20	16	2	20	1	29	9

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

To date, we have received informed consent from 190 eligible GW veterans.

Task 5b. Assess subjects by obtaining demographics, medical history, self-report questionnaires, neuropsychological testing, Fitbit tracker, blood draw and saliva samples from 500 study participants.

One hundred and eighty seven study participants have been seen in-person to date, however all study protocols for the clinical studies have been finalized and are listed in the tables below and in Table 1 above. To initially reduce COVID-19 risks, we removed some of the cognitive tests were that involved the most touching and sharing of materials back and forth and included a different executive function task. We also instituted plexiglass between the tester and the study participants as well as PPE including masks and gloves. These amendments that were instituted last year have remained in place for consistency. All study protocols that can be done remotely will also be done that way including study surveys. We will continue recruitment by re-contacting participants from GWIC and other GW studies at the SF VAMC, Miami VAMC and Bronx VAMC who have agreed to be re-contacted for

future studies. The tables below lists the complete survey and cognitive data that is collected at the prospective sites. To date, 190 participants have completed the study protocol.

Table 6. BBRAIN study surveys

Name	Description
Demographics	Subjects report information on age, education, gender, ethnicity, marital status, handedness, corrective lenses, hearing aid usage (SENSE), GW duty service (active vs. reserve/National Guard), military rank and current military status
SF36V	Veterans' version of the SF36 which assesses functional health-related quality of life in 8 domains and provides overall summary scores for physical and mental health status.
Kansas Gulf War and Health Questionnaire	Queries veterans about demographics, military and deployment history, and chronic symptoms and diagnoses required to ascertain Kansas GWI and CMI case status.
Medical Conditions	A checklist with 21 medical conditions that the subject is asked to rate if they have ever had the condition, how it was diagnosed (self or doctor) and when it was diagnosed.
Current Medications list	A list of current medications which participants are taking listing the medication and what symptoms it is taken for.
Profile of Mood States (POMS)	The POMS is a self-report assessment of current mood. The indicators of importance are current fatigue, confusion, tension and depression.
3 Question DVBIC TBI screening tool	A 3 question screening for deployment related TBI causes and symptoms from the Defense and Veterans Brain Injury Center.
American Academy of Neurology mTBI guidelines	Questions are asked regarding history of mild TBI (mTBI) during three time periods (before, during, after GW) and number of mTBIs.
Kansas Gulf War Experiences and exposures	A questionnaire that assesses veteran-reported experiences and exposures during their deployment to the 1991 Gulf War.
Structured Neurotoxicant Assessment Checklist (SNAC)	The SNAC assesses the degree of past and current exposure to neurotoxicants during civilian and military occupations and includes questions pertaining to recent occupational and environmental exposures.
Pittsburg Sleep Quality Index (PSQI)	PSQI assesses sleep quality during the past month. It covers domains of sleep quality, latency, duration, efficiency, disturbances, medications and daytime dysfunction. Total global scores range from 0-21.
Multidimensional Fatigue Inventory Questionnaire (MFI-20)	20 item self-report fatigue instrument that covers general fatigue, physical fatigue, mental fatigue, reduced motivation and reduced activity.
McGill Pain Questionnaire	A pain questionnaire that includes 3 sections including what the pain feels like, change over time and strength of pain. Scores range from 0-78.
Every day Cognition Scale	A self-report survey measuring aspects of everyday cognition related to memory, language, visuospatial and executive functioning.
Brief Pain Inventory (BPI) short form	The BPI is a brief 9 question self-report survey of severity, location and impact of pain on daily functioning.
Visual Analog Pain and Fatigue Scales	Participants measure their pain and fatigue by drawing a vertical line on a horizontal scale from 'not at all' to 'worst pain or fatigue ever'.
Fatigue Severity Scale	A brief survey where statements of fatigue affecting daily activities is endorsed on a scale from 1 to 7 for disagree to completely agree.
PTSD Checklist for DSM-5 (PCL-5)	A 20-item self-report measure that assesses the 20 <i>DSM-5</i> symptoms of PTSD. It screens and provides a provisional PTSD diagnosis.
Center for Epidemiologic Studies Depression Scale (CES-D)	A 20-item scale whichs measures depressive feelings and behaviors during the past week. Provides a depression quotient.

COVID-19 Questionnaire	A 6 item questionnaire where the subject is asked to provide information on prior positive tests of COVID-19 and COVID-19 vaccination status.
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Table 7. BBRAIN Neuropsychological Test Battery

Test Name	Description	Outcome Measure
I. Executive System Functioning		
Controlled Oral Word Association Test (COWAT)	Spontaneous generation of words from letters F, A and S and animals category.	Total correct words generated
D-KEFS Category switching	Number of correct words generated switching between two categories	Total number of words Total number of correct category switches
D-KEFS Color-Word Interference Test (Stroop)	The Stroop is a test that measures the ability to inhibit competing responses in the presence of salient conflicting information. This ability can be called response inhibition, or behavioral inhibition. Secondly, the test also measures processing speed of verbal (words) and nonverbal (colors) stimuli.	Total Errors Self-corrected errors Uncorrected errors
D-KEFS Category switching	Number of correct words generated switching between two categories	Total number of words Total number of correct category switches
II. Tests of Attention, Vigilance and Tracking		
Trail-making Test (Reitan & Wolfson, 1985)	Timed connect-a-dot task to assess attention and motor control requiring sequencing (A).	Time to Completion Self-Corrected errors Uncorrected errors
Continuous Performance Test (Connors' CPT3)	Target letter embedded in series of distractors; to assess sustained attention and reaction time.	Reaction Time, Total Omission and Commission Errors
III. Tests of Motor Function		
Finger Tap Test	Continuous tapping of computer key with alternate hands; assesses simple motor speed.	Number of taps
IV. Tests of Visuospatial Function		
Rey-Osterrieth Complex Figure Test	Copy of a complex figure	Total correct out of 36
V. Tests of Memory		
California Verbal Learning Test (CVLT- II; Delis et al., 2000)	List of 16 nouns from 4 categories presented over multiple learning trials with recall after interference; assesses memory and learning strategies.	Total Trials 1-5 Long Delay
Rey-Osterrieth Complex Figure Test	Immediate and delayed recall of a complex figure	Total recall out of 36
VI. Tests of Motivation and General Intellectual Ability		
CVLT – Forced Choice Recognition	Forced choice of word pairs for each of the 16 target items.	Total correct

Wide-Range Achievement Test - Reading	Measures word reading skills and word decoding through letter identification and word recognition.	Total Correct Scaled Score
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Task 5c. Obtain participants urine and stool sample kits back by mail to BUSPH for biorepository storage.

BUSPH serves as a biorepository storage (BS) site for urine and stool sample collection. Urine and stool sample collection kit are handed to participants on the day of their in person appointment at the four prospective sites. Subjects provide their first morning urine and ship it overnight to the BUSPH Exposure Assessment laboratory for analysis and storage. Subjects also collect stool samples using detailed instructions provided on the day of appointment and also ship it to BUSPH Exposure Assessment laboratory for analysis and storage. This procedure has been working well for the study participants. To date, we have received more than 280 samples total and are continuing to follow up communication with those participants who have yet to return their samples.

Task 5d. Upload Fitbit sleep, heart rate variability and blood pressure data to BUSPH DCC to merge with other datasets.

The BUSPH BEDAC maintains the tracking database for the Fitbit sleep, heart rate variability and blood pressure data collection and transfer. The database resides on a secure, password-protected SQL server at the Boston University Medical Campus (BUMC). Because the server is part of the BUMC network, only connections from users authenticated from the domain controller will be accepted, thus providing a secure environment for all project data. The database is automatically backed up on a nightly basis. Fitbits capable of collecting heart rate variability and sleep metrics have been purchased and given to the study participants. The BEDAC team has created a secure website for participants to upload their Fitbit data to promote higher compliance with obtaining Fitbit data. To date, we have received data from 96 participants and are continuing to follow up with those participants who have yet to send their Fitbit data.

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

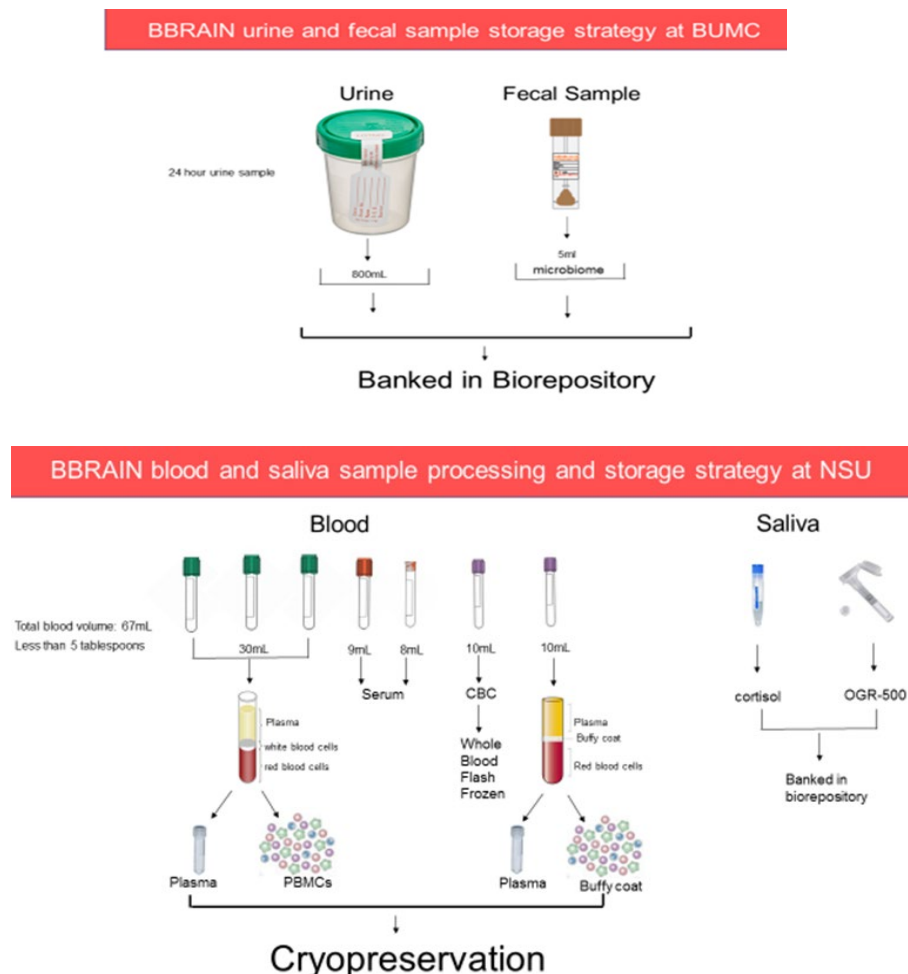
Neuropsychological protocols have been collected for 190 study participants to date and any new study staff at all sites have been trained for all test administration and standardized scoring procedures at the Boston Administrative Core. As data is collected, quality control procedures remain in place 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. Regular monthly check-ins between the BU Administrative Core and study administrators are in place to answer any neuropsychological testing questions that may arise.

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

One hundred and eighty seven participant blood and saliva samples have been sent to

NOVA Southeastern University for this study. The analysis of cytokine, chemokine and cortisol measurements has included testing for neuroendocrine and immune alterations and for hypothalamic pituitary adrenal axis abnormalities. Specifically, blood samples have been sent to NOVA Southeastern University for analysis of proinflammatory cytokine and chemokines and RNA analysis. Multiplex Quansys ELISA system have been used with an existing cytokine platform created by Dr. Klimas' research laboratory. Dr. Klimas will measure 16 cytokines in plasma. Saliva samples will continue to be analyzed for DNA and cortisol measurements. See figure below for aliquotting and storage scheme for BBRAIN.

TASK 6. PROCESS, STORE AND MANAGE SAMPLES FOR A TOTAL OF 500 NEW PARTICIPANTS (MONTHS 6-33)



As previously mentioned, we have completed 190 subjects to date. Samples from these 190 subjects were sent to the NOVA (NSU) and BUMC laboratories for processing and storage and study protocols have been running smoothly. The INIM NSU lab is the biorepository site for all blood and saliva samples collected throughout the study. We have obtained all the required approvals from IRB and the exempt status for HRPO.

Dr. Abreu, and her successor Dr. Kristina Aenelle, have worked closely with Dr. Sullivan on the logistics of this project in weekly meetings to ensure the project is on task and running efficiently. Reagents needed, such as blood collection tubes, saliva kits, and shipping kits are routinely purchased and distributed to each site. Regular inventory is tracked at each study site and new supplies are ordered as needed. Dr. Abreu established a protocol for the various study sites. The study coordinators were trained on blood and

saliva sample collection and the sample shipment process. Study coordinators were also trained on urine and stool sample collection to be shipped to Boston University. Sample accessioning, barcoding, and sample processing was reviewed with the laboratory technician at the INIM NSU lab. Blood and saliva samples are routinely accessioned, processed, and aliquoted for storage. Saliva, plasma, and serum samples are stored at -80°C freezers. Flash-frozen whole blood, buffy coat, and isolated peripheral blood mononuclear cells (PBMCs) are stored in liquid nitrogen. Sample tracking is maintained in WebLDMS, a laboratory information management system (LIMS) for managing collections of biological specimens.

Task 6a. Process and store blood, saliva, urine and stool samples received from 4 recruitment sites at two biorepository sites

We processed and stored the blood, saliva, urine and stool samples from 187 participants and will continue with our study protocols.

Task 6b. Perform local laboratory analyses (CBC) for 500 participants

We have performed local laboratory analyses for our first 190 participants and will continue this protocol for the next 310 participants.

Task 6c. Extract DNA and RNA from 500 saliva samples and store in repository

We have performed multiple batch analysis of DNA and RNA from saliva samples. These analysis will continue to be run in batches at Nova.

Task 6d. Run batched assays for cortisol and cytokine results (18 plex cytokine assay)

We have performed multiple batch assays for cortisol and cytokine results from saliva samples. These analysis will continue to be run in batches at Nova.

Task 6e. Provide Sample request forms and track information regarding the use of samples

To date we have received 17 requests for samples/data to that have been sent to our steering committee for approval. 16 of the 17 requests have been approved and we have shared brain imaging data, CSF samples and blood samples as well as cognitive data, TBI data and health symptom data with participating investigators. The urine and stool samples will soon be shared as well for existing requests.

Task 6f. Ship samples to requesting investigators

We have shipped CSF and blood samples to the requesting investigators who have been approved to date through the online BBRAIN sample request form. We will soon ship urine and stool samples that have been requested and approved.

Table 8. Approved Data Requests from the BBRAIN Repository

Institution	Lead Investigator	Project Title	Samples Requested	Shared?
Baylor College of Medicine	Marjin Lijffijt, PhD	Glutamate receptor and Kynurenin pathway functioning in the pathobiology of GWI	1.5mL CSF samples	Yes
Boston University of Medicine	Bang-Bon Koo, PhD	Comparison of complex diffusion markers with glial PET imaging in GWI	T1 structural, diffusion, and TSPO PET scans collected from MGH under Dr. Loggia's PET study group	Yes
Baylor College of Medicine	Marjin Lijffijt, PhD	Glutamate receptor and Kynurenin pathway functioning in the pathobiology of GWI	Blood and CSF samples for additional analyses using neurofilament light	Yes
The Roskamp Institute	Laila Abdullah, PhD	Gulf War pesticide metabolite mediated autoimmune dysfunction in Gulf War Illness	750mL Plasma samples	Yes
Boston Children's Hospital	Yandming Ou, PhD	Brain Aging in Gulf War Veterans	Structural and Diffusion MRI Scans	Yes
Texas A&M Health Science Center	Ashok Shetty, PhD	Pilot Study on Microglia	Human induced pluripotent Stem cells – case and control	Yes
UCSF & San Francisco VA Health Care System	Linda Chao, PhD	Investigating the Incidence of Mild Cognitive Impairment in GW veterans	Structural MRI scans, demographics, exposure status	Yes
UC-Irvine	Saurabh Chatterjee, PhD	Studying the unique virome signature in Gulf War Illness to treat dysbiosis and symptom persistence	stool, plasma, serum, PBMC	Yes
University of Texas Health Science Center at Houston	Deborah Little, PhD	Expanding Characterization and Application of Clinical MRI Markers in Gulf War Illness	Structural and diffusion MRI scans, demographics, neuropsychological testing, GW exposure data, medical history, health symptoms, lab results	Yes
Nova Southeastern university	Travis Craddock, PhD	Machine Learning and Network Modeling in Gulf War Illness Paradigm	Demographics, neuropsychological testing, GW exposure data, medical history, health symptoms, lab results	Yes
The Roskamp Institute	Laila Abdullah, PhD	Validating blood biomarkers of brain immune and metabolic dysfunction in Gulf War Illness	Whole blood, PBMC, plasma, PET imaging, MR Spectroscopy, demographics, neuropsychological testing, GW exposure data, medical history, health symptoms, lab results	In Process
Columbia University	Ian Lipkin, MD	Metabolomics in Gulf War Illness: A Systems Biology Approach to Dissecting Mechanisms of Disease	150 plasma samples of GWI cases and controls and 100 plasma samples of ME/CFS cases	Yes
Boston University School of Public Health	Junenette Peters, ScD	Metal Exposure Assessment in Gulf War Veterans Update	Blood and urine samples	Yes
UCSF/San Francisco VA Health Care System	Linda Chao, PhD	An investigation of the relationship between toxicant exposures during GW deployment and prodromal PD	Demographics, neuropsychological test results, GW exposures	Yes
UCSF & San Francisco VA Health Care System	Linda Chao, PhD	Investigating the Incidence of Mild traumatic brain injury	Plasma	In Process
NIH, NHLBI	Jay Chung, MD, PhD	VDAC oligomer formation in Gulf War Illness	PBMCs	In Process

TASK 7: MERGE DATA AND PERFORM INTERIM ANALYSES (MONTHS 6-24)

Data merging has been performed from cleaned data sets and interim analyses have been completed.

7a. Merge Clinical datasets data from prior studies for brain imaging, cognitive, demographic and health symptom report data

Retrospective data sets were merged for neuroimaging, cognitive, demographic and health symptom outcomes by the BEDAC data management team and interim data analysis was performed. Multiple reports of these data have been presented at conferences and are in draft for publication. Brief reports on sleep quality found that over 80% of Gulf War veterans in our sample reported poor sleep quality (Figure 1, Table 9). In addition, the PSQI sleep quality subdomains showed a positive correlation between sleep habits (disturbance, latency and dysfunction) and poorer cognitive functioning on Trail making Test B, CPT3 omission errors and the DKEFS Color-Word interference Test (Table 10). Preliminary data shows that veterans with GWI had significantly poorer performance on all neuropsychological tests than healthy controls, indicating deficits in sustained attention and verbal memory (Table 11, Figure 2). Further, these cognitive decrements were associated with environmental exposures during the Gulf War (Table 12). This data is currently being prepared for publication.

Figure 1. Self-Reported sleep quality in all Gulf War veterans

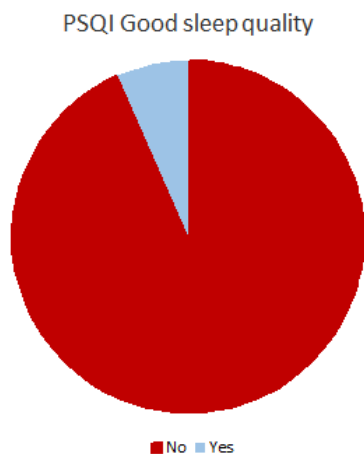


Table 9. Health Survey comparisons in GWI cases vs controls

Health Surveys	case Adjusted Mean	control Adjusted Mean	p-value	Effect Size
MFI-20 Fatigue Score (Range 20-100)	70.85	54.46	<.0001	1.11
PSQI Total Score	12.80	8.44	<.0001	1.06
McGill Pain Score (Range 0-78)	32.11	19.23	<.0001	0.91

*analyses adjusted for PTSD

Table 10. Neuropsychological outcomes correlated with PSQI subdomains

SPEARMAN CORRELATION COEFFICIENTS PROB > R UNDER H0: PARTIAL RHO=0				
N=197	Sleep quality	Sleep latency	Sleep disturbance	Sleep aid medications
TRAIL MAKING B	-0.06 0.35	0.18 0.01	0.22 0.002	0.12 0.09
CPT3 OMISSION ERRORS	-0.02 0.7	0.18 0.009	0.15 0.03	0.08 0.2
DKEFS	-0.07 0.29	0.09 0.21	0.15 0.03	-0.042 0.55

Significantly more GWI cases self-reported poor sleep quality compared to healthy controls (81% vs 19%, $p < 0.001$, Figure 1) In addition, PSQI total score was significantly higher for GWI cases compared with controls (13 vs 7 total score, $p < 0.001$, Table 10). Spearman correlations were then compared with PSQI and cognitive outcomes PSQI total score was significantly correlated with a slower Trail making B total time ($p < 0.05$) (not shown). An increased CPT3

omission errors and DKEFS color-word interference trial 4 ($p < 0.05$) was correlated with sleep disturbance PSQI sleep latency was correlated with slower Trail making B and more CPT3 omission errors ($p < 0.05$). In addition, less DKEFS color-word interference errors was associated with veterans taking sleep aid medication ($p < 0.05$).

Table 11. Retrospective Neuropsychological Measures from BBRAIN Repository by GWI Status

		GW Cases (n=312)	Controls (n=99)
		Means	
<i>CPT 3</i>	Omissions*	49.72	47.05
	Commissions**	53.13	48.17
<i>CVLT-II</i>	Correct in trials 1-5**	46.34	50.82
	Correct in SD Recall**	9.36	10.61
	Correct in LD Recall*	9.69	10.94
<i>D-KEFs Stroop</i>	Trial 1 time (sec)**	32.71	29.51
	Trial 2 time (sec)**	24.21	21.53
	Trial 3 time (sec)**	62.43	56.85
	Trial 4 time (sec)**	67.43	60.63
<i>Trails</i>	Trail A time (sec)**	32.00	26.79
	Trail B time (sec)**	73.73	63.22

*p<0.05
**p<0.001

Figure 2. Retrospective Neuropsychological Measures from BBRAIN Repository by GWI Status

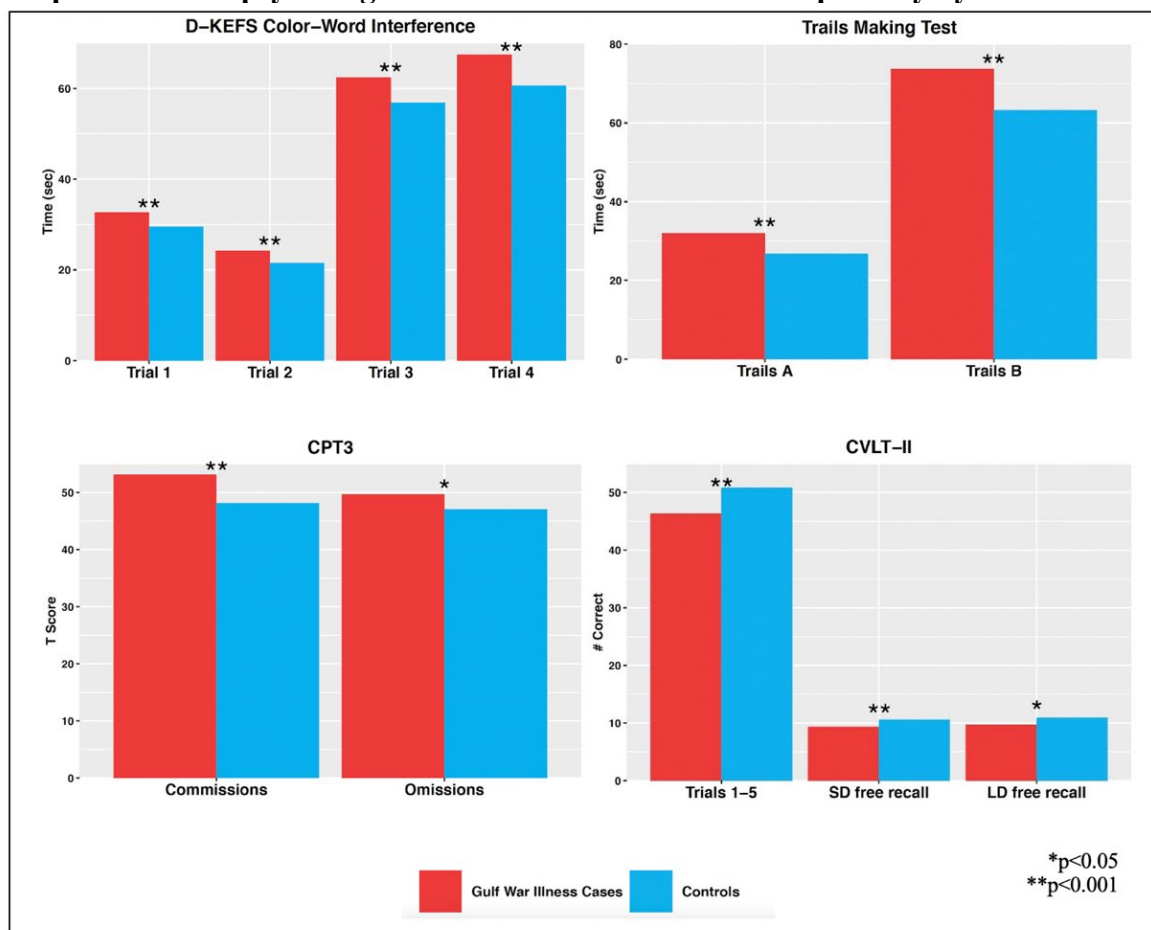


Table 12. Retrospective Neuropsychological data by exposure status

Exposures			Controls	<7 days Exposed Cases	7+ days Exposed Cases
			Mean		
Heard chemical alarms sounded	CPT 3	Omissions*	49.09	49.71	48.29
		Commissions*	45.4	52.67	55.67
	CVLT-II	# correct in long-delay free recall*	11.79	9.77	8.9
	Trails	Trail A(sec)*	22.94	32.33	34.38
		Trail B(sec)*	59.65	71.04	80.17
Saw Smoke from oil well fires	CVLT-II	# correct in short-delay free recall*	11.2	9.71	8.79
	Trails	Trail A(sec)*	23.2	32.15	33.78

7b. Perform machine learning and/or meta-analytic techniques on common data elements from prior study epidemiological, cognitive and brain imaging data

Common data elements are being compiled from the prior clinical datasets that have been shared and will then be combined with brain imaging outcomes for Dr. Koo to perform machine learning analyses. A review paper of cognitive outcomes was published in collaboration with NOVA Southeastern investigators while waiting for the common data elements to be available for planned meta-analysis studies (Jeffrey et al., 2019).

7c. Discussion of results and preparation of abstracts for meeting presentations and initial manuscript for publication

Study results have been prepared for abstracts and presented at meetings. Planned upcoming presentations are in progress as well as initial manuscripts for publication.

7d. Annual reports of progress will be written

Four annual progress reports have been written and submitted for review.

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

- **Research Staff Training:** personnel who will be using the web systems for either direct data capture, participant tracking, or viewing reports have been trained to ensure uniformity of procedures, to achieve the ultimate aim of ensuring high quality protocol implementation and data collection. As a large academic community, Boston University also provides opportunities for students to get real world experience. The BBRAIN can tap into this talent pool by taking practicum and intern students in the fields of health communication and promotion, who as part of an academic program, will create a media and outreach program for the network. Doctoral students in related fields can also be brought on to aid in this project. Finally, as has been exhibited with

the GWIC, the network will be financially continued through spin-off grants in GWI and similar fields through DOD, VA and NIH and by providing the tools for talented young researchers to aid us in tackling and solving the problem of GWI. For example, in summer of 2019 we had a student from Middlebury College, VT working with us a summer intern who helped develop the BBRAIN website. This year, we had two doctoral students working with us to learn cognitive evaluations and to assist with subject recruitment efforts.

- **Participation in conferences, workshops, and seminars:** BBRAIN researchers will continue to attend conferences, workshops and seminars to present research findings.
 - In February 2021, Dr. Sullivan and the BU research team presented on multiple findings from retrospective and prospective BBRAIN data at the International Neuropsychological Conference.
 - On August 23rd, 2021, Dr. Sullivan was invited to present on the BBRAIN network to Congressional staffers at the Capitol Hill Gulf War Illness Forum hosted by Veterans for Common Sense.
 - On February 25th, 2022, Dr. Sullivan was invited as a keynote speaker to present the BBRAIN network at the Gulf War, Operation Desert Shield virtual meeting that was co-led by CDMRP and VA.
 - In July 2022, Dr. Sullivan and the BU research team presented on cognitive data and health symptom findings from retrospective and prospective BBRAIN data at the International Neuropsychological Conference.

- **How results will be disseminated to communities of interest.**

Research staff will use some of the outreach structure already in place from the Boston GWI consortium. The Facebook page has over 3,000 followers, many of whom regularly interact with and share page content. This allows researchers to freely spread information among the already existing GW network on social media. GWIC also has a Twitter account and study website that will be used to disseminate information to the community and refer to the BBRAIN social media sites. Boston University also has a very proactive media team that produces text and video news stories that get widely shared. Research staff will also be aided by the Veteran Working Group in getting news out about the BBRAIN to fellow veterans and their communities. As the research team has done for GWIC, they will write professional publications that state what samples are available and who to contact. This was done for the stem cell repository that is now part of BBRAIN and was very well received in the research community. To date, a media story was written about BBRAIN in April 2019 at <https://www.bu.edu/sph/2019/04/08/professor-awarded-3-2m-to-establish-biorespository-network-for-gulf-war-illness-research/> and a new larger story about BBRAIN came out on Veterans Day 2019 at <https://www.bu.edu/sph/2019/11/13/the-brink-5/> to help with subject recruitment efforts and in letting investigators know about samples that will be shared for other relevant studies. Most recently, an article highlighting the BBRAIN study 30 years after the Gulf War was published in November 2021: https://www.bu.edu/sph/news/articles/2021/30-years-after-gulf-war-veterans-still-battle-health-issues-caused-by-toxic-wounds/?fbclid=IwAR1_3yibizQ_PvH1Onlii4tlqlqvMsXB3tk0SmbiWneBY44jE02c_IvGH3s

- **Plan to do during the next reporting period to accomplish the goals:** In the next reporting period, we plan to:
 - Continue to obtain Biorepository data for analysis and catalogue available biospecimens for sharing from Repository Resource Sites.
 - Continue to obtain imaging data from retrospective sites and perform post-processing of and machine learning analysis with combined common data elements.
 - Continue recruitment at the three prospective sites. The Bronx VA will discontinue recruitment and data collection. We will continue to process, store and manage samples for a total of 320 new participants.
 - We believe that continuing our media stories and working with new web advertising companies will help us reach our target enrollment by year 5.

4. IMPACT

- **Impact on the development of the principal discipline(s) of the project:**
 - *“Nothing to Report.”*
- **Impact on other disciplines:**
 - BBRAIN investigators will collaborate with researchers external to the consortium on 10 DoD, 4 VA applications and 8 additional study sites (Harvard University, Georgetown University, Roskamp Institute, Boston VA, Bronx VA, San Francisco VA, U-Alabama, Mass General Hospital). Each site has valuable and unique samples to share within and outside the repository network that would not otherwise be available to the research community. It is the goal of the BBRAIN to continue the expert infrastructure of scientific, laboratory and data management currently within the GWIC, include other expert scientist collaborators, and to hasten biomarker discovery and treatment development for GWI on multiple aspects of GWI disciplines (immune, neurological, respiratory, gastrointestinal etc) as well as other disciplines including comorbid conditions of ME/CFS, IBS and fibromyalgia. We also have investigators interested in studying the risk of toxicant induced Parkinson’s disease and brain cancer and heavy metals exposure with our research network that we will continue to pursue.
- **Impact on technology transfer:**
 - Data shared from the biorepository will allow more investigators across the U.S to perform more research in the area of Gulf War Illness and other related health conditions. Results from studies conducted with BBRAIN samples will also make it easier for investigators to secure funding for supplemental and diverse GWI related studies and to share them for public use. This has already been shown in Dr. Sullivan and Klimas contributing our biorepository knowledge and plans for BBRAIN with the National Academy of Science (NAS) Gulf War Respiratory Health Working Group meeting in October 2019. We have also created a stem cell line with organoid brain tissue available to other researchers that is the first in this field.
- **Impact on society beyond science and technology:**
 - The demographic, exposure history, clinical data symptom assessment, diagnostic outcomes and other data gathered from prospective and retrospective sites will aid in the comprehensive understanding of the pathobiology of GWI. Results of our analysis may uncover and increase the diagnostic and treatment capability for GWI. The biorepository can also become a model for the establishment of other biorepositories in the field. Researchers may research out to us for questions regarding the setup and management of the biorepository. In fact, we have already spoken to CDC investigators studying ME/CFS and the NAS GW respiratory health working group for advice about biorepositories.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

Nothing to Report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Nothing to Report

Changes that had a significant impact on expenditures

Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report

6. PRODUCTS:

- **Publications, conference papers, and presentations**

- **Journal publications.**

We have published a methods paper on BBRAIN study to share the knowledge of our resource with the GWI research community. CDE that are utilized in BBRAIN were also published.

- Sullivan, K.; O'Callaghan, J.P. *Advancing the Role of Neuroimmunity and Genetic Susceptibility in Gulf War Illness*. Brain Sci. **2022**, *12*, 1068. <https://doi.org/10.3390/brainsci12081068>
- Bose D, Chatterjee S, Older E, Seth R, Janulewicz P, Saha P, Mondal A, Carlson JM, Decho AW, Sullivan K, Klimas N, Lasley S, Li J, Chatterjee S. *Host gut resistome in Gulf War chronic multisymptom illness correlates with persistent inflammation*. Commun Biol. 2022 Jun 7;5(1):552. doi: 10.1038/s42003-022-03494-7.
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- Yates PL, Patil A, Sun X, Niceforo A, Gill R, Callahan P, Beck W, Piermarini E, Terry AV, Sullivan KA, Baas PW, Qiang L. *A cellular approach to understanding and treating Gulf War Illness*. Cell Mol Life Sci. 2021 Nov;78(21-22):6941-6961. doi: 10.1007/s00018-021-03942-3. Epub 2021 Sep 27. PMID: 34580742
- Oberlin, S., Nkiliza, A., Parks, M. *et al*. Sex-specific differences in plasma lipid profiles are associated with Gulf War Illness. *J Transl Med* **20**, 73 (2022). <https://doi.org/10.1186/s12967-022-03272-3>
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Fletcher, V. Kalasinsky, E. Kokkotou, K. Lidie, D. Little, W. Loging, M. Morris, L. Nathanson, M.D. Nichols, G. Pasinetti, D. Shungu, P. Waziry, J. VanLeeuwen, J. Younger, and N. Klimas, *A common language for Gulf War Illness (GWI) research studies: GWI common data elements*. Life Sci, 2021.

- Keating D, Zundel CG, Abreu M, Krengel M, Aenlle K, Nichols MD, Toomey R, Chao LL, Golier J, Abdullah L, Quinn E, Heeren T, Groh JR, Koo BB, Killiany R, Loggia ML, Younger J, Baraniuk J, Janulewicz P, Ajama J, Quay M, Baas PW, Qiang L, Conboy L, Kokkotou E, O'Callaghan JP, Steele L, Klimas N, Sullivan K. *Boston biorepository, recruitment and integrative network (BBRAIN): A resource for the Gulf War Illness scientific community*. Life Sci. 2021 Nov 1;284:119903. doi: 10.1016/j.lfs.2021.119903. Epub 2021 Aug 26. PMID: 34453948. Link: <https://www.sciencedirect.com/science/article/pii/S0024320521008900?via%3Dihub>
- Guan Y, Cheng CH, Chen W, Zhang Y, Koo S, Krengel M, Janulewicz P, Toomey R, Yang E, Bhadelia R, Steele L, Kim JH, Sullivan K, Koo BB. *Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning*. Brain Sci. 2020 Nov 20;10(11):884. doi: 10.3390/brainsci10110884. PMID: 33233672
- Jeffrey MG, Krengel M, Kibler JL, Zundel C, Klimas NG, Sullivan K, Craddock TJA. *Neuropsychological Findings in Gulf War Illness: A Review*. Front Psychol. 2019 Sep 26;10:2088. doi: 10.3389/fpsyg.2019.02088. eCollection 2019. Review. PMID: 31616335. Link: <https://www.frontiersin.org/articles/10.3389/fpsyg.2019.02088/full>

▪ **Books or other non-periodical, one-time publications.**

Nothing to Report.

▪ **Other publications, conference papers, and presentations.**

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. Boston, Biorepository and Integrative Network for GWI. GWI State of the Science 30th Anniversary of the Operation Desert Storm meeting, virtual, August 18, 2020.

Conference Posters:

- Dylan Keating, Jenna Groh, Maxine Krengel, Rosemary Toomey, Linda Chao, Emily Quinn, Julianne Dugas, Kimberly Sullivan. *Toxic Wounds are Associated with Cognitive Decrements in Women Veterans of the 1991 Gulf War*. Abstract submitted to International Neuropsychology Society conference (INS), February 2023.
- Jenna R Groh, Dylan Keating, Rabindra Kadel, Julianne N Dugas, Emily Quinn, Maxine Krengel, Kimberly Sullivan. *Longitudinal Cognitive Functioning in Gulf War veterans with and without*

Gulf War Illness: Data Mining from the BBRAIN Repository. Abstract submitted to the International Neuropsychological Society Annual Meeting in San Diego 2023.

- Dylan Keating, Jenna Groh, Maxine Krengel, Rosemary Toomey, Linda Chao, Emily Quinn, Julianne Dugas, Kimberly Sullivan. *Neuropsychological Outcomes Vary by Sex in Neurotoxicant Exposed Veterans with Gulf War Illness*. Abstract submitted to International Neuropsychology Society conference (INS), February 2023.
- Dylan Keating, Maxine Krengel, Rosemary Toomey, Emily Quinn, Rabindra Kadel, Kimberly Sullivan. *Mood functioning in Gulf War veterans: findings for the Boston, Biorepository, Recruitment and Integrative Network for GWI (BBRAIN)*. Abstract accepted to the American Public Health Academy conference (APHA), November, 2022.
- Alexa Friedman, Dylan Keating, Jeff Carlson, Charlotte JeanMichel, Emily Sisson, Steve Coughlin, Vahe' Heboyan, Glenn Markenson, Lizette Mendez, Patricia Janulewicz Lloyd, Maxine Krengel, Kimberly Sullivan. *Reproductive and Developmental Outcomes Following Deployment: Gulf War and Gulf War-Era Women Veterans*. Poster presented at Military Health Systems Research Symposium (MHSRS), September 2022.
- Dylan Keating, Maxine Krengel, Rosemary Toomey, Linda Chao, Layla Abdullah, Lea Steele, Patricia Janulewicz, Emily Quinn, Julianne Dugas, Kimberly Sullivan. *Toxic wounds and cognition in US Gulf War veterans from the BBRAIN repository network*. Poster presented at International Neuropsychology conference (INS), July 2022.
- Jenna Groh, Chia-Hsin Cheng, Bang-bon Koo, Ronald Killiany, Kimberly Sullivan. *Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers*. Poster presented at the International Neuropsychological Society (INS) Conference in Barcelona, Spain, July 2022.
- Charlotte JeanMichel, Alexa Friedman, Dylan Keating, Emily sisson, Julianne Dugas, Timothy Heeren, Rosemary Toomey, Lea Steele, Patricia Janulewicz, Maxine Krengel, Nancy Klimas, Kimberly Sullivan. *Self-reported sleep quality and cognitive outcomes in US veterans with Gulf War Illness*. Poster presented at International Neuropsychology conference (INS), July 2022.
- Jenna Groh, Chia-Hsin Cheng, Bang-bon Koo, Ronald Killiany, Kimberly Sullivan. *Defining and Characterizing GWI Pathobiology using Longitudinal Brain Imaging Biomarkers*. Poster presented at the Massachusetts Neuropsychological Society (MNS) Science Symposium and Student Poster Session, May 2022.
- Dylan Keating, Alexa Friedman, Jenna Groh, Charlotte Jean Michel, Maria Abreu, PhD, Emily Quinn, Maxine Krengel, PhD, Kimberly Sullivan, PhD. *Neuropsychological Functioning and Interferon Gamma Levels in Veterans with Gulf War Illness from the Gulf War Illness Consortium (GWIC)*. Poster presented at International Neuropsychology Conference (INS), February 2022.
- **Website(s) or other Internet site(s)**
 - <http://sites.bu.edu/bbrain/>
 - https://wwwapp.bumc.bu.edu/BEDAC_BBRAINRetro
- **Technologies or techniques**
 - **REDCap**: This primary platform utilized in the BBRAIN study is a freely available platform used extensively for collaborative multi-site research. It can be used at all sites to collect existing data and assist with data mining strategies.

- **Laboratory Data Management System (LDMS):** Samples received by the biobank laboratory will be processed in LDMS. The LDMS (Frontier Science Foundation) system allows for the seamless management of bio-specimens from collection, processing, shipment, and storage processes. Specimens are barcoded and accessioned to ensure the integrity of the aliquot is maintained even over decades of storage.
- **Inventions, patent applications, and/or licenses**
Nothing to Report.
- **Other Products**
Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **Individuals that have worked on the project:**

Name: Kimberly Sullivan	Project Role:	<i>PI of Biorepository Network and Boston site</i>
	Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0001-7940-6123
	Nearest person month worked:	<i>12</i>
	Contribution to Project:	<i>Oversee the biorepository at BUSPH; oversee the recruitment and participation at BUSPH (200 participants); oversee data and sample coordination of all prospective and retrospective BBRAIN sites.</i>
	Funding Support:	<i>BBRAIN Award, W81XWH-18-1-0549</i>
Name: Nancy Klimas	Project Role	<i>Co-Investigator, PI of Miami Site</i>
	Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-1459-3268
	Nearest person month worked:	<i>12</i>
	Contribution to Project:	<i>Oversee the resource site at the Miami VA medical center; oversee recruitment and participation (100 participants).</i>
	Funding Support:	<i>BBRAIN Award, W81XWH-18-1-0549</i>

Name: Julia Golier	Project Role	<i>Co-Investigator, PI of Bronx VA Site</i>
	Researcher Identifier (e.g. ORCID ID):	
	Nearest person month worked:	<i>12</i>
	Contribution to Project:	<i>Oversee site at Bronx VA (100 participants); serve as neuroendocrine expert for BBRAIN; oversee cortisol study protocols, analyses, and outcomes.</i>
	Funding Support:	<i>BBRAIN Award, W81XWH-18-1-0549</i>
Name: Linda Chao	Project Role	<i>Co-Investigator, PI of San Francisco VA Site</i>
	Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0002-8593-2434
	Nearest person month worked:	<i>12</i>
	Contribution to Project:	<i>Serve as imaging expert for BBRAIN; oversee site in San Francisco (100 participants); share prior brain imaging data, saliva samples, and demographic, exposure, and survey data.</i>
	Funding Support:	<i>BBRAIN Award, W81XWH-18-1-0549</i>

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report.

- **What other organizations were involved as partners?**
 - **Organization Name:** Harvard University and New England School of Acupuncture
 - **Location of Organization:** Boston, Massachusetts
 - **Partner's contribution to the project** (*identify one or more*)
 - **Financial support;** *Nothing to Report*
 - **In-kind support:** *Nothing to Report*
 - **Facilities:** *Nothing to Report*
 - **Collaboration:** Lisa Conboy, BBRAIN consultant affiliated with both listed institutions. Dr. Lisa Conboy is a social epidemiologist and is well-published in the area of Complementary and Alternative Medicine. She is an Instructor in Medicine at Harvard Medical School, and Research Director at the New England School of Acupuncture. Dr. Conboy has been involved in studies investigating diagnostic biomarkers and predictors of therapeutic responses in inflammatory bowel disease, irritable bowel syndrome (IBS) and GWI. Drs. Conboy have previously shared blood samples from their acupuncture study for a study of CNS autoantibodies in GWI in collaboration with Drs. Sullivan and colleagues. She has also collaborated in the Boston area PI recruitment network sharing study flyers and recruitment information with their GW veteran cohort of other local studies. Dr. Conboy will continue to participate in the Boston PI

Recruitment Network as well as contribute retrospective repository blood samples and data from her successful acupuncture trial with GW veterans.

- **Personnel exchanges:** *Nothing to Report*
- **Other.** *Nothing to Report*

1. **Organization Name:** Harvard University and Beth Israel Hospital
2. **Location of Organization:** Boston, Massachusetts
3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support;** *Nothing to Report*
2. **In-kind support:** *Nothing to Report*
3. **Facilities:** *Nothing to Report*
4. **Collaboration:** Drs. Efi Kokkotou, BBRAIN Consultant affiliated with both institutions. She has previously shared blood samples from her acupuncture study for a study of CNS autoantibodies in GWI in collaboration with Drs. Sullivan and colleagues. She has also collaborated in the Boston area PI recruitment network sharing study flyers and recruitment information with their GW veteran cohort of other local studies. Dr. Kokkotou will share samples from her large biorepository of IBS and GWI samples from DOD and NIH funded studies (W81XWH-09-0064 and K01AT004916).
5. **Personnel exchanges:** *Nothing to Report*
6. **Other.** *Nothing to Report*

1. **Organization Name:** VA Boston Healthcare System
2. **Location of Organization:** Boston, Massachusetts
3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support;** *Nothing to Report*
2. **In-kind support:** *Nothing to Report*
3. **Facilities:** *Nothing to Report*
4. **Collaboration:** Drs. Christopher Brady and Neil Kowall, BBRAIN Consultant and Co-Investigator, affiliated with this institution. The VA Biorepository Brain Bank (VABBB), Gulf War Veterans' Illnesses Biorepository (GWVIB) is a collaborative effort between multiple VAs (Boston-coordinating center/diagnostic neuropathology, Tucson-biorepository) and academic medical center affiliates (Harvard, BU and the University of Arizona). GWVIB VA Boston/BU/Harvard staff are professionals that bring essential skills to the project via their collaborative work on the GWVIB and other projects: the Boston University Alzheimer's Disease Center (Dr. Kowall, Director); the Center for the Study of Chronic Traumatic Encephalopathy (Dr. Ann McKee,

M.D., Director), and the VISN1 Neuropathology Laboratory (Dr. McKee, Director). Tissue is stored at the Southern Arizona VA Healthcare System (SAVAHCS) in Tucson, AZ. GWVIB Tucson/U. of Arizona staff is composed of biorepository storage, processing, management and tissue disbursement experts from the Pathology departments at SAVAHCS and the U. of Arizona. The VABBB has developed SOPs for tissue collection, processing, quality control, storage and disbursement, and developed a comprehensive tissue database annotated with relevant clinical and histopathological data. This collaboration will be leveraged for the BBRAIN and Drs. Kowall and Brady will advise Dr. Sullivan on best practices in multi-site biorepository management.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** Boston University Medical Campus

2. **Location of Organization:** Boston, Massachusetts

3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Drs. Bang Bon Koo, Ronald Killiany and Maxine Krengel, BBRAIN Co-Investigators affiliated with Boston University Medical Campus. Dr. Bang-Bon Koo, a clinical faculty member in the Anatomy and Neurobiology department at BUMC will assist with machine-learning analyses in GWI biomarker and diagnostic analyses. Dr. Ronald Killiany, director of the BUMC Center for Biomedical Imaging will serve as the Imaging Core Director and will oversee compilation of imaging data and data mining/post-processing samples. Dr. Bang-Bon Koo, a clinical faculty member in the Anatomy and Neurobiology department at BUMC will also be available to assist with machine-learning analyses in GWI biomarker and diagnostic analyses. Dr. Maxine Krengel currently leads the Ft. Devens cohort research effort and has published several recent publications in collaboration with Drs. Sullivan and Patricia Janulewicz on long-term health outcomes in GW veterans from the DOD funded Time 5 survey (GW100046). Dr. Krengel will share repository data from the Ft. Devens Cohort survey and in-person assessment studies for collaborative BBRAIN studies.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** CDC NIOSH

2. **Location of Organization:** Morgantown, West Virginia

3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Dr. Jim O'Callaghan, BBRAIN Co-Investigator affiliated with CDC NIOSH. Dr. James O'Callaghan serves as Distinguished Consultant, Centers for Disease Control (CDC) and Prevention, and Head of the Molecular Neurotoxicology Laboratory in the Toxicology and Molecular Biology Branch of the Health Effects Laboratory Division at the CDC-NIOSH. He is an expert neurotoxicologist who is a member of the Boston GWIC (GW120037) and formerly served on the VA Research Advisory Committee on Gulf War Veterans' Illnesses (RAC-GWVI). He has a very active and well-funded animal GWI research program aimed at establishing the relationship among toxicant-induced changes in gene expression, growth-associated phosphorylation cascades and signaling events associated with astrocytic hypertrophy and microglial activation. He will share mouse brain samples from his GWI model for the BBRAIN studies and repository.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** Roskamp Institute/ Tampa VAMC

2. **Location of Organization:** Sarasota, Florida

3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Drs. Laila Abdullah and Ghania Ait Ghezalia, BBRAIN Consultants affiliated with Roskamp Institute/ TAMPA VAMC. Dr. Laila Abdullah is a senior neuroscientist at the Roskamp Institute, a non-profit Research Center. Dr. Abdullah has identified novel therapies and biomarkers for GWI by applying omics (proteomics, lipidomics, and metabolomics) technology to target lipid metabolism. Dr. Abdullah has been working on identifying lipid metabolism pathways associated with immune/inflammation and metabolic disturbances in the brains of GWI mouse models. In collaboration with Drs. Sullivan, Klimas, and Ait-Ghezalia, Dr. Abdullah has been evaluating blood lipids as potential biomarkers of GWI and has successfully validated some aspects of lipid disturbances in the GWI mouse model against lipid disturbances observed in blood from veterans with GWI using Boston GWIC repository samples. Dr. Abdullah will contribute her expertise in 'omics' research, share omics data and biological samples from animal and clinical studies of

GW. Samples and data will be shared for BBRAIN through CDMRP awards (GW130045, GW150056) and a VA Merit award (RX002260-01A1).

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** Drexel University
2. **Location of Organization:** Philadelphia, Pennsylvania
3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Drs. Peter Baas and Liang Qiang, BBRAIN Co-Investigators affiliated with Drexel University. Drs. Peter Baas and Liang Qiang also Boston GWIC members from Drexel University are axonal transport, neuronal microtubule and stem cell experts. They have most recently developed a cutting-edge bank of human induced pluripotent (hiPSC) stem cells from GW veterans, in order to conduct mechanistic studies and high-throughput testing of potential therapies. The stem cell biorepository will be shared with BBRAIN and outside collaborators. Drexel and BUSPH researchers have established a repository of hiPSCs from peripheral blood mononuclear cells (PBMCs) directly from GW veterans. They have utilized contemporary stem cell technology to convert somatic cells from GW veterans into pluripotent cell lines that can be differentiated into various cell types, including neurons, glia, muscle, or other relevant cell types. They have generated hiPSCs from 2 groups of age-matched individuals: GW veterans with GWI versus those without GWI. The sets were validated for comparable and appropriate hiPSC induction, neuronal and glial differentiation, neural cell survival over time, and basic electrophysiological properties of the terminally differentiated cells. Two high profile papers have been published about this hiPSC repository. These cell lines are immortal and will be a resource for GWI researchers to pursue mechanistic hypotheses and therapies for GWI as a result of DOD funding (GW140086) to Drexel and BUSPH investigators.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** Mass General Hospital
2. **Location of Organization:** Boston, Massachusetts
3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Dr. Marco Loggia, BBRAIN Consultant affiliated with Mass General Hospital and Harvard University. Dr. Marco Loggia is an Assistant Professor of Radiology at Harvard Medical School, the Associate Director of the Center for Integrative Pain NeuroImaging (CiPNI) at Massachusetts General Hospital, and Faculty in the MGH/HSY Athinoula A. Martinos Center for Biomedical Imaging. He is a pain expert and has been funded for a ground-breaking study of positron emission tomography (PET) markers of glial activation in GWI working in collaboration with GWIC and other Boston area investigators (GW130100). Dr. Loggia will share blood samples and imaging, cognitive and demographic data from his DOD funded [11C]PBR28 PET/MR study of glial activation in GWI and BBRAIN will analyze his stored blood samples for cytokine markers of microglial activation to compare with his PET study outcomes.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** Georgetown University

2. **Location of Organization:** Washington, DC

3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Drs. James Baraniuk, BBRAIN Consultant is affiliated with Georgetown University. Dr. James Baraniuk is Professor of Medicine at Georgetown University and a practicing rheumatologist. He specializes in fatigue and pain management in patients with GWI, fibromyalgia and chronic fatigue syndrome (CFS)⁹. Dr. Baraniuk has been funded for multiple DOD studies of biomarkers of GWI including plasma, cerebrospinal fluid and brain imaging markers pre-and-post exercise challenge and treatment trials of GWI. Dr. Baraniuk and the GWIC are the only DOD funded sites that we are aware of that have highly valuable CSF samples in repositories. Dr. Baraniuk will share CSF, blood and brain imaging data from GWI, CFS and healthy control groups from DOD and NIH funded studies (CDMRP GW080053; GW140064; GW060044). Collaborative studies utilizing both Dr. Baraniuk's and Sullivan's CSF samples will be planned.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

1. **Organization Name:** University of Alabama
2. **Location of Organization:** Birmingham, Alabama
3. **Partner's contribution to the project** (*identify one or more*)

1. **Financial support:** *Nothing to Report*

2. **In-kind support:** *Nothing to Report*

3. **Facilities:** *Nothing to Report*

4. **Collaboration:** Dr. Jared Younger, BBRAIN Consultant is affiliated with University of Alabama. Dr. Jarred Younger is Associate Professor at the University of Alabama at Birmingham (UAB), and Director of the Neuroinflammation, Pain and Fatigue Laboratory. Dr. Younger's group specializes in intensive longitudinal studies where biosamples are collected daily. This unique dataset provides measurement of self-reported symptom severity over time and biomarkers in blood. These data and samples will be shared with BBRAIN researchers. Dr. Younger maintains sera and plasma stored in 0.5mL aliquots. Crosssectional studies and 150 intensive longitudinal (daily) samples are available. Half of the samples are plasma and half sera from 6,000 GWI samples, 7,200 Healthy Controls samples, 11,200 Chronic Fatigue Syndrome samples and 4,800 Fibromyalgia samples will be shared as well as several hundred brain scans from healthy control, fibromyalgia and CFS groups.

5. **Personnel exchanges:** *Nothing to Report*

6. **Other.** *Nothing to Report*

8.SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**

Nothing to report

- **QUAD CHARTS:** *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES.***

Appendix:

Please see attached publications:

Sullivan, K.; O'Callaghan, J.P. *Advancing the Role of Neuroimmunity and Genetic Susceptibility in Gulf War Illness*. *Brain Sci.* **2022**, *12*, 1068. <https://doi.org/10.3390/brainsci12081068>

Bose D, Chatterjee S, Older E, Seth R, Janulewicz P, Saha P, Mondal A, Carlson JM, Decho AW, Sullivan K, Klimas N, Lasley S, Li J, Chatterjee S. *Host gut resistome in Gulf War chronic multisymptom illness correlates with persistent inflammation*. *Commun Biol.* 2022 Jun 7;5(1):552. doi: 10.1038/s42003-022-03494-7.

Oberlin, S., Nkiliza, A., Parks, M. *et al.* Sex-specific differences in plasma lipid profiles are associated with Gulf War Illness. *J Transl Med* **20**, 73 (2022). <https://doi.org/10.1186/s12967-022-03272-3>

Cheng CH, Alshelh Z, Guan Y, Sullivan K, Loggia ML, Koo BB. *Association of the tissue microstructural diffusivity and translocator protein PET in Gulf War Illness*. *Brain Behav Immun Health.* 2021 Oct 6;18:100364. doi: 10.1016/j.bbih.2021.100364. eCollection 2021 Dec. PMID: 34693367

Yates PL, Patil A, Sun X, Niceforo A, Gill R, Callahan P, Beck W, Piermarini E, Terry AV, Sullivan KA, Baas PW, Qiang L. *A cellular approach to understanding and treating Gulf War Illness*. *Cell Mol Life Sci.* 2021 Nov;78(21-22):6941-6961. doi: 10.1007/s00018-021-03942-3. Epub 2021 Sep 27. PMID: 34580742

Cohen, D.E., K.A. Sullivan, R.B. McNeil, N.G. Klimas, R. McNeil, W. Ashford, A. Bested, J. Bunker, A. Cheema, D. Cohen, D. Cook, J. Cournoyer, T. Craddock, J. Golier, A. Hardie, D. Helmer, J.B. Lindheimer, P.J. Lloyd, K. Kerr, M. Krengel, S. Nadkarni, S. Nugent, B. Paris, M. Reinhard, P. Rumm, A. Schneiderman, K.J. Sims, L. Steele, M. Turner, K. Sullivan, L. Abdullah, M. Abreu, M. Abu-Donia, K. Aenlle, J. Arocho, E. Balbin, J. Baraniuk, K. Block, M. Block, B. DeBeer, B. Engdahl, N. Filipov, M.A. Fletcher, V. Kalasinsky, E. Kokkotou, K. Lidie, D. Little, W. Loging, M. Morris, L. Nathanson, M.D. Nichols, G. Pasinetti, D. Shungu, P. Waziry, J. VanLeeuwen, J. Younger, and N. Klimas, *A common language for Gulf War Illness (GWI) research studies: GWI common data elements*. *Life Sci*, 2021.

Keating D, Zundel CG, Abreu M, Krengel M, Aenlle K, Nichols MD, Toomey R, Chao LL, Golier J, Abdullah L, Quinn E, Heeren T, Groh JR, Koo BB, Killiany R, Loggia ML, Younger J, Baraniuk J, Janulewicz P, Ajama J, Quay M, Baas PW, Qiang L, Conboy L, Kokkotou E, O'Callaghan JP, Steele L, Klimas N, Sullivan K. *Boston biorepository, recruitment and integrative network (BBRAIN): A resource for the Gulf War Illness scientific community*. *Life Sci.* 2021 Nov 1;284:119903. doi: 10.1016/j.lfs.2021.119903. Epub 2021 Aug 26. PMID: 34453948.

Guan Y, Cheng CH, Chen W, Zhang Y, Koo S, Krengel M, Janulewicz P, Toomey R, Yang E, Bhadelia R, Steele L, Kim JH, Sullivan K, Koo BB. *Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning*. *Brain Sci.* 2020 Nov 20;10(11):884. doi: 10.3390/brainsci10110884. PMID: 33233672

Jeffrey MG, Krengel M, Kibler JL, Zundel C, Klimas NG, Sullivan K, Craddock TJA. *Neuropsychological Findings in Gulf War Illness: A Review*. *Front Psychol.* 2019 Sep 26;10:2088. doi: 10.3389/fpsyg.2019.02088. eCollection 2019. Review. PMID: 31616335. Link: <https://www.frontiersin.org/articles/10.3389/fpsyg.2019.02088/full>

Editorial

Advancing the Role of Neuroimmunity and Genetic Susceptibility in Gulf War Illness

Kimberly Sullivan ^{1,*}  and James P. O'Callaghan ^{2,*}¹ Department of Environmental Health, Boston University School of Public Health, 715 Albany St. T4W, Boston, MA 02118, USA² Molecular Neurotoxicology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, Centers for Disease Control and Prevention-NIOSH, 1000 Frederick Lane, Morgantown, WV 26508, USA* Correspondence: tty@bu.edu (K.S.); jdo5@cdc.gov (J.P.O.)

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Gulf War Illness (GWI) is a chronic multi-symptom disorder affecting as many as 30% of veterans of the 1991 Gulf War. Over the past 30 years since the war ended, an increasing body of research has accumulated on the pathobiology of this disorder. Overall, most evidence is suggestive of in-theater neurotoxicant exposures as a major underlying cause of chronic health symptoms [1]. As reflected in the titles of the two Special Issues, neuroimmune mechanisms are thought to be the primary targets of the neurotoxicant exposures that instigate the multiple symptoms modulated by underlying genetic susceptibility. Much has been learned about potential molecular targets of the disease, but a broad array of validated biomarkers of GWI are needed before the efficacy of treatments can be fully realized. Multiple animal models are now being utilized to elucidate the molecular mechanisms of GWI and evaluate potential therapeutics. The neuroimmune (i.e., brain-immune) mediators implicated in both animal studies and clinical assessments of ill veterans are suggestive of neuroinflammatory responses that likely underlie the symptoms of GWI. Recent PET imaging of neuroinflammatory responses in ill GW veterans further affirms the likely neuroimmune basis of GWI [2], emphasizing the need to expand neuroimaging analyses of GW veterans. To that end, several of the papers in the two issues demonstrate novel approaches to human brain image analyses [3,4]. The diverse imaging methodologies presented in these studies represent comprehensive approaches to achieving an in-depth understanding of brain structures/cell types affected in GWI, devising and implementing appropriate treatments. This includes using common data elements across the field [5].

A strong role for genetic susceptibility was also reinforced by findings presented in the two issues, both with respect to ill veterans (e.g., PON1 variants, immune genetic variants) [6,7] but also in novel genetic strains of mice exposed to GW-relevant neurotoxicants [8]. Among Gulf War veterans that served in the same unit, some returned ill while others did not. If genetics underlies this susceptibility, identifying the affected gene products offers hope for restoring function through genetic interventions in the future [9].

The incidence and severity of GWI may be affected by factors outside of nervous system targets that are not necessarily involved in genetic susceptibility. For example, in the first issue, the potential role of the gut microbiome in GWI was demonstrated, raising the possibility of modulating the illness through diet and selected antimicrobial therapies [10,11]. Potential objective biomarkers of GWI were identified based on blood-based central nervous system autoantibodies [12]. In addition, sex differences in CNS autoantibodies were identified in the second issue, suggesting that men and women veterans may benefit from different therapeutic strategies [13]. Finally, the following were introduced to the field with samples and data freely available to GWI researchers: a much-needed brain tissue biorepository [14], and a biorepository of existing and newly obtained biospecimens, brain imaging, and corresponding demographics, health symptom surveys and neurotoxicant exposure histories [15].

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Host gut resistome in Gulf War chronic multisymptom illness correlates with persistent inflammation

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Chronic multisymptom illness (CMI) affects a subsection of elderly and war Veterans and is associated with systemic inflammation. Here, using a mouse model of CMI and a group of Gulf War (GW) Veterans' with CMI we show the presence of an altered host resistome. Results show that antibiotic resistance genes (ARGs) are significantly altered in the CMI group in both mice and GW Veterans when compared to control. Fecal samples from GW Veterans with persistent CMI show a significant increase of resistance to a wide class of antibiotics and exhibited an array of mobile genetic elements (MGEs) distinct from normal healthy controls. The altered resistome and gene signature is correlated with mouse serum IL-6 levels. Altered resistome in mice also is correlated strongly with intestinal inflammation, decreased synaptic plasticity, reversible with fecal microbiota transplant (FMT). The results reported might help in understanding the risks to treating hospital acquired infections in this population.

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Antibiotic resistance has emerged as a threat to public health on the local and global scale^{1,2}. Resistance to antibiotics is conferred on bacteria by ARGs. All of the ARGs collectively form a resistome, and usually are carried by the opportunistic pathogens humans may encounter, thus increasing the risk of acquired infections that are difficult to treat with currently approved antibiotics^{3–6}. The evolution of drug resistance in such pathogens is driven by chromosomal mutation and the acquisition of ARGs. Since most of the ARGs are linked to MGEs, they can transfer easily through horizontal gene transfer (HGT) among different clones, taxa and habitats^{7–10}.

The human gut harbors multiple commensal microorganisms and forms a good reservoir of ARGs. The gut environment predisposes ARG transfer, sometimes leading to emergence and spread of specific bacterial clones carrying genes of resistance and/or virulence. The most frequently reported genes are those directed against tetracycline, β -lactams, aminoglycosides, and glycopeptides. Tetracycline and glycopeptide resistant genes are most common in fecal samples of human^{4,11–13}. On the other hand, human microbiota is also influenced by environmental factors like exposure to chemicals (pesticide, biocide) along with antibiotics that can alter the microbial composition and affect colonization resistance to pathogens. The selection pressure due to the exposure to such chemicals have resulted in emergence and increase of ARGs in both environment as well as gut microbiome^{14–18}. A report from Sun et al., shows that changes in living environment can alter the human gut microbiota and resistome¹². Another similar study by Gao et al. suggested that exposure to Triclosan resulted in gut microbiome and resistome alteration that persisted over a prolonged exposure^{19,20}.

Numerous studies have reported that elderly and war Veterans often suffer from multiple syndromes with inflammatory phenotypes and are more susceptible to resistance against a large number of antibiotics^{21–23}. Veterans have been reported to be resistant to amoxicillin, β -lactams, fluoroquinolones, methicillin and most importantly the carbapenem group of antibiotics^{24,25}. In our present study, we aimed to analyze the ARG and MGE patterns in a Gulf War Illness (GWI) mouse model as well as in a cohort of GW Veterans. GWI is a complex multisymptom illness often closely associated with chronic multisymptom illnesses but now classified exclusively as GWI. The above referred condition are reported by a section Veterans who returned from Operation Desert Shield/Desert Storm in 1990–1991. Symptoms reported by the GW Veterans include fatigue, headache, cognitive dysfunction, musculoskeletal pain, respiratory and gastrointestinal dysfunctions more often characterized by a persistent systemic inflammation with higher IL-6, TNF-R1 and IL-1 β blood levels. These symptoms have been linked to chemical exposures experienced during the war^{26–28}.

Several studies in preclinical mouse models of GWI have reported that exposure to chemicals such as insecticides and anti-nerve gas agents resulted in gut microbial dysbiosis. There has been a decrease in the relative abundance of several beneficial bacteria. Recently, a study in GWI Veterans also reported similar alteration of gut microbiome^{29–32}. With reported studies on microbial dysbiosis patterns in the preclinical animal models as well as the GW Veterans well established, and with studies suggesting the potential of environmental chemicals in increasing antibacterial resistance, we hypothesized that exposure to environmental pesticides and pyridostigmine bromide (PB) may also lead to an alteration of ARGs and MGEs expression, an important constituent of the gut resistome. In the present study we used both GW Veteran stool samples and fecal pellets from a GWI persistence mouse model administered with representative GW chemicals to study the alteration in gut resistome. We also studied the possible associations between gut resistome and GWI

proinflammatory pathology by using a prolonged fecal microbiota transfer regimen that aimed to recolonize the mouse gut with microbiota from a healthy donor.

Results

Characterization of antimicrobial resistance in mouse fecal samples. We performed whole-genome shotgun sequencing, metagenomic assembly, and functional gene annotation on fecal samples collected from three groups (Control, GWI, and GWI_FMT) to construct ARG profiles associated with the GWI mice model and the FMT treatment. We detected 455 unique ARGs across all groups with 538 ± 16 (mean $\pm$ standard error) total ARGs in Control, 514 ± 15 total ARGs in GWI, and 567 ± 19 total ARGs in GWI_FMT. Performing PERMANOVA revealed a significant deviation in the ARG profiles across sample groups ($p = 0.0001$, $R^2 = 34\%$). This indicated that the resistome profile of each sample group were distinct from each other. To get a clearer picture of the specific changes in the resistome, we performed differential abundance analysis of the ARGs to identify significantly changed genes (DESeq2, negative binomial generalized linear models (GLMs), Wald's test, $p < 0.05$). Comparing these selected ARGs between sample groups, we observed a significant decrease in their sum relative abundances from Control to GWI ($p = 0.02021$, Welch two-sample t -test) and a significant increase when comparing GWI to GWI_FMT group ($p = 0.00005425$, Welch two-sample t -test, Fig. 1a).

Looking to connect ARG changes with gut microbiome composition, we performed procrustes rotation of the principal component analyses (PCAs) of the resistome and microbiome profiles which showed a significant correlation ($p = 0.0001$, PROTEST, $M^2 = 0.4302$, Fig. 1b), confirming that resistome changes were in fact linked to microbial dysbiosis. Further, principal coordinate analysis of ARG profiles showed clustering of profiles according to sample group with the first two axes accounting for 49% of the variance in the ARG profiles. The GWI_FMT group formed an independent cluster from both GWI and Control groups (ape, pcoa, PCoA1 accounting for 34% of variance in ARG profile, Fig. 1c).

We also examined the profiles of MGEs constituting the mobilome in each sample group. We detected 77 unique MGEs evenly distributed across all groups with 77 ± 3 total MGEs in Control, 74 ± 2 total MGEs in GWI, and 81 ± 3 total MGEs in GWI_FMT. PERMANOVA showed significant differences in the mobilomes with respect to sample group, supporting the observed deviations in resistome profile which we previously linked to changes in the microbiome composition ($p = 0.0001$, $R^2 = 24\%$). We selected differentially abundant MGEs (DESeq2, negative binomial GLMs, Wald's test, $p < 0.05$), compared their sum relative abundances as previously described for ARGs. The changes observed in the sum relative abundances between Control to GWI ($p = 0.4717$, Welch two-sample t -test) and between GWI and GWI_FMT ($p = 0.1119$, Welch two-sample t -test, Fig. 1d) were not statistically significant. Hence, on administration of representative GW chemicals in GWI mice model, we did not see an increase in the relative abundance of ARGs and the changes observed in the relative abundances of MGEs were not significant.

Distribution of ARGs and MGEs across the different groups in GWI mouse model. We detected multiple ARGs imparting resistance to antimicrobial classes which have been marked as highly important and critically important by the World Health Organization (WHO) (AGISAR, 2018). In our analysis, glycopeptide resistance genes were the most abundant ARG observed across all three mice groups (not statistically significant). Using

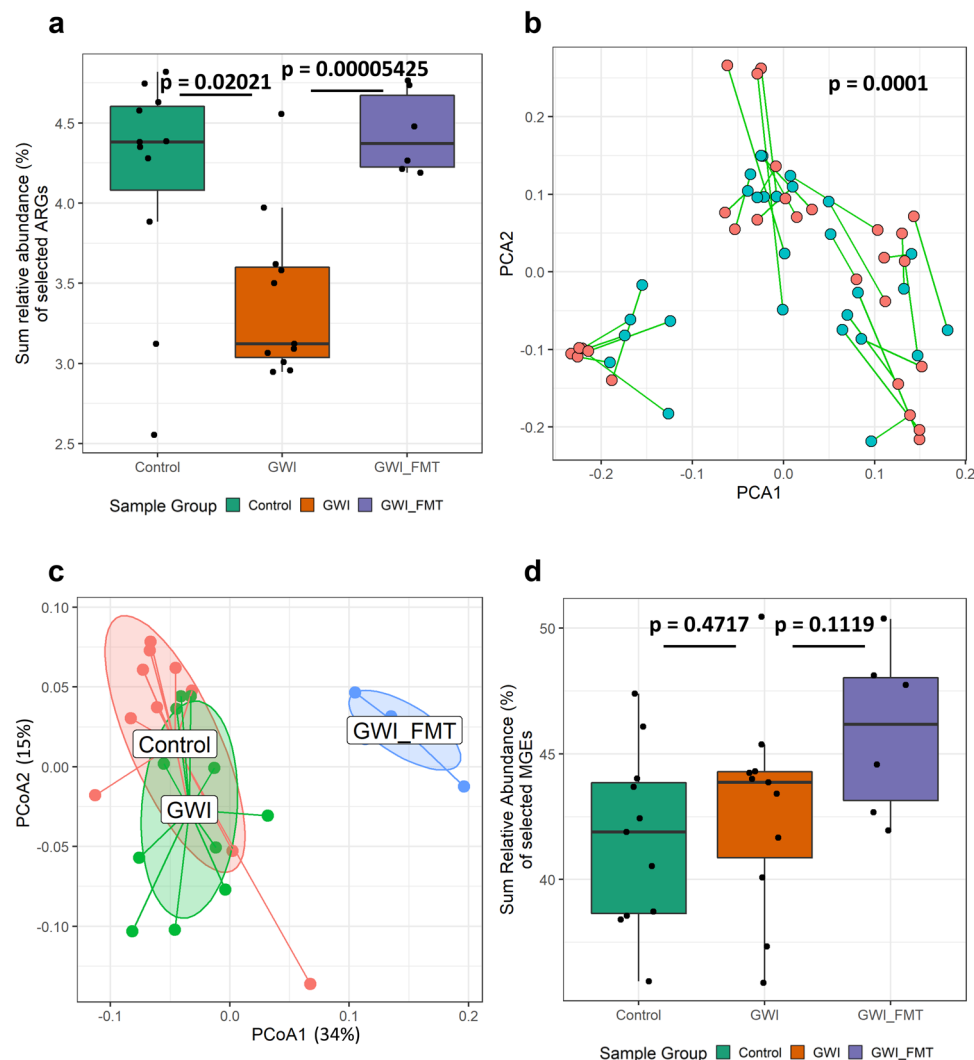


Fig. 1 Distribution of ARGs and MGEs in persistence GWI mouse models. **a** Box plots showing sum relative abundance (%) of selected antibiotic resistance genes (ARGs) in Control (adult C57BL/6 J mice administered with vehicle, $n = 11$ biologically independent samples), GWI (adult C57BL/6 J mice administered with GW chemicals, $n = 11$ biologically independent samples) and GWI_FMT (adult C57BL/6 J mice group exposed to GW chemicals followed by Fecal Microbiota Transfer (FMT) treatment, $n = 6$ biologically independent samples). Median values of 4.38% in Control, 3.12% in GWI, and 4.37% in GWI_FMT indicated by solid black lines. **b** Procrustes rotation analysis comparing resistome (blue) and microbiome (red) changes using PCA ordinations, (X-axis: PCA1, Y-axis: PCA2, PROTEST, $M^2 = 0.4302$, $p = 0.0001$). **c** PCoA of Bray-Curtis dissimilarity between ARG and Taxa profiles across sample groups. Principal coordinate axes PCoA1 (34%) and PCoA2 (15%) are calculated to explain 49% of variance detected. Lines connect points from the center of gravity of each sample group. **d** Box plots showing sum relative abundance (%) of selected mobile genetic elements (MGEs) Control, GWI and GWI_FMT. Boxes in the box plots indicate interquartile range. Error bars in the box plots extend to the most extreme values within 1.5 times the interquartile range. Outlier data points are represented by black dots drawn outside of the box plot. p -values were determined using PROTEST or Welch's t -test where $p < 0.05$ was considered statistically significant.

differential abundance analysis, we identified the individual ARGs which were significantly altered (DESeq2, negative binomial GLMs, Wald's test, $p < 0.05$). Among these ARGs, *vanXYN* (ARO: 3002969), and *adeS* (ARO: 3000549) were significantly increased in the GWI group compared to the Control group (DESeq2, negative binomial GLMs, Wald's test, $p < 0.05$, Fig. 2a). This result is suggestive of a potential effect of GW chemicals on the individual ARGs. FMT treatment decreased the abundance of *vanXYN* and *adeS* genes as observed in the GWI_FMT group over the GWI group, however it was not statistically significant. Interestingly, there were 6 unique ARGs which were only present in the mouse GWI group.

When comparing the resistant drug classes across the three groups, it was observed that glycopeptide antibiotics followed by aminoglycosides, peptide, phenicol, and diaminopyrimidine

antibiotics were among the resistant drug classes that were detected in the 3 experimental mice groups ($p = 0.0001$, PERMANOVA, $R^2 = 33\%$, Fig. 2b). Based on their mechanisms of resistance, antibiotic efflux, antibiotic target alteration, antibiotic target protection, and antibiotic inactivation mechanisms were among the antibiotic resistance mechanisms that were detected in the 3 experimental mice groups ($p = 0.0001$, PERMANOVA, $R^2 = 31\%$, Fig. 2c).

ARGs have a high propensity to mobilize from one bacterium to another through the MGEs by horizontal gene transfer⁹. We thus investigated the presence of different MGEs and MGE types among the three mice groups. We observed that relative abundances of *ISCR8*, *ISCRsp1*, and *Tn916-orf6* increased (not statistically significant) in the GWI group compared to Control but significantly decrease in the GWI_FMT group (DESeq2,

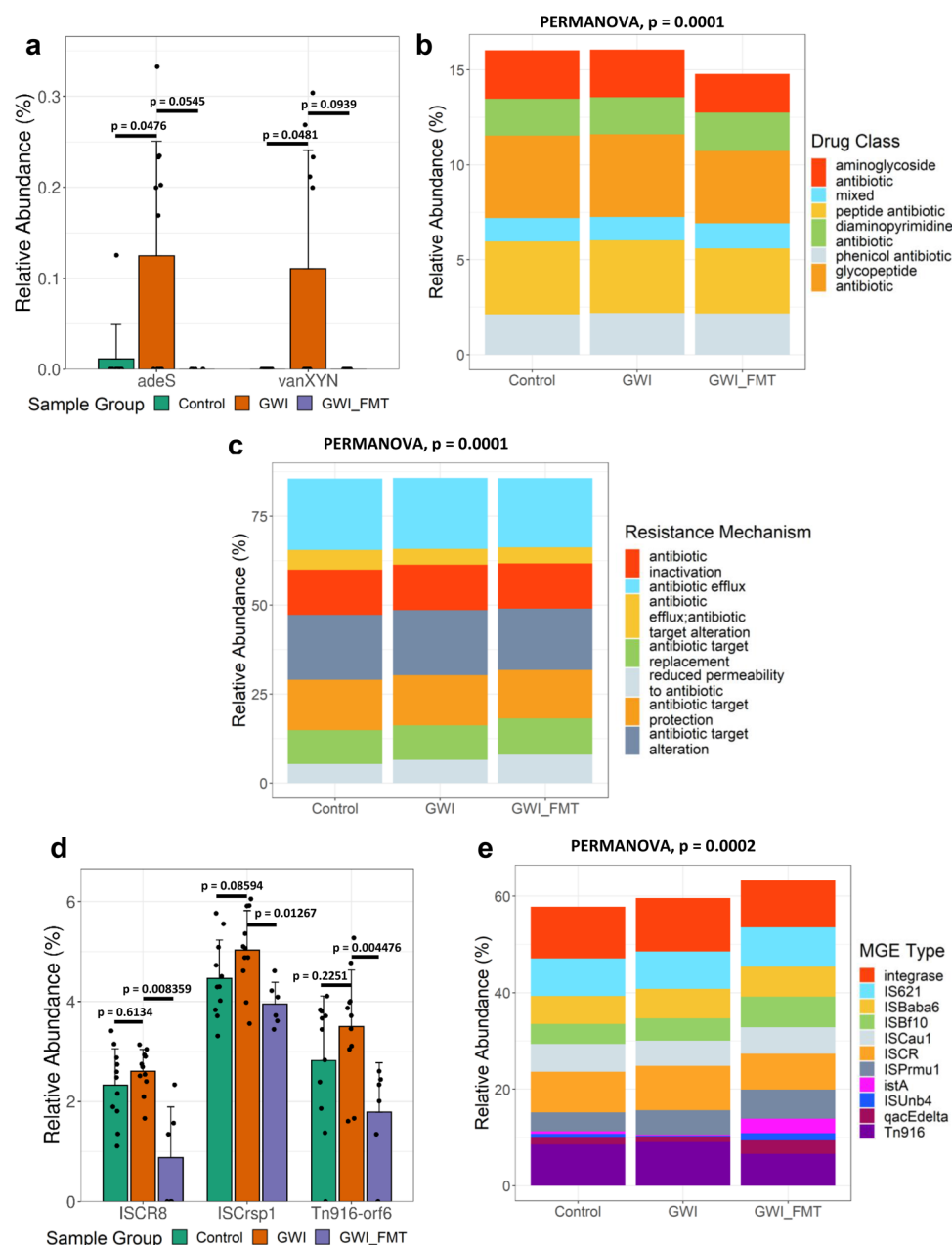


Fig. 2 Classification of selected ARGs and MGEs in persistence GWI mouse model. **a** Grouped bar graph showing relative abundance (%) of selected ARGs in Control (adult C57BL/6 J mice administered with vehicle, $n = 11$ biologically independent samples), GWI (adult C57BL/6 J mice administered with GW chemicals, $n = 11$ biologically independent samples), and GWI_FMT (adult C57BL/6 J mice group exposed to GW chemicals followed by FMT treatment, $n = 6$ biologically independent samples) groups. **b** Stacked bar analysis of relative abundance (%) of drug classes resistances and **c** mechanisms of resistance. **d** Grouped bar graph showing relative abundance (%) of selected MGEs in Control, GWI and GWI_FMT groups. **e** Stacked bar analysis of relative abundance (%) of MGE types. Data represented as Mean $\pm$ SD (SD: Standard deviation) for the bar graphs. p -values were determined using Wald's test or PERMANOVA where $p < 0.05$ was considered statistically significant.

negative binomial GLMs, Wald's test, $p < 0.05$, Fig. 2d). Transposase, insertional sequences *ISCR* and integrase were among the abundant MGE types detected in all the samples across the 3 mice groups (Fig. 2e). Multivariate analysis of the MGE type profiles showed a significant deviation and correlation pattern that was dependent on sample groups ($p = 0.0002$, PERMANOVA, $R^2 = 33\%$).

Demographic Information of GW Veterans. In this study, we obtained samples from 33 GW Veterans. Out of these 33

participants, 28 met the required Kansas GWI Criteria and were categorized into the Hum_GWI group. The remaining 5 participants did not meet the criteria and were considered as the Hum_Control group. There were no major differences based on age, height, weight, or body mass index of the participants in both groups (Supplementary Table 2). In the Hum_GWI group, 61% of the participants reported diarrhea and nausea, 57% reported abdominal pain or cramping, 46% reported irritable bowel syndrome (IBS). In the Hum_Control group, there were no reports of diarrhea or IBS, but 40% of participants reported nausea and 20% reported abdominal pain or cramping.

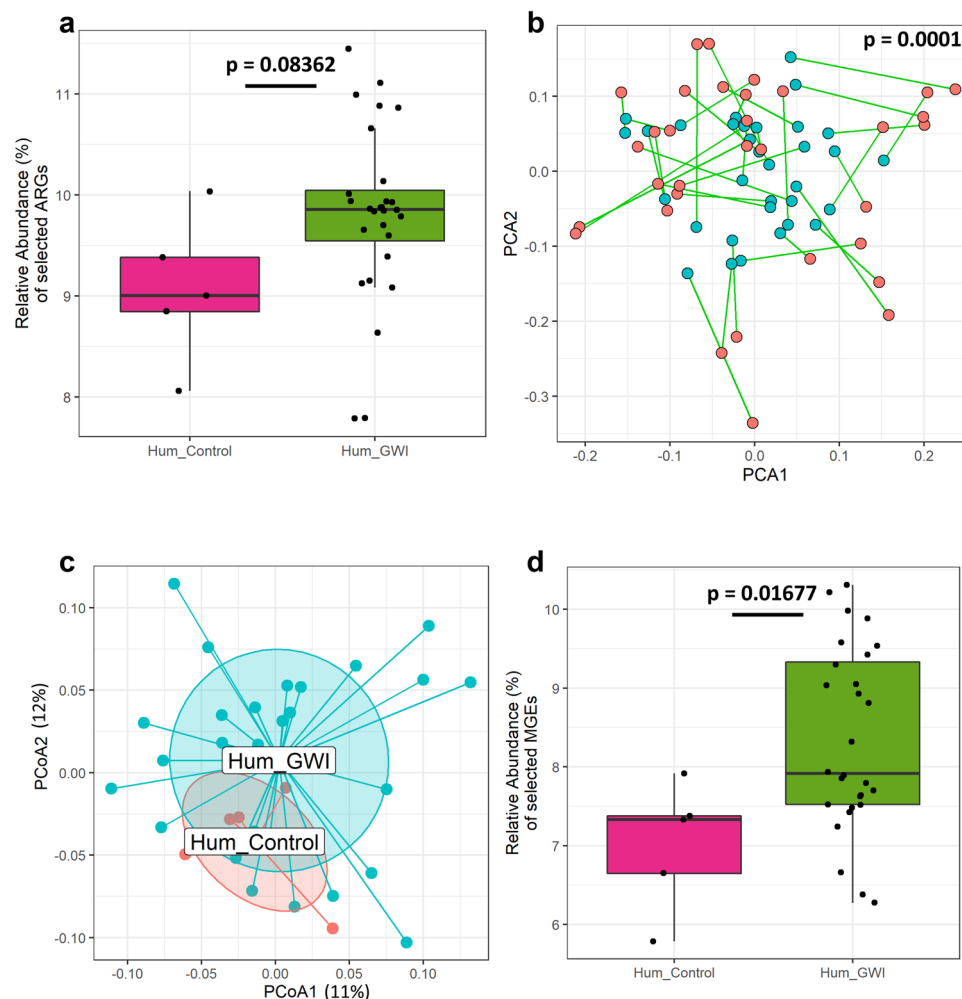


Fig. 3 Distribution of ARGs and MGEs in GWI Veteran groups. **a** Box plot showing relative abundance (%) of ARGs in Hum_Control (Veteran group without GWI symptom, $n = 5$ biologically independent samples) and Hum_GWI (Veteran group with GWI symptom, $n = 28$ biologically independent samples). Median values of 9.0% in Hum_Control and 9.86% in Hum_GWI are indicated by solid black lines. **b** Procrustes rotation analysis comparing resistome (blue) and microbiome (red) changes using PCA ordinations, (X-axis: PCA1, Y-axis: PCA2, PROTEST, $M^2 = 0.6665$, $p = 0.0001$). **c** PCoA of Bray-Curtis dissimilarity between ARGs and Taxa. Principal coordinate axes PCoA1(12.7%) and PCoA2 (11.5%) account for 24.2% of detected variance. **d** Box plot showing relative abundance (%) of selected MGEs in Hum_Control and Hum_GWI. Boxes in the box plots indicate interquartile range. Error bars in the box plots extend to the most extreme values within 1.5 times the interquartile range. Outlier data points are represented by black dots drawn outside of the box plot. p -values were determined using PROTEST or Welch's t -test where $p < 0.05$ was considered statistically significant.

Characterization of antimicrobial resistance gene in Veteran samples. We studied the gut resistome pattern in GW Veterans. We performed whole-genome shotgun sequencing, metagenomic assembly, and functional gene annotation as with the mouse samples. We detected 604 unique ARGs with 224 ± 11 and 238 ± 6 total occurrences in the Hum_Control group and Hum_GWI groups respectively. Comparing the ARG profiles of the two groups, we observed only minor and insignificant deviation in the resistome profiles between the two groups ($p = 0.2826$, PERMANOVA, $R^2 = 3.4\%$). When investigating relative abundance of significantly changed ARGs (DESeq2, negative binomial GLMs, Wald's test, $p < 0.05$), we observed that the sum relative abundance of selected ARGs increased but not statistically significant in the Hum_GWI group compared to the Hum_Control group ($p = 0.08362$, Fig. 3a). Procrustes analysis showed a significant correlation of bacterial taxa and the ARGs ($p = 0.0001$, PROTEST, $M^2 = 0.6665$, Fig. 3b). PCoA analysis showed clustering of the Hum_Control and Hum_GWI groups with major overlap on the first two axes accounting for only 24% of the total variance in the dataset (Fig. 3c). Considering the

minor divergence indicated by PERMANOVA and the low proportion of explained variance, this result might have stemmed from the increased complexity of the human gut microbiome compared to that of our mouse model.

When studying the transferability, we identified 110 unique MGEs across both groups with 80 ± 5 and 86 ± 2 total MGEs in the Hum_Control group and the Hum_GWI groups respectively. We observed minor variation in the MGE profiles of the two groups which was shown to not be significantly dependent on the sample group variable ($p = 0.3757$, PERMANOVA, $R^2 = 3.3\%$). The relative abundance of selected MGEs were significantly increased in Hum_GWI group compared to the Hum_Control ($p = 0.01677$, Welch two-sample t -test, Fig. 3d).

Distribution of selected ARGs and MGEs in human samples. Our results showed that the relative abundance of individual ARGs was significantly higher in Hum_GWI group compared to Hum_Control group. The glycopeptide resistance gene was found to be the most abundant ARG in both groups of GW Veteran

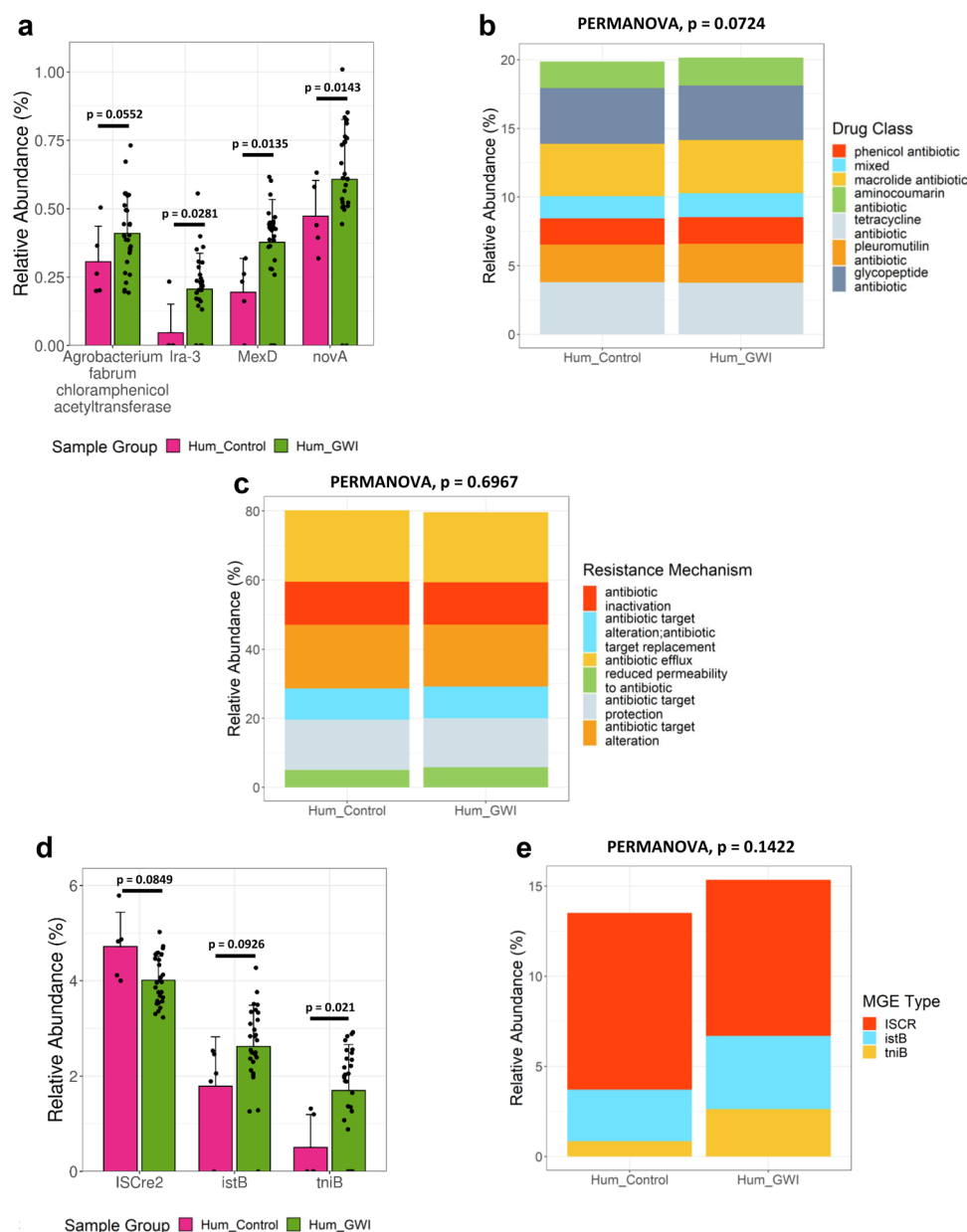


Fig. 4 Classification of selected ARGs and MGEs in GWI Veterans groups. **a** Grouped bar graph showing relative abundance (%) of selected ARGs in Hum_Control (Veteran group without GWI symptom, $n = 5$ biologically independent samples) and Hum_GWI (Veteran group with GWI symptom, $n = 28$ biologically independent samples). Stacked bar analysis of relative abundance (%) of **b** drug classes and **c** mechanisms of resistance. **d** Grouped bar graph showing relative abundance (%) of selected MGEs in Hum_Control and Hum_GWI groups. **e** Stacked bar analysis of relative abundance (%) of MGE types. Data represented as Mean $\pm$ SD (SD: Standard deviation) for the bar graphs. p -values were determined using Wald's test or PERMANOVA where $p < 0.05$ was considered statistically significant.

samples (not statistically significant). Based on differential abundance analysis, we found that the abundance of 3 ARGs were significantly increased in the Hum_GWI group compared to the Hum_Control group (DESeq2, negative binomial GLMs, Wald's test, $p < 0.05$, Fig. 4a). These ARGs were *lra-3* (ARO: 3002510), *mexD* (ARO: 3000801) and *novA* (ARO: 3002522). *Agrobacterium fabrum chloramphenicol acetyltransferase* (*afca*) (ARO: 3004451) increased in Hum_GWI but it was not statistically significant. These ARGs confer resistance against multiple critically important antibiotics which are used as human medicine (WHO, AGISAR 2018). Furthermore, there were 250 unique ARGs found in Hum_GWI group which were absent in

Hum_Control group. Changes in the drug classes and antibiotic resistance mechanism were not statistically significant between the 2 groups (Fig. 4b, c).

The abundance of individual MGEs were also altered in Hum_GWI group compared to Hum_Control group. The MGE *tniB* significantly increased in the Hum_GWI group ($p = 0.021$, DESeq2, negative binomial GLMs, Wald's test, Fig. 4d). Although a change in the relative abundance of *istB* and *ISCR2* was observed in Hum_GWI group over the Hum_Control group, it was not statistically significant. Among the MGE types, transposons, and most importantly insertional sequence *ISCR* was increased in the Hum_GWI group (not statistically

significant, Fig. 4e). ISCR has been reported to cause increased mobilization of ARGs among bacteria, presence of this type of mobilome in GW Veterans raises the concern of increased antibiotic resistance³³. According to our procrustes analysis in GW Veteran samples, changes in the resistome are correlated with the altered gut microbiome, hence increases in MGEs like ISCR might increase the chances of ARG mobilization which could be fatal for the GW Veteran health due to increase in resistance to antibiotics. Multivariate analysis of the MGE class profiles revealed minor deviation between sample groups which was not significant ($p = 0.1422$, PERMANOVA, $R^2 = 4.5\%$). Further investigations in GW Veterans are required to prove the resistance against the individual ARGs.

Expression study of antimicrobial resistance genes in mouse and GW Veteran samples by q-RTPCR analysis. To confirm the expression of the ARGs observed by the gene annotation studies in the previous sections (differential abundance by DESeq2), we performed q-RTPCR analysis of the same ARGs, using DNA extracted from the mouse (Fig. 5a) and GW Veteran fecal samples (Fig. 5b).

In the mouse samples, expression of *vanXYN* was significantly increased by 17.65-fold in the GWI group compared to the Control group ($p < 0.001$, two-way ANOVA with Bonferroni's post hoc test). Expression of *vanXYN* (12.87 fold compared to the Control group) significantly decreased in the GWI_FMT group compared to the GWI group ($p = 0.002$, two-way ANOVA with Bonferroni's post hoc test). Similarly, expression of *adeS* was significantly increased by 23.59-fold ($p < 0.001$, two-way ANOVA with Bonferroni's post hoc test) in the GWI group compared to the Control group. The expression of *adeS* (12.63 fold compared to the Control group) significantly decreased ($p < 0.001$, two-way ANOVA with Bonferroni's post hoc test) in the GWI_FMT group compared to the GWI group. Although q-RTPCR analysis results showed similar trend like the gene annotation study, i.e., significant increase in expression of the respective ARGs in the mice GWI group and a decrease (statistically significant by q-RTPCR) in GWI_FMT group, further experimental proof would be required to confirm that FMT administration indeed was successful in decreasing the individual ARG expressions.

In the GW Veteran samples, expression of *mexD*, *novA*, and *afca* was significantly increased by 3.68, 5.59, and 2.81-fold respectively in the Hum_GWI group compared to the Hum_Control group (for *mexD* $p < 0.001$, for *novA* $p < 0.001$, for *afca* $p < 0.001$ between Hum_Control and Hum_GWI group, two-way ANOVA with Bonferroni's post hoc test, Fig. 5b).

Gastrointestinal, systemic, and neuronal inflammation and its association with ARGs and Drug Classes in mouse GWI model samples and GW Veteran samples. Gastrointestinal and neuronal inflammation is reported in GW Veterans and in preclinical GWI mice models due to the influence of GW chemicals^{31,34–38}. Increases in systemic inflammatory markers were also reported in GW Veterans and mouse models^{31,38–40}. Following our study of the abundance and expression of ARGs and MGEs, we sought to identify a link between ARGs and biomarkers of GWI pathology which would aid in establishing a role of gut resistome in influencing host health. The purpose of this study was also to show whether a predictive insight can be made regarding future susceptibility to infectious diseases related to hospital acquired infections in GW Veterans, elderly, and immunocompromised individuals. Results in mice showed that the expression of IL-1 β in the small intestine significantly increased in GWI group ($p < 0.001$, one-way ANOVA with Tukey's post hoc test) compared to the Control group, as shown through immunoreactivity

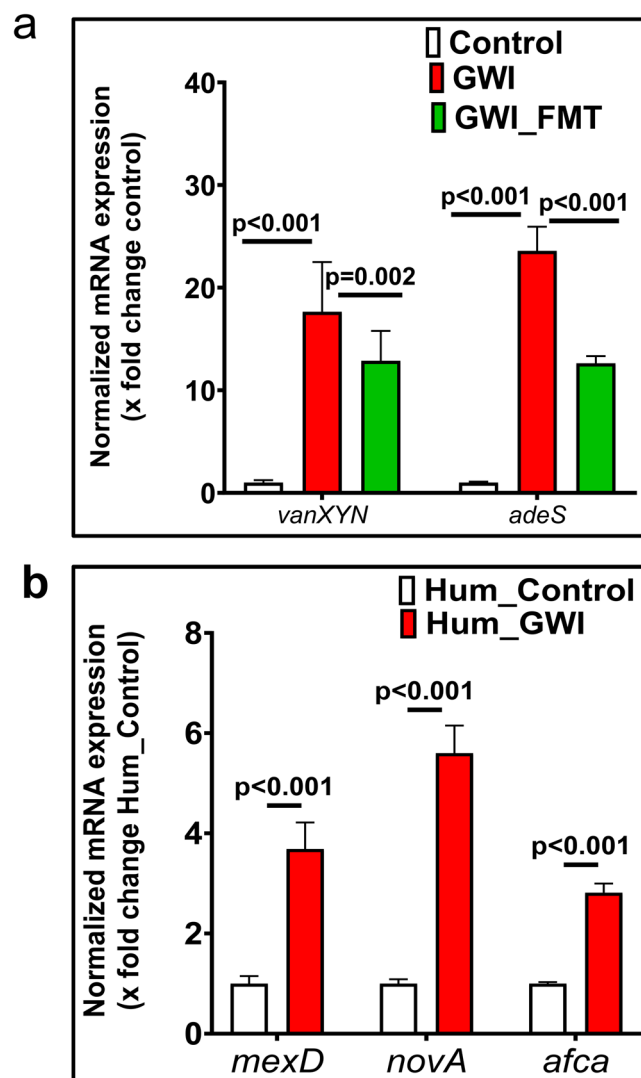
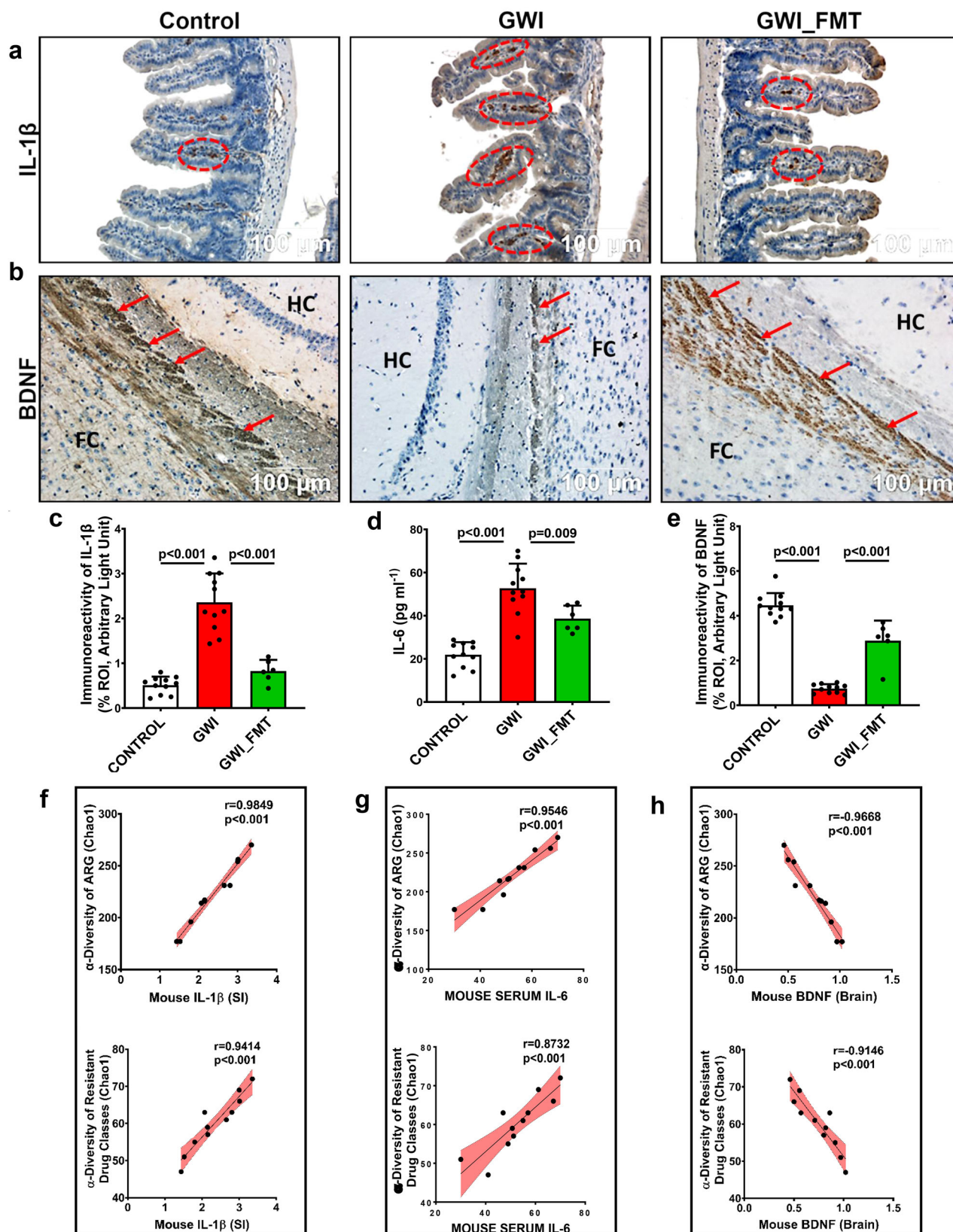


Fig. 5 Relative expression levels of antimicrobial resistance genes.

Relative mRNA expression of ARGs. **a** Between mouse experimental groups Control ($n = 11$ biologically independent samples), GWI ($n = 11$ biologically independent samples) and GWI_FMT ($n = 6$ biologically independent samples). **b** Between GW Veteran groups Hum_Control ($n = 5$ biologically independent samples) and Hum_GWI ($n = 28$ biologically independent samples). The mRNA expression was calculated as fold change against control (in Fig. 6a) and against Hum_Control (in Fig. 6b). Data are represented as mean $\pm$ SEM (SEM: Standard Error Mean). p -values were determined using by two-way ANOVA with Bonferroni's post hoc test for GWI mice groups and GW Veteran groups where $p < 0.05$ was considered statistically significant.

of the cytokine in the villi (Fig. 6a, c). Treatment with FMT significantly decreased the expression of IL-1 β in the GWI_FMT mice group compared to the GWI group ($p < 0.001$, one-way ANOVA with Tukey's post hoc test, Fig. 6a, c). To study the association between IL-1 β expression and ARG diversity, we performed a correlation analysis. Results showed a positive correlation ($r = 0.9849$, $p < 0.001$ and $r = 0.9414$, $p < 0.001$ respectively, Pearson regression analysis) between α -diversity of ARGs, resistant drug classes and increased IL-1 β in GWI mice group, suggesting that alteration of the gut resistome profile had a significant association with gastrointestinal inflammation (Fig. 6f).

We observed a significant increase in serum IL-6 level in the GWI mice group when compared to the Control. Further, there



was a significant decrease in serum IL-6 levels in GWI_FMT groups when compared to GWI group ($p < 0.001$ between GWI and Control, $p = 0.009$ between GWI and GWI_FMT, one-way ANOVA with Tukey's post hoc test, Fig. 6d). We also observed that α -diversity of ARGs and resistant drug classes were positively correlated ($r = 0.9546$, $p < 0.001$ and $r = 0.8732$, $p < 0.001$,

Pearson regression analysis) with increased systemic IL-6 level in the GWI mice group (Fig. 6g).

Our previous studies have shown that a decrease in synaptic plasticity marker brain derived neurotrophic factor (BDNF) played a key role in brain pathology in GW chemical exposed mice⁴¹. Results showed that the expression of BDNF significantly decreased

Fig. 6 Gastrointestinal, systemic, brain inflammation and its correlation with antibiotic resistance genes and the resistant drug classes in GWI mice model. **a** Representative immunohistochemistry image showing immunoreactivity of proinflammatory cytokine IL-1 β (marked by red circle) in mouse experimental groups Control ($n = 11$ biologically independent samples), GWI ($n = 11$ biologically independent samples) and GWI_FMT ($n = 6$ biologically independent samples). Images were taken at 20X magnification. Scale bar = 100 μ m. **b** Representative immunohistochemistry image showing immunoreactivity of synaptic plasticity marker BDNF (marked by red arrows) in Control ($n = 11$ biologically independent samples), GWI ($n = 11$ biologically independent samples), GWI_FMT ($n = 6$ biologically independent samples) mice groups. Images were taken at 20X magnification. Scale bar = 100 μ m. FC Frontal Cortex, HC Hippocampus. **c** Bar graph depicting immunoreactivity of IL-1 β . Data are represented as mean $\pm$ SD (SD: Standard deviation) of %ROI (mean value calculated from two different fields in each sample). **d** Bar graph depicting the serum IL-6 level at pg/ml in Control, GWI, GWI_FMT mice groups. Data represented as mean $\pm$ SD. **e** Bar graph depicting immunoreactivity of BDNF. Results are represented as mean $\pm$ SD of %ROI (mean value calculated from 2 different fields in each sample). **f** Correlation plot between α -diversity (Chao1) of resistant ARGs and drug classes and immunoreactivity of IL-1 β in mouse GW group in small intestine section. **g** Correlation plot between α -diversity (Chao1) of resistant ARGs and drug classes and serum IL-6 level in mouse GW group. **h** Correlation plot between α -diversity (Chao1) of resistant ARGs and drug classes and immunoreactivity of BDNF in mouse GW group in brain section. r -value was determined by Pearson's Regression analysis. Pearson's linear regression is shown in red with 95% confidence bands. Statistical significance was analyzed by one-way ANOVA (Tukey's post hoc test) where $p < 0.05$ was considered statistically significant.

in GWI group when compared to Control. Further, the levels of BDNF as determined by morphometry from immunohistochemical analysis showed that there was a significant increase in the BDNF levels in the GWI_FMT mice groups when compared to GWI group ($p < 0.001$ between Control vs GWI; $p < 0.001$ between GWI vs GWI_FMT respectively, one-way ANOVA with Tukey's post hoc test, Fig. 6b, e). Interestingly, a negative correlation was observed between BDNF and ARGs and resistant drug classes ($r = -0.9668$, $p < 0.001$ and $r = -0.9146$, $p < 0.001$ respectively, Pearson regression analysis) suggesting that increased ARG- α -diversity may have a strong influence on observed neuroinflammation in the GWI mice group (Fig. 6h).

Furthermore, we studied the level of systemic IL-6 in the serum collected from the respective GW Veterans in this study. Results showed a significantly increased level of serum IL-6 in the Hum_GWI group compared to the Hum_Control group ($p = 0.046$, Welch's t -test; Fig. 7a). Correlation analysis between serum IL-6 level and the α -diversity of ARGs and resistant drug classes ($r = 0.9173$, $p < 0.001$ and $r = 0.4840$, $p = 0.042$ respectively, Pearson regression analysis) in GW Veterans showed a positive correlation (Fig. 7b, c). This results may be suggestive of a link between altered gut resistome and increased systemic IL-6 in the human samples. However, further experimentation is required to confirm this hypothesis.

Discussion

In the present study, we report an alteration of host gut resistome in chronic multisymptom illness as observed in a subsection of Gulf War Veterans. We also report the association of systemic inflammation with an altered gut resistome that in turn is further correlated with increased circulating proinflammatory cytokine IL-6, intestinal pathology and neurotrophic factor BDNF in preclinical GWI mice model. Most importantly, we have shown that increased systemic IL-6 had strong association with altered human gut resistome in GW Veterans. IL-6 has been shown to be a pleiotropic cytokine and is key to gastrointestinal disturbances, and cognitive deficits^{42,43}. Recent advances in understanding the host resistome improved our knowledge about evolution, origins and emergence of antibiotic resistance though the field is continuously evolving⁴⁴. Previously, our knowledge of resistome included proto-resistance and silent-resistance genes⁴⁴. Silent-resistance genes do not cause phenotypic resistance until they are transferred via MGEs, or a mutation occurs in the associated regulatory elements. Gut microbiome is usually associated with a silent resistome, as they have the potential to contribute in clinical resistance through mobilization⁴⁴. Our study supports the existing evidence that the resistome is highly dependent on the gut microbiome and indicates to a possible association between changes in the bacteriome and the gut resistome, particularly in

GWI groups, both in the case of mouse models and Veterans with GWI as studied by PERMANOVA and procrustes analysis (Figs. 1b and 3b).

Exposure to environmental chemicals like pesticides, biocides, aerosols have been reported to the increase in antimicrobial resistance via HGT which have long-lasting changes and increase in these resistant bacterial population in human due to selection pressure leads to treatment failure with available antibiotics^{12,16,17,19,45–48}. This led us to our hypothesis that exposure to a mixture of environmental hazards (war theater) in GW Veterans as well as representative GW chemical exposure in GWI mice model may affect the gut resistome. Our results suggested that representative GW chemicals PB and permethrin (Per) may change the ARGs significantly, but the changes observed in individual MGE profiles in GWI group of the mouse model when compared to the control group were not significant. One of the limitations in this study includes our inability to experimentally confirm that the GW chemical-treated mice were indeed resistant to the classes of antibiotics or the individual ARGs that has been found in this study. However, we would also like to state that to the best of our knowledge no GWI mice model could exactly mimic the health condition of the GW Veterans. Different combinations of GW chemicals along with different routes of administration needs to be included during the various GWI mice model studies to further elucidate the mechanisms proposed. Future studies need to overcome these limitations for in-depth mechanistic links to gut resistome in GWI mice models.

Previous reports stated that elderly individuals and Veterans had increased resistance to sulfonamide, macrolide, β -lactam antibiotics followed by tetracycline as well as fluoroquinolones especially found in *Acinetobacter baumannii* isolates^{49,50}. Reports also stated that fluoroquinolones and cephalosporin usage should be prescribed in a limited manner among the elderly and Veterans as they have higher susceptibility in developing resistances upon treatment with such high generation antibiotics⁵⁰. However, there is very little evidence about the ARGs in Veterans, especially GW Veterans suffering from CMI or collectively referred as GWI who were deployed 30 years back and are presently in the age range of 50–60 years. The present study attempted to report the alteration of ARG and MGE signature in GW Veterans belonging to the aforementioned age group. Another limitation of the present study includes the inability to confirm whether the gut resistome alteration is a result of GW chemical exposures alone. GW Veterans may have been exposed to multiple courses of antibiotics at different points in their lifetime, along with this factors like diet, metabolic conditions like obesity that might prompt an increase in the altered gut resistome. A further limitation in our analysis of gut resistome in GW Veterans may be whether the GW Veterans recruited in our study

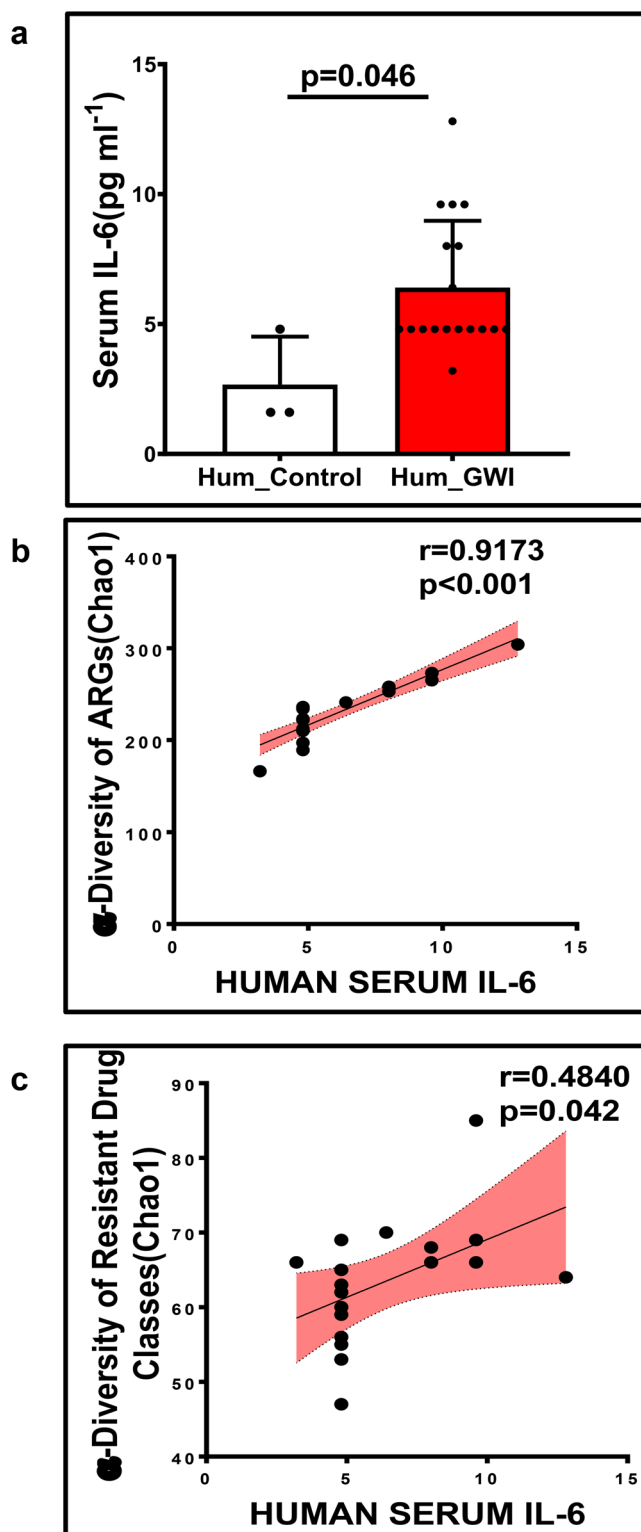


Fig. 7 Systemic IL-6 level and its correlation with antibiotic resistance genes and the resistant drug classes in GW Veteran samples. **a** Bar graph depicting the serum IL-6 level at pg/ml in Veteran control group Hum_Control ($n=3$ biologically independent samples) and Hum_GWI ($n=18$ biologically independent samples) groups. Correlation plot between α -diversity (Chao1) of **b** resistant ARGs and **c** resistant drug classes and serum IL-6 in Hum_GW group. Data are represented as mean $\pm$ SD (SD standard deviation). r -value was determined by Pearson's regression analysis. Pearson's linear regression is shown in red with 95% confidence bands. Statistical significance was analyzed by Welch's two-tailed t -test where $p < 0.05$ was considered statistically significant.

Vancomycin is used to treat bacterial infections like methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, and *Enterococcus spp.*⁵¹. Recently it has been reported that administration of Vancomycin in patients at Veterans Affairs Hospitals have been ineffective against these clinically important bacteria and causing nephrotoxicity^{52–55}. Increased glycopeptide resistance as detected in our GW Veteran samples might be suggestive of the reason behind the ineffectiveness of Vancomycin antibiotic used to treat a section of Veterans. A further study of the resistant drug classes as well as mechanisms of resistance could be helpful to confirm this result.

FMT has been reported to restore the beneficial microbiome in pathological conditions like inflammatory bowel disease (IBD), type 2 diabetes, nonalcoholic steatohepatitis and neurological disorders where gut microbial dysbiosis plays a direct role in the disease progression^{56–62}. Studies have also reported that FMT administration have decreased the ARGs in patients with cirrhosis and *Clostridium difficile* infection⁶². In the present study, we aimed to study if FMT could be a therapeutic by decreasing antimicrobial resistance in GWI condition in preclinical GWI mice model. We observed a decrease in relative individual ARGs and MGEs in GWI_FMT group compared to GWI group in the GWI mice model (Fig. 2a, d). FMT treatment was able to significantly ameliorate GW chemical induced gastrointestinal inflammation and systemic inflammation and significantly increase synaptic plasticity marker expression. Moreover, future dosing considerations are required in FMT treatment since prolonged FMT treatment may cause stress alteration of gut microbiome and resistome. This also emphasizes the need for screening the fecal samples of healthy donors for FMT which has been a limitation⁶³. Hence, these criteria should be considered in using FMT as a therapeutic measure to ameliorate gut resistome alteration and systemic inflammation in GW Veterans through carefully designed clinical trials.

Interestingly, we observed the accumulation of ARGs that are highly mobile and transferable. The mice in the preclinical GWI model were never exposed to any classes of antibiotics during the entire course of the study, but they showed a spontaneous acquisition of unique ARGs in GW chemical exposed group but not in Control and GWI_FMT groups. This transfer may occur due to intrinsic or extrinsic transfer by HGT method based on MGE patterns. This study also suggested that GW chemical exposure may be responsible for the alteration and appearance of ARGs and MGEs in mice since they have been maintained in a controlled environment with very limited risk of exposure to a multifaceted environment, though the presence of these ARGs needs to be further elucidated.

We were able to identify diverse types of MGEs in which transposons were majorly observed in both mouse and GW Veteran samples. A study by Parnanen et al. stated that transposase constitutes the most abundant MGE class²⁰. This study completely is consistent with our observations about MGEs.

were actually resistant to the ARGs and antibiotics reported. The limitation is also confounded by the small sample size of Veterans that may have caused low significance of our ARG and MGE results. To overcome these limitations, future studies with a large cohorts with sufficient power would help to obtain more in-depth knowledge about the antibiotic resistance in GWI Veterans. We identified that glycopeptide resistance was high in GWI compared to other mouse groups as well as in GW Veterans (not statistically significant). Glycopeptide group of antibiotics especially

Higher abundance of MGEs belonging to transposon group along with integrons, insertional sequence might indicate a probability for inter-bacterial ARG transfer which might increase the adverse consequences due to increase in antimicrobial resistance in GWI pathology^{9,64,65}. To track the ARG transfer through MGE, further detailed investigation is needed to identify the ARGs associated with the MGEs.

Studies have also reported that low dose antibiotics have increased the abundance of a single pathogenic bacteria, however, difference in ARGs were not significant compared to control groups⁶⁶. This study also showed change in immunological markers but association between ARGs and immunological markers have not been established⁶⁶. Also, IBD phenotype is known to be associated with microbiome dysbiosis which led to upregulation of antibiotic resistance⁶⁷, a condition also observed in GW Veterans⁶⁸. We have shown an association between gastrointestinal, systemic, and neuronal inflammation observed in GWI pathology and diversity of ARGs and resistant drug classes in a GWI mice model. Our results also indicated an association between systemic inflammation reflected by increased serum IL-6 and ARG diversity in GW Veterans. Interestingly, association between diversity of ARGs and systemic proinflammatory marker IL-6 will be an important benchmark in future studies of chronic multisymptom illness and other related pathology. Future mechanistic studies are needed to establish the exact role of ARGs in GWI pathophysiology by using germ free and gnotobiotic models combined with the use of short chain fatty acid treatment regimens such as butyrate therapy.

Limitations of the study. Modeling the chronic multisymptomatic condition of GWI in rodent models to mimic the health condition of the present-day GW Veterans has been challenging. Due to the complex environmental chemical exposures during the Persian Gulf War, it is imperative that incorporation of other chemicals like organophosphates, N,N-diethyl-meta-toluamide (DEET), depleted uranium exposure, vaccines, oil smoke along with Per and PB would strengthen the translatability of the results in GWI. The research in GWI should consider the differences in the routes of exposure of these environmental mixtures such as the dermal route for administration of Per, a widely used insecticide. Small sample size of the GWI persistence mouse model as well as GWI Veteran cohorts may have affected the resistome analysis, hence an incorporation of a larger sample size along with studies with significant statistical power in future mechanistic analyses of the gut resistome will help us in better understanding the changes ARGs and MGEs. This is especially important in detecting the minute changes related to the resistant drug classes, antibiotic resistance mechanisms and MGE types. In the GWI mice model, inclusion of an FMT-only control would help us to clearly distinguish the effects of the above treatment regimen on gut resistome. A modification of the treatment plan of FMT in the murine model that takes into account the additional stress-induced changes in the gut resistome that might have occurred, would be pertinent to the present line of investigation. Future studies would also need to screen the fecal samples from the healthy mice-Control groups to minimize the transfer of ARGs to the recipient group. The present study should not be conceived as an attempt to integrate the mouse data with that of the data from the human cohorts as there are remarkable differences in the exposure patterns as underlined in our previous sections. The human cohort was included to investigate if resistome alteration occurred in GWI conditions as an effect of microbiome dysbiosis, as reported in earlier studies. The GW Veterans have been exposed to multiple environmental chemical exposures singly or in combination. In addition, diet, sedentary lifestyle after

deployment, metabolic conditions like obesity and exposure to multiple courses of antibiotics and treatment regimens may have contributed to higher resistome diversity. Another significant limitation might be the inability to confirm experimentally if the GWI Veterans or the mice exposed to representative GWI chemicals were actually resistant to the antibiotics observed from the gene annotation studies. These limitations could have been overcome by exposing GWI mice to representative antibiotics following a bacterial infection or an acute challenge with bacteria that cause pathogenesis. Inclusion of these set of experiments might have supported our hypothesis that a resistome signature can predict phenotypic disease susceptibility later in life. Also, inclusion of the previously stated factors like diet and obesity in the future GWI mouse model study along with use of humanized and germ-free mice might improve the translatability of the results obtained from the animal studies. We were also limited in linking the MGEs with the ARGs as well as identifying the mechanism that connected the GWI pathology with altered gut resistome which we plan to study in the immediate future. The present study is a preliminary report. The observations can help build a stronger hypothesis that points to the role of the host gut resistome in GWI disease pathology. Further studies with larger sample sizes may establish causality and help in determining risk of GWI Veterans to antibiotics and other co-morbidities.

In conclusion, to the best of our knowledge the present study is the first to investigate gut resistome alterations in GWI. Further, a preliminary association was established between an altered resistome and systemic IL-6 levels in a translatable mouse model that has broad implications in the general population suffering from similar ailments though the actual causality is yet to be established. It is expected that 78 million of the US population is expected to be in the elderly category by 2030. Most of them have a history of prolonged antibiotic use, a case similar with our aging Veterans. The elderly population belonging to the age group of 50–60 years have increased risk of acquiring antibiotic resistance due to several factors like impaired immune functioning, immunosenescence, and exposure to multi-drug resistant bacteria due to multiple visits to clinics and hospitals^{69–71}. In the present study, the GW Veterans were of the same age group (53–56 years) and studies have reported that the present day GW Veterans have a maladaptive immune system primarily due to GW chemical exposure and aging^{72,73}. The scenario is also significant owing to old age associated hospitalizations and increased chances of hospital acquired infections. Based on the above facts and our study results, we can predict that GW Veterans may have a very high chance of acquiring antimicrobial resistance. In addition, FMT can be used as a therapeutic strategy against the increased antibiotic resistance in Veterans and elderly to attenuate a possible altered resistome. Knowledge gained from the microbiome and resistome profiles from GW Veterans can be very helpful for treating a variety of bacterial diseases or hospital acquired infections (following surgery) that may require antibiotic treatment in the Veterans and can be a useful tool for a personalized medicine approach.

Methods

Per and PB were purchased from Sigma-Aldrich. Primary antibodies anti-interleukin-1 β (IL-1 β), anti-brain derived neurotrophic factor (BDNF) were purchased from Santacruz Biotechnology (Dallas, TX, USA). Species specific biotinylated secondary antibodies and streptavidin-HRP (Vectastain ABC Kit) were purchased from Vector laboratories (Burlingame, CA, USA). All other chemicals used in the present study were purchased from Sigma unless specified. Animal tissues were sent for paraffin embedding and sectioning to AML Laboratories (St. Augustine, FL, USA). Fecal samples from experimental mice groups and GW Veterans were sent to COSMOSID (Germantown, MD, USA) for whole-genome sequencing.

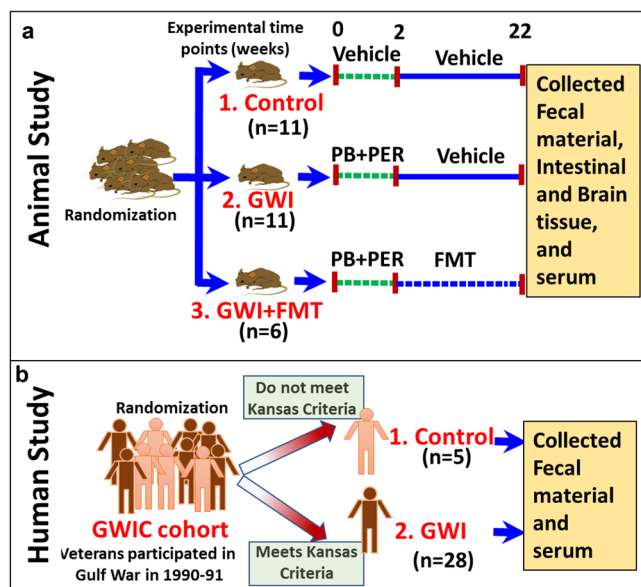


Fig. 8 A schematic representation of the experimental cohorts.

a Experimental mouse model of Gulf War Illness (GWI). **b** Study design of Veteran participants in Boston Gulf War Illness Consortium (GWIC) based on Kansas GWI criteria.

Animals. C57BL/6J wild type, male mice of 10 weeks age were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The mice were maintained in accordance with local IACUC standards and National Institute of Health guidelines for human care and use of laboratory animals. All animal experimental procedures were approved by University of South Carolina at Columbia, SC (Animal protocol number 2419-101345-072318, approved on 7/23/2020). All the mice had ad libitum access to food and water and were housed at 22–24 °C with 12 h light/12 h dark cycles. The mice were sacrificed after the animal experiments. Organs including frontal cortex and distal part of small intestine were collected after dissecting the mice and fixed in Bouin's solution and 10% neutral buffered formaldehyde respectively. Serum was collected from fresh blood of mice by performing cardiac puncture after anesthesia. The fecal pellets were collected from colon, and it was stored at –80 °C for whole-genome sequencing (WGS).

Mouse model of Gulf War illness. After 1 week of acclimatization, the mice were randomly distributed into three groups. The first group received vehicle (0.6% dimethyl sulfoxide) for 2 weeks and were denoted Control ($n = 11$). The second and third mice groups denoted GWI ($n = 11$) and GWI_FMT ($n = 6$) were treated with Per [200 mg/kg body dissolved in DMSO and phosphate buffer saline (PBS)] and PB (2 mg/kg dissolved in PBS) by oral gavage tri-weekly for 15 days. After 2 weeks of GW chemical exposure, GWI group mice were allowed to persist for 20 weeks. Fecal microbiota transplant was administered in GWI_FMT after GW chemical exposure. 100 mg of fecal pellets were collected from healthy C57BL/6J mice of same age group as the GWI_FMT mice. The pellets were homogenized in 1 ml of PBS and centrifuged at $3000 \times g$ for 5 min. 100 μ l of supernatant was dosed in each mouse on alternate days of a week for 20 weeks^{74–76} (Fig. 8a).

Human subjects: GW Veterans with GWI and controls. The Boston Gulf War Illness Consortium (GWIC) performs preclinical and clinical studies to understand the pathophysiology behind the complex symptoms in GW Veterans to aid in designing of possible therapeutic strategies⁷⁷. Veterans were included as participants in GWIC studies based on requirement that they had to be deployed in the GW i.e., from August 1990 to July 1991. The GWIC used the Kansas GWI criteria as the case definition which requires the Veterans to have symptoms in 3 out of 6 broadly defined group of symptoms (neurological, pain, gastrointestinal, skin, respiratory, fatigue) to meet criteria of CMI in GW Veterans known as Gulf War Illness (GWI)^{31,78}. GW Veterans who do not meet Kansas criteria are deemed the control group.

The Veterans who participated in the GWIC, underwent multiple tests including neuropsychological assessments, health surveys, biological specimen collection, and brain imaging^{27,77}. The present study was conducted as a GWIC call-back study in which we aimed to reassess 150 of the GWIC participants. For this study, data from the first 33 recruited subjects from the microbiome call-back study has been analyzed. The recruitment of participants was via telephone on completion of GWIC study protocol. After filling out a brief questionnaire regarding screening, the participants were sent a stool collection kit which was then shipped back to the study investigators (Fig. 8b).

Collection of demographical, deployment exposure, and health symptom information from GWIC participants. The full GWIC protocol has been previously published in Steele et al., 2021⁷⁷. Briefly, the GWIC participants had to answer to surveys regarding demographics and health condition which included Multi-dimensional Fatigue Inventory (MFI-20), Pittsburgh Sleep Quality Index and McGill Pain Inventory^{79–81}. The Structured Neurotoxicant Assessment Checklist (SNAC) and Kansas Gulf War and health Questionnaire and Kansas Gulf War Experiences surveys were given to obtain details about self-reported exposures. The survey regarding health condition provided the details if the participants had an ascertained diagnosis of the medical conditions reported by them^{31,78,82}. As part of the call-back study, the Veterans also filled out questionnaires about their current and recent gut health and use of antibiotics or probiotics.

Collection of stool samples from participants. The GWIC participants of the microbiome study were mailed a Second Genome stool collection kit (Second Genome, San Francisco, CA, USA). The kit was a self-collecting kit which contained a bar-coded vial with stabilizing solution for long term preservation of nucleic acids in stool during transportation and storage. Once received from the subjects, the stool samples were stored at –20 °C and upon collection of significant sample numbers, they were sent for WGS by COSMOSID. The protocol was approved by Institutional Review Board at Boston University School of Public Health (proposal no. GW170068) on 4/15/2021. Sample collection from GWIC participants were done following all ethical guidelines and informed consent was also obtained.

DNA extraction and whole-genome shotgun sequencing. Briefly, the total DNA from mouse and human samples were isolated and purified using ZymoBIOMICS Miniprep kit. DNA was quantified using Qubit dsDNA HS assay (ThermoFisher, Waltham, MA, USA). Illumina Nextera XT library preparation kit was used with modifications for preparing DNA libraries. Illumina HiSeq 4000 and Illumina NextSeq 550 platform was used to perform WGS for mice and human samples respectively, following protocol optimized by vendor. 2x150bp of read length and an average insert size of 1400 bp were used for sequencing. DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) with Nextera Index Kit (Illumina) for mice and IDT Unique Dual Indexes for human samples, with total DNA input of 1 ng. Genomic DNA was fragmented using a proportional amount of Illumina Nextera XT fragmentation enzyme. Combinatory dual indexes were added to each sample followed by 12 cycles of PCR to construct libraries. DNA libraries were purified using AMPure magnetic Beads (Beckman Coulter) and eluted in QIAGEN EB buffer. DNA libraries were quantified using Qubit 4 fluorometer and Qubit™ dsDNA HS Assay Kit. Upon data arrival, raw data were backed up to Amazon AWS and run through fastqc and a multiqc report was generated⁸³. The multiqc report was checked to ensure read depth thresholds were met, and that there were no abnormalities with read quality, duplication rates, or adapter content. Taxonomic results were checked on the COSMOSID-Hub Microbiome platform to ensure there were contamination or barcoding issues.

We obtained a total of 536.92 million (M) sequencing reads with per sample averages of 9.33 M reads in the Control samples, 8.49 M reads in the GWI samples, 10.12 M reads in the GWI_FMT samples, 11.32 M reads in the Hum_Control samples, and 11.17 M reads in the Hum_GWI samples.

Metagenomic analysis and assembly. MetaPhlAn v3.0.7⁸⁴ was used to profile the taxonomic composition of each sample with default parameters. The resulting relative abundance tables were then merged with the provided python tool, “merge_metaphlan_tables.py”. A custom python script was used to filter the data to contain only species level identifications and prepare the operational taxonomic unit (OTU) table for statistical analysis. The metaWRAP v1.3.2⁸⁵ pipeline was used to process and assemble raw sequencing reads from each sample. First, the “read_qc” module was used with default parameters to trim sequencing adapters and bases with low PHRED scores. To decontaminate the data, reads mapping to the human reference genome GRCh38.p12 (RefSeq Acc: GCF_000001405.38) and the mouse reference genome GRCm38.p6 (RefSeq Acc: GCF_000001635.26) were removed by the metaWRAP “read_qc” module. After decontamination and quality control, we recovered a total of 532.43 million (M) sequencing reads with per sample averages of 9.22 M reads in the Control samples, 8.24 M reads in the GWI samples, 9.96 M reads in the GWI_FMT samples, 11.28 M reads in the Hum_Control samples, and 11.13 M reads in the Hum_GWI samples. Decontaminated reads were used for de novo assembly using metaSPAdes⁸⁶ as contained in the metaWRAP “assembly” module with default parameters. Resulting contigs were binned by the “binning” module which uses three binning methods, metaBAT2 v2.12.1⁸⁷, MaxBin2 v2.2.6⁸⁸, and CONCOCT v1.0.0⁸⁹ to produce three sets of bins. The “bin_refinement” module was used to refine these three bin sets to produce a single set of best bins. Finally, the single bin set was used by the “bin_reassembly” module which extracts the reads mapping to each bin and uses them for a second round of de novo assembly to improve the completion and reduce the contamination of the bins.

Antimicrobial resistance gene family and MGE identification. Contigs produced through metaWRAP were used for open reading frame (ORF) finding using Meta-Prodigal v2.6.3⁹⁰ with parameters “-c -p meta”. ORFs were then clustered with CD-

HIT v4.8.1^{91,92} with parameters “-c 0.95 -s 0.90” corresponding to 95% sequence identity threshold over 90% of the shorter ORF length. Next, ORFs were mapped to the Comprehensive Antibiotic Resistance Database (CARD) v3.1.0⁹³ and a recently published custom Mobile Genetic Element (MGE) database composed of 278 distinct genes and over 2000 unique gene sequences²⁰ using the tool “nhmmer” from the HMMER v3.3.1⁹⁴ software with parameters “-E 0.001 -incE 0.001” corresponding to an e-value threshold of 0.001 for matches. A custom python script was used to filter multiple hits to select the single best hit for each ORF. Finally, Microsoft Excel was used to generate count data for ARGs and MGEs.

Laboratory methods

Immunohistochemistry. The fixed mouse small intestine and frontal cortex tissues were paraffin embedded and 5 µm thick sections were done for immunohistochemistry. Deparaffinization were performed following previous protocol⁴¹. Antigen retrieval was performed using epitope retrieval solution and steamer (IHC world, Woodstock, MD, USA). Three percent hydrogen peroxide was used for blocking endogenous peroxidase activity for 20 mins. Serum blocking was performed using 10% goat serum for 1 h. Tissue sections were incubated with primary antibodies for IL-1β and BDNF at 1:200 dilution for overnight in humidified chamber at 4 °C. After incubation, the tissue sections were washed three times with PBS containing 0.05% Tween 20 solution. Tissues were probed with species specific biotinylated antibodies (1:200 dilution) followed by incubation with horse radish conjugated streptavidin (1:500 dilution). 3,3'-diaminobenzidine was used as chromogenic substrate solution and counterstaining was performed using Mayer's hematoxylin. The stained tissues sections were mounted using Aqua Mount (Lerner Laboratories, Kalamazoo, MI, USA). The images were acquired using Olympus BX63 microscope (Olympus, Center Valley, PA, USA). Morphometry was performed using Cellsens Software from Olympus America (Center Valley, PA, USA).

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) was performed to measure ARG expression in DNA extracted from mouse and human stool samples. Gene specific primers were designed using Primer3 (version 0.4.0) and IDT, purchased from Sigma (St. Louis, MO, USA) (Supplementary Table 1). SYBR Green Supermix (BioRad, Hercules, CA, USA) was used in CFX96 thermal cycler (BioRad, Hercules, CA, USA). The samples (both mouse and human) were run in triplicates for each gene. Ct or threshold cycle values of all ARG genes were normalized with 16S as internal control. $2^{-\Delta\Delta Ct}$ method was used to calculate the relative fold change of the ARGs.

ELISA. Serum IL-6 level was estimated using serum collected from the mice groups using commercially available kit from Proteintech (Rosemont, IL, USA). Serum IL-6 of Veteran participants in this study was measured using commercially available ELISA kit from Abcam (Boston, MA, USA). The ELISA was performed according to the manufacturer's protocol.

Statistical analysis and reproducibility. Analyses were performed using R v3.6.3 (1). ARG, MGE, and OTU count data were normalized based on library size using the “estimateSizeFactors” function from the “DESeq2” package⁹⁵ with the parameters “type = poscounts”. Normalized count data were then log transformed with the base R function “log2”. Permutational analysis of variance (PERMANOVA) was calculated using the “adonis” function from the package “vegan” (2) with Bray-Curtis dissimilarity and 9999 permutations. Welch two-sample *t*-test was implemented using the base R function “t.test” with the parameters “conf.level = 0.95, alternative = two.sided” indicating 95% confidence level and two-tailed testing. PCA ordinations of ARG and Taxonomy abundance data were performed using the “rda” function from “vegan”. Procrustes analysis was performed using PCA ordinations with the function “protest” from “vegan” with 9999 permutations. Principle coordinate analysis (PCoA) was performed with the “pcoa” function from the “ape” package with Bray-Curtis dissimilarity. Statistical analysis for q-RT-PCR, immunohistochemistry and ELISA was done using Prism (Graphpad, San Diego, CA). One-way ANOVA was used for comparing between the three mice groups and Welch's *t*-test was used for comparison between the Veteran groups for statistical analysis of immunohistochemistry and ELISA. Chao1 α-diversity was calculated using the “chao1” function from the “fossil” package. Correlation analyses between α-diversity and selected biomarkers were performed using Pearson's correlation implemented by the base R function “cor”. All visualizations were rendered using the “ggplot2” package unless otherwise described. For all analyses, *p* < 0.05 was considered statistically significant, and data are represented as mean ± standard error of mean or mean ± standard deviation. Each figure and data set has been provided with individual *P*-values for better clarity.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Microbiome and resistome sequence data that support the findings of this study have been deposited in GenBank with the accession code: PRJNA734321 and PRJNA843121

(<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA843121>) with the link. All data generated or analyzed during this study are included in this published article (data generated for this study is provided in the Supplementary Data file and public databases-GenBank). Any other data such as certain metagenomic sequences that are not publicly available due to space constraints are available from the corresponding author on reasonable request.

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Author contributions

Conceptualization: S.C.; experimentation: D.B., S.C., E.O., P.S., R.S.; data curation: D.B., S.C., E.O., J.L., S.C., P.J., K.S.; formal analysis: D.B., S.C., E.O.; funding acquisition: S.C.; investigation, methodology, resources: S.C., R.S., A.M., P.S., D.B., S.C., E.O., J.M.C.; software: D.B., S.C., E.O.; supervision: S.C., R.S., J.L.; writing—original draft: D.B., S.C., E.O. and S.C.; writing: S.C.; review and editing: S.C., N.K., K.S., P.J., S.L., A.D. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interest.

Additional information

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Sex-specific differences in plasma lipid profiles are associated with Gulf War Illness

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Abstract

Background: Nearly 250,000 veterans from the 1990–1991 Gulf War have Gulf War Illness (GWI), a condition with heterogeneous pathobiology that remains difficult to diagnose. As such, availability of blood biomarkers that reflect the underlying biology of GWI would help clinicians provide appropriate care to ill veterans. In this study, we measured blood lipids to examine the influence of sex on the association between blood lipids and GWI diagnosis.

Methods: Plasma lipid extracts from GWI (n = 100) and control (n = 45) participants were subjected to reversed-phase nano-flow liquid chromatography-mass spectrometry analysis.

Results: An influence of sex and GWI case status on plasma neutral lipid and phospholipid species was observed. Among male participants, triglycerides, diglycerides, and phosphatidylcholines were increased while cholesterol esters were decreased in GWI cases compared to controls. In female participants, ceramides were increased in GWI cases compared to controls. Among male participants, unsaturated triglycerides, phosphatidylcholine and diglycerides were increased while unsaturated cholesterol esters were lower in GWI cases compared to controls. The ratio of arachidonic acid- to docosahexaenoic acid-containing triglyceride species was increased in female and male GWI cases as compared to their sex-matched controls.

Conclusion: Differential modulation of neutral lipids and ratios of arachidonic acid to docosahexaenoic acid in male veterans with GWI suggest metabolic dysfunction and inflammation. Increases in ceramides among female veterans with GWI also suggest activation of inflammatory pathways. Future research should characterize how these lipids and their associated pathways relate to GWI pathology to identify biomarkers of the disorder.

Keywords: Gulf War Illness, Neutral lipids, Biomarkers, Mass spectrometry, Sex-specific effect, Plasma

Introduction

Approximately 30% of the 700,000 veterans of the 1991 Gulf War (GW) suffer from Gulf War Illness (GWI), a chronic multi-symptom condition characterized by cognitive impairment, fatigue, and pain [1]. Veterans' exposure to chemical warfare agents, pesticides, and pyridostigmine bromide are among the major contributors to GWI etiology [1–4]. Currently, there are no biomarkers

for indicating the underlying pathology of this condition. Advances in GWI research suggest lipid dysfunction, bioenergetics deficits, and inflammation as key contributors to its ongoing pathology [5, 6]. Blood biomarkers of these biological processes would be a cost-effective and minimally invasive avenue to assist clinicians with accurate detection of GWI.

Evidence suggests that GW pesticides affect cellular lipid metabolism and lipid homeostasis [2, 7–11]. Altered lipid homeostasis can result in an abnormal accumulation of lipids that promote cellular inflammation [12, 13]. Abnormal plasma and brain lipid profiles after GW pesticide exposure in rodents and in veterans with GWI have

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been shown to accompany inflammation, neurobehavioral and bioenergetic deficits [14–18]. As such, altered plasma lipid profiles may provide a biomarker signature for the bioenergetic deficits and inflammation associated with GWI.

It is expected that since the pesticides to which GW veterans were exposed tend to disturb lipid homeostasis, owing to their high affinity for lipids, neutral lipid classes, such as triglycerides (TG), diglycerides (DG), and cholesterol esters (CE), and phospholipid (PL) classes would be affected in GWI [2, 9–11, 19, 20]. The degree of unsaturation of the fatty acids within the PL classes is known to alter metabolic and inflammatory systems, with many saturated fatty acid (SFA)-containing PL positively associated with chronic metabolic conditions whereas polyunsaturated fatty acid (PUFA)-containing PL are associated with inflammation [21–24]. Among these, PUFA containing the omega-3 fatty acid, docosahexaenoic acid (DHA), generate anti-inflammatory or inflammation-resolving lipid metabolites and PUFA containing the omega-6 fatty acid, arachidonic acid (AA), can produce metabolites that promote pro-inflammatory responses [23–25].

Changes in lipid profiles were also expected in relation to the sex of GW veterans, as many classes of plasma lipids are significantly associated with sex [26]. Women are up to four times more likely than men to be diagnosed with myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS), a chronic multi-symptom disease clinically similar to GWI that has been shown to have sex-linked differences in blood lipid profiles [27, 28]. Female GW veterans have a higher prevalence of GWI diagnosis and severe GWI-related symptoms than male GW veterans [29]. These findings suggest potential sex-linked differences in pathophysiological responses to GW-era chemical exposures, necessitating sex-stratified analyses.

We hypothesized that blood lipid levels would be affected both by GWI status and the sex of GW veterans. We evaluated the levels of several major blood lipid classes and their molecular species in three cross-sectional cohorts of participants classified as GWI or control. This study will further our understanding of plasma lipids as potential biological markers to track the ongoing inflammation and metabolic dysfunction of GWI, which may help to provide better care and management of this illness.

Materials and methods

Cohort characteristics

All protocols were conducted in compliance with the relevant guidelines and regulations and were approved by institutional review boards (IRB) at each institution.

Informed consent was obtained from all participants. All plasma utilized within this study were from the following case–control veteran cohorts: (1) the Roskamp Neurology Clinic (RNC) cohort (n=36), (2) the Boston Gulf War Illness Consortium (GWIC), and (3) the Fort Devens cohort (n=58) [3, 30, 31]. Additional healthy control samples were provided by Dr. Klimas to supplement the control population of the study. GW veteran controls and the healthy civilian controls were grouped together for analysis in the GWIC cohort (n=51). In all cohorts, either the Center for Disease Control (CDC) chronic multi-symptom illness definition or the Kansas GWI criteria were used to diagnose GWI [32, 33]. These criteria required that GW veterans must show symptoms in at least 3 of 6 symptom domains (fatigue/sleep problems, somatic pain, neurological/cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms, and skin abnormalities) for GWI diagnosis. Exclusionary criteria have been described previously, primarily that participants were excluded if they reported prior diagnosis with a confounding medical condition [17, 30, 31]. Controls were either veterans deployed to the 1991 GW or healthy civilian controls, who did not meet the Kansas GWI criteria or any of the exclusionary criteria. Additional information about the self-report of cardiovascular health status was collected on RNC and GWIC participants. Self-report of any heart conditions, diabetes, high blood pressure or high cholesterol was combined into a single variable to assess cardiovascular risk factors as a group. Reporting of any one or more of these risk factors was coded as presence and a lack of reporting on any cardiovascular risk factors was coded as absence.

Data on the sex of the participants were gathered via self-report, with the options being either male or female. As per the National Institute of Health, sex refers to biological differences between males and females [34]. As per the World Health Organization, gender refers to the characteristics of men and women that are socially constructed [35]. Therefore, this paper evaluating biological lipid levels uses exclusively male/female terminology for participants rather than men/women terminology, as data on the gender of the participants was not collected.

Participant recruitment, blood collection, and blood storage procedures have been described previously [17, 18, 31]. All samples were collected using the same written standard operating procedures for performing phlebotomy, plasma separation and aliquoting. All participants had fasted prior to blood collection. There was no requirement of time of collection.

Due to low numbers in ethnic groups other than Caucasian, we combined participants who were African American, Aleutian Eskimo, American Indian, Asian, Pacific Islander, multiracial or other races into one

category, called Non-Caucasian. Only one African American veteran with GWI was present in the RNC cohort. Of the eleven African American participants in GWIC, three were control participants and eight were veterans diagnosed with GWI. Of the six African American participants in the Fort Devens cohort, four were control participants and two were veterans diagnosed with GWI.

Lipidomic analysis and quantification

Plasma samples were randomized prior to use via a group-block design, to ensure that the experimenters were blinded to participants' group membership and other characteristics. The extraction protocol and liquid chromatography–mass spectrometry (LC–MS) run settings have been previously described by our laboratory [18, 36]. Briefly, lipid extracts from 10 μ L of plasma spiked with class-specific internal standards (to control for variability in lipid extractions) were resuspended in 200 μ L of 70:30 LC–MS mobile phase A:B [36, 37]. Mobile phase A was composed of 42% water, 31% acetonitrile, 27% isopropanol, 10 mM ammonium formate, and 0.1% formic acid. Mobile phase B was composed of 90% isopropanol, 10% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid. For LC–MS analysis, an Easy-nanoLC 1000 chromatography pumping system was used with a nanoflex electrospray ionization (ESI) source on a Thermo LTQ/orbitrap mass spectrometer.

Resuspended lipid extracts (4 μ L) were injected onto an Acclaim PepMapTM 100 (75 μ m $\times$ 2 cm, nanoViper, C18, 3 μ m, 100 Å) trapping column attached to an Acclaim PepMapTM RSLC (75 μ m $\times$ 15 cm, nanoViper, C18, 2 μ m, 100 Å) analytical column for reversed-phase chromatographic separation of lipid species. The chromatographic gradient started at 30% B, then increased linearly from 1 to 40 min from 50 to 98% B, plateauing at 98% B from 40 to 50 min, and then decreasing to 30% B for 50 to 65 min. All samples were run in triplicate, accompanied by a blank and a quality control sample in each run. Full-scan mass spectrometry (MS) data was acquired in positive ion mode, over a mass range of 300–2000 m/z at a resolution of 30,000. A maximum inject time of 200 ms was used with 6 microscans/acquired scan. Thermo Xcalibur Qual Browser was used to examine the individual chromatographic data of samples for lipid retention times. Lipid peak areas were integrated using Tracefinder™ software, using target compound lists of analytes of interest, identifying them based on m/z and retention time. A high mass accuracy window of 5 mmu was used for detecting each individual lipid species. We detected several major classes of lipids including the phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), the neutral lipid classes TG, DG, and CE and the sphingolipid classes sphingomyelin (SM), ceramides

(Cer) and hexosylceramides (HexCer), which were quantified as previously described [28, 36]. The quality control (QC) samples were then used to normalize between batches of data ([sample] $\times$ [batch QC/normalizing QC]). Once the coefficients of variance (CV) were calculated per class for the whole cohort, lipid species with an average CV > 25 were excluded from further analysis. Lipid species with less than 90% coverage were also excluded from our analyses.

To categorize lipids based on their degree of unsaturation, totals were calculated by adding up values for all lipid species measured belonging to that lipid class. Saturated fatty acids (SFA) were calculated by totaling the lipids with no double bonds; monounsaturated fatty acids (MUFA) were calculated by totaling the lipids with one double bond. Polyunsaturated fatty acids (PUFA) were calculated by totaling all measured lipid species with three or more double bonds. For lipid classes known to contain AA or DHA, their respective categorizations were made by totaling individual species known to contain the relevant type of fatty acid, as based on previous MS/MS confirmations.

Statistical analysis

Chi-square and one-way analysis of variance (ANOVA) tests were used, as appropriate, to compare between the three cohorts and to compare GWI and healthy controls for descriptive purposes. Each lipid species was assessed for normality and those found to be skewed were log transformed for parametric analysis. All lipidomics data were analyzed using mixed linear modeling (MLM) as described previously to examine the independent effects of sex and GWI diagnosis as well as to account for random effects associated with repeated measurements in lipidomics and metabolomics datasets [17, 36–39]. The Benjamini-Hochberg (B-H) correction procedure was performed to correct for multiple testing and the false discovery rate (FDR) limit was set at 0.05. The webtool MetaboAnalyst 5.0 was used to perform a hierarchical clustering analysis of the individual lipids with a coverage superior to 90% [40]. Clusters were determined based on the similarity of lipids as expressed by the Euclidean distance. Lipids that were adjacent based on the Ward algorithm were combined in clusters and an ANOVA was applied to select the top 50 lipids significantly modulated by sex and diagnosis represented in the dendrogram and heatmap.

Results

Basic demographics

General demographic characteristics of participants from the three cohorts at the time of the blood collection are presented in Table 1. The difference in ages between the

Table 1 Descriptive table of demographic variables of the three cohorts: RNC, GWIC, and Fort Devens

	RNC (n = 36) Mean (SD)	GWIC (n = 51)	Fort Devens (n = 58)
Age**	55.4 (1.2)	50.3 (1.1)	55.9 (1.2)
BMI	29.4 (5.5)	30.8 (4.8)	–
	N (%)		
GWIC Cases	31 (86.1)	31 (60.8)	38 (65.5)
Female	10 (27.8)	7 (13.7)	13 (22.4)
Race*			
White/Caucasian	31 (86.1)	34 (66.7)	50 (86.2)
Non-Caucasian	5 (13.9)	17 (33.3)	8 (13.8)
Cardiovascular risk factors	19 (55.9%)	21 (50.0%)	
Diabetes	6 (17.6%)	4 (9.3%)	–

The mean age and standard deviation (SD) are reported. Significant differences are indicated by either single asterisks ($p < 0.05$) or double asterisks ($p < 0.01$).

Table 2 Descriptive table of demographic variables of GWI cases and controls from the RNC, GWIC, and Fort Devens cohorts combined

	Controls (n = 45) Mean (SD)	GWI (n = 100)
Age	55.6 (10.8)	53.1 (7.1)
BMI	30.1 (3.8)	30.0 (5.4)
	N (%)	
Female*	14 (31.1%)	16 (16.0%)
Race		
White/Caucasian	35 (77.8%)	80 (80.0%)
Non-Caucasian	10 (22.2%)	20 (20.0%)
Cardiovascular risk factors (Yes)	8 (50.0%)	32 (53.3%)
Diabetes	1 (6.3%)	9 (15.0%)

Significant differences are indicated by single asterisks ($p < 0.05$).

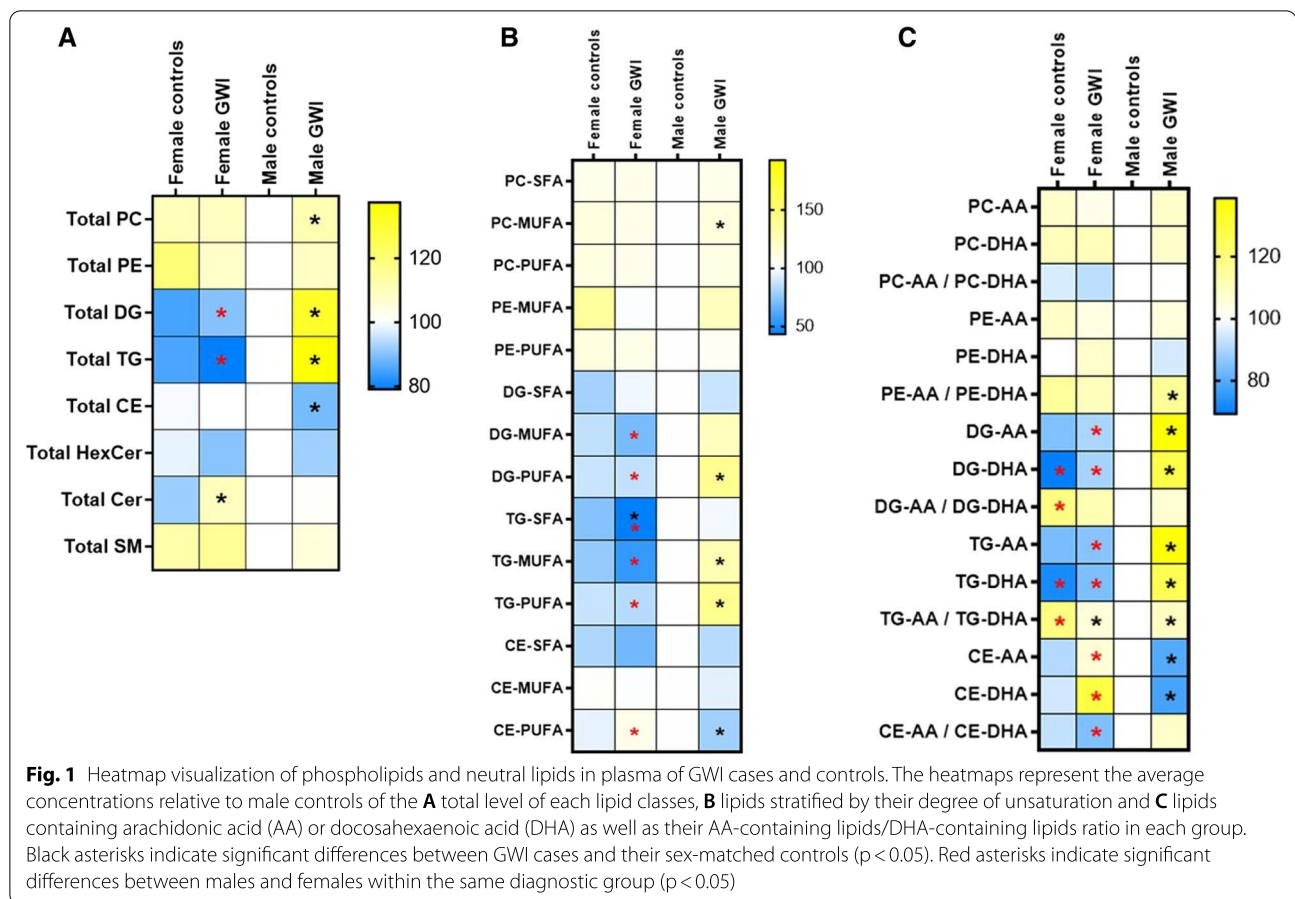
RNC participants and the GWIC participants was found to be significantly different ($p < 0.01$). The Fort Devens and GWIC cohorts were found to be significantly different for race ($p < 0.05$). Overall, the GWIC cohort was younger and had a higher proportion of non-Caucasian participants than the other two cohorts (Table 1). The percentage of female participants ranged from 13.7% to 27.8% across the 3 cohorts (Table 1).

When all cohorts were combined (Table 2), there were no significant differences for age and race between GWI cases and controls. However, the sex ratio was found to be significantly different between GWI cases and controls, as 16% of GWI cases were female compared to 31% of controls ($p < 0.05$). In a subset of participants, there were no differences between GWI cases and controls for

BMI, the presence of diabetes and the presence of cardiovascular risk factors. There were no differences between males and females for the presence of diabetes or cardiovascular risk factors ($p > 0.05$). Similarly there were no significant differences between males and females for BMI (males: 30.1 ± 5.3 SD and females: 29.4 ± 4.2 SD). Given the known associations between sex and blood lipid levels and differences in the proportion of male and female sex between GWI and control participants, subsequent analyses were stratified by sex to account for its confounding effects.

Sex-specific differences were observed for the degree of unsaturation and AA and DHA content of several major classes of lipids in veterans with GWI

Figure 1 shows heatmaps of plasma profiles of eight different lipid classes that are differentially affected by sex and diagnostic differences among male and female participants (see Additional file 1: Fig. S1 for individual values). The total amount of PC, DG and TG were significantly increased while the total amount of CE was significantly decreased in male GWI compared to male controls (Fig. 1A). Among female participants, only total Cer were significantly increased in GWI compared to controls. We also evaluated the degree of fatty acid unsaturation in individual lipid species since neutral lipids are considered reservoirs of PUFA lipids (Fig. 1B). Compared to male controls, male veterans with GWI had a significant increase in the unsaturated lipids, consisting of DG-PUFA, TG-MUFA, TG-PUFA and PC-MUFA, while CE-PUFA was significantly decreased. Among female participants, TG-SFA levels were significantly lower in GWI compared to controls. The PUFA-containing lipid

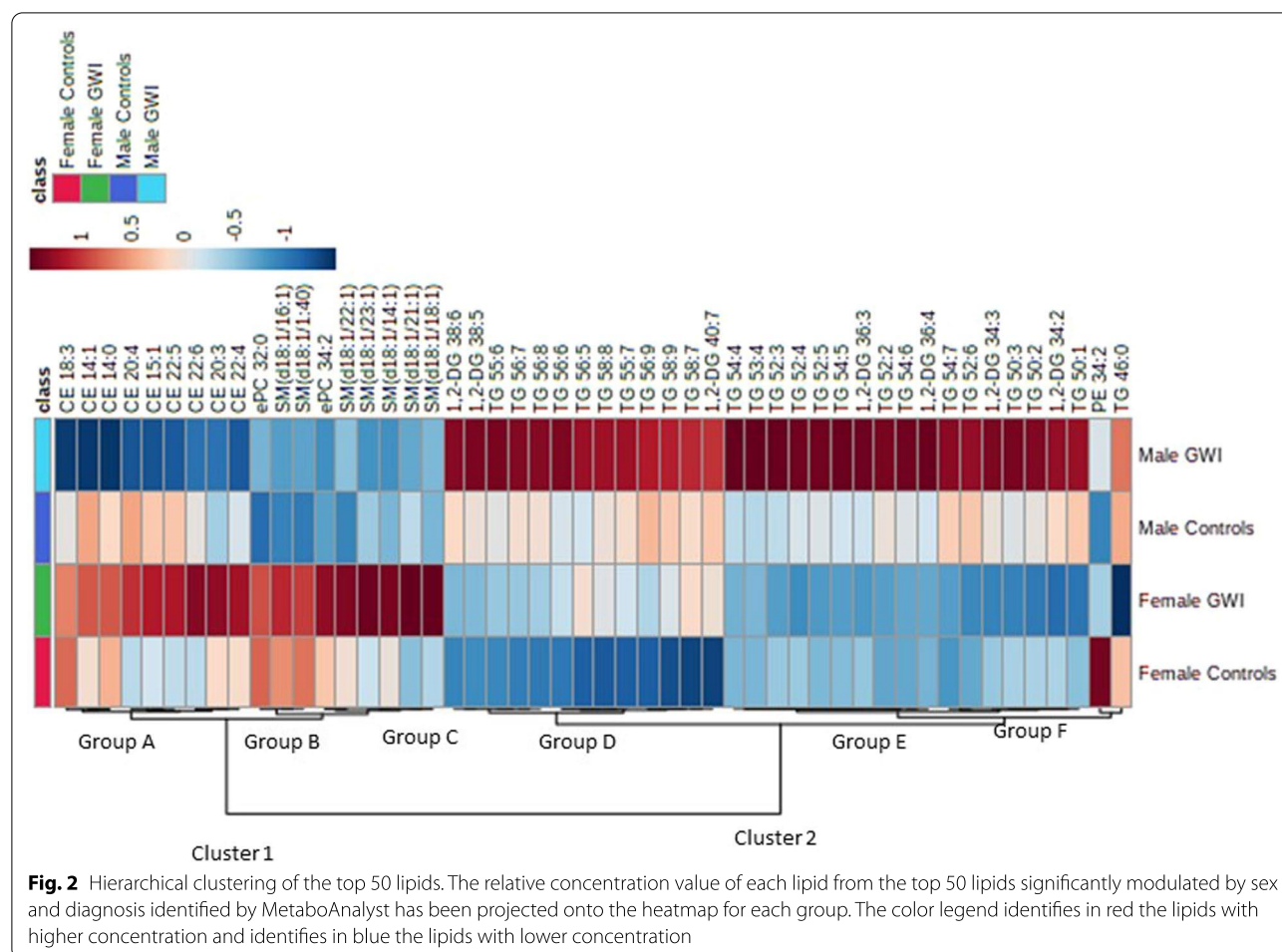


species were further grouped by their AA or DHA-content for each lipid class (Fig. 1C). Levels of DG-AA, DG-DHA, TG-AA and TG-DHA were significantly increased while CE-AA and CE-DHA were significantly decreased in male GWI cases compared to male controls. The ratios of PE-AA/PE-DHA and TG-AA/TG-DHA were both significantly increased in male GWI compared to male controls. The TG-AA/TG-DHA ratio was also significantly increased in female GWI compared to female controls. Independent effects of the presence of cardiovascular risk factors on these lipid profiles are presented in Additional file 2: Fig. S2.

Hierarchical clustering analysis identifies sex-specific lipid species signature of GWI

Hierarchical clustering analyses identified two major clusters (cluster 1 and cluster 2) within the top 50 lipid species significantly modulated by sex and diagnosis (Fig. 2). Lipids within both clusters were affected by sex differences, irrespective of GWI diagnosis. Cluster 1 was primarily composed of the neutral lipid class CE and the sphingolipid class SM and showed a phenotype

of increased levels in female GWI and decreased levels in male GWI. Cluster 2 was primarily composed of the neutral lipid classes DG and TG, which were increased in male participants compared to female participants, irrespective of GWI diagnosis. Lipids associated with each cluster further separated into 3 subgroups. The CEs of group A show controls to be generally similar regardless of sex while the lipids differed in concentration in the GWI veterans based on sex. Lipids associated with groups B and E were both principally influenced by sex regardless of the diagnostic status. Group B was composed of an ether PC and SM lipids, while Group E was composed of DGs and TGs. Lipids in group C differed between female GWI and female controls. In group D, neutral lipids of the male and female GWI groups were both increased compared to their sex-matched controls. Both group D and E were composed of the neutral lipids, DG and TG, and notably differed in their range of degree of unsaturation, with lipids in group D consisting of more double bonds and those in group E consisting of fewer double bonds. Group F consisted of 2 lipid species that are atypical of



any other group, but do not match each other either. PE34:2 is highly increased in the female controls and decreased in all other groups, while TG 46:0 is highly decreased in the female GWI group and increased in all other groups.

Of the top 50 lipids significantly modulated by sex and diagnosis, more lipid species were significantly modulated in male GWI cases (Fig. 3A) than in female GWI cases (Fig. 3B) as compared to their respective sex-matched controls. Among male participants, TG and DG were significantly increased by 1.18-fold at minimum (TG 58:9, $\log_2FC = 0.24$) and all CEs were significantly decreased by 0.88-fold at minimum (CE 20:3, $\log_2FC = -0.19$) in GWI veterans versus controls.

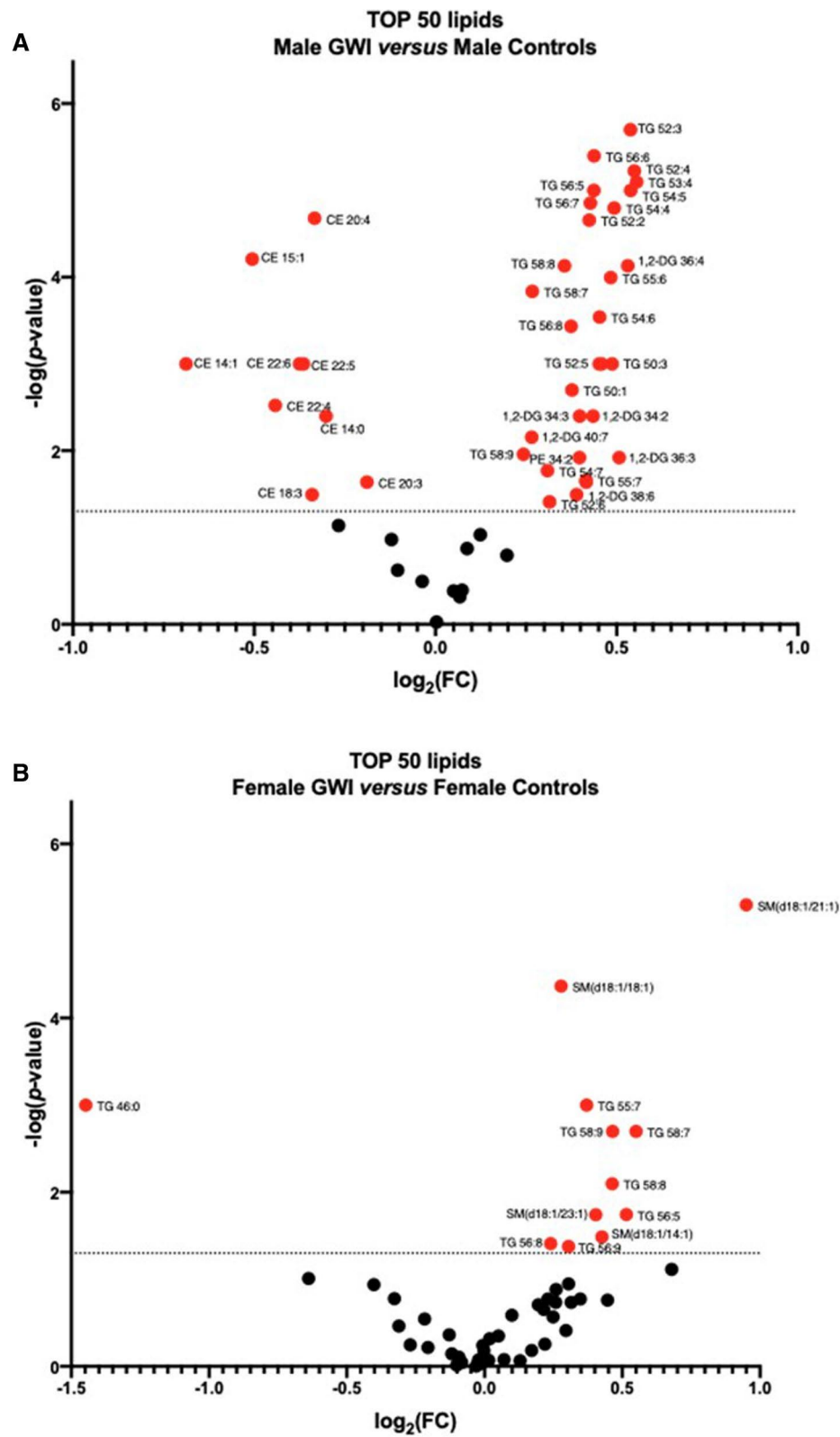
A few TGs and SMs were significantly increased by a minimum of 1.18-fold change (TG 56:8, $\log_2FC = 0.24$) in female GWI participants compared to female controls, except for TG 46:0, which was significantly decreased by 0.37-fold ($\log_2FC = -1.45$).

Discussion

Previous animal studies have shown that exposure to GW chemicals, such as those to which GW veterans were exposed, disturbs lipid homeostasis and contributes to inflammatory and metabolic dysfunction that are seen in veterans with GWI [7–9, 14, 15, 41]. To evaluate the translational significance of prior mechanistic studies, the current study analyzed plasma lipid profiles of veterans

(See figure on next page.)

Fig. 3 Fold Change of the top 50 lipids by sex. Relative changes of the top 50 lipids significantly modulated by sex and diagnosis in males (A) and females (B) between GWI participants and GW cases are shown by fold change (FC) on the volcano plots. Each dot represents one individual lipid from the list of top 50 lipids identified by MetaboAnalyst. The red dots indicate the significant changes relative with p -value threshold set at 0.05 (horizontal dotted line)



with GWI and control participants. Most of the differences pertained to the neutral lipids (CE, TG and DG), where the degree of unsaturation and ratios of AA-to-DHA were changed in GWI compared to controls. Our study shows that male veterans with GWI had significant increases in total neutral lipid content compared to male controls. Female veterans with GWI had elevated levels of Cer and lower levels of SFA TG species as compared to female controls. These sex-specific plasma lipid compositions in GWI veterans require further attention as they may indicate progressive metabolic and inflammatory processes, particularly in male veterans with GWI.

In the plasma, neutral lipids primarily appear in lipoprotein compartments due to their hydrophobic nature. The neutral lipid class of TG are major dietary fats, but they are also synthesized in the liver [42]. Because dietary TGs are digested to free fatty acids and monoglycerides in the intestines, plasma TG levels within lipoprotein particles are reflective of TG synthesis in liver via acylation with DG [43]. In plasma, an excess of TG, such as seen in the male veterans with GWI, is thought to reflect an overproduction of very low-density lipoprotein (VLDL) corresponding with the production of CE-depleted low-density lipoprotein (LDL) particles [42]. This production of CE-depleted LDL may explain the significant decrease in CE in male veterans with GWI. In the current study, female participants, irrespective of their diagnosis, had lower levels of TG and DG compared to their male counterparts. This has also been reported by the Genetics of Lipid-Lowering Drugs and Diet Network (GOLDN) study of plasma lipidome, who attributed this to normal sex-linked lipid differences [26]. In terms of the clinical implications of elevated TG in male veterans with GWI, elevated plasma TG is thought to be a risk factor for cardiovascular disease [44]. Elevated TG and the generation of CE-depleted LDL particles are also the defining features of dyslipidemia associated with insulin resistance and type 2 diabetes mellitus [42].

Veterans with GWI may be more sedentary and exercise less than healthy controls due to their condition, so this TG increase could reflect heightened body weight in veterans with GWI. A positive association between high TG and elevated body mass index (BMI) has been previously reported [45, 46]. As early as 1995, it was observed that veterans with severe GWI had slightly higher BMI than non-cases [33]. However, BMI did not differ between GWI and controls (in a subsample) and therefore unlikely to explain the observed differences in lipid profiles. A recent health survey of GW veterans from the Fort Devens cohort suggested increases in the prevalence of high blood pressure, diabetes, and cardiovascular disease over the last 30 years [4]. The Fort Devens Cohort was also assessed to have significantly higher rates of

diabetes in both male and female GW veterans than the general population [47]. However, differences between GWI and controls were not evaluated in this prior study. In our current study, a lack of difference in cardiovascular risk factors between controls and GWI suggested that observed differences in lipid profiles between the two groups were not simply a reflection of the presence of cardiovascular risk factors in this study cohort. As many of the neutral lipid changes may be indicative of subsequent risk of cardiovascular disease with age, further assessment of blood lipids by clinicians may be helpful for monitoring cardiovascular health as veterans with GWI age.

In the current study, SFA-containing TG species were reduced in female veterans with GWI. The role of SFA in promoting cardiovascular disease remains controversial as the source of these SFA-containing lipids and the individual identity of each SFA influences their potential role in promoting cardiovascular disease [21, 48]. As the SFA-TG species measured ranged from TG 46:0 to TG 54:0, and therefore consisted of 46 to 54 carbons split amongst 3 fatty acid chains, it is likely that the SFA-TG species measured contained long-chain fatty acids, which are over 12 carbons in length [48]. However, their significance in the clinical presentation of female GWI is unclear. Since these same TG-SFA species were elevated among those with cardiovascular risk factors, future studies aimed at different aspects of dietary modification in male vs. female GW veterans may be required to better understand the role of SFA-containing TG species in the health, particularly cardiovascular health, of veterans with GWI.

Our previous studies using subsets of samples from these cohorts showed increases in proinflammatory cytokines, interleukin-1 beta (IL-1 β), interferon gamma (IFN- γ) and IL-6, and increases of peripheral immune cells among veterans with GWI compared to controls [18]. Others have shown that veterans with GWI have increases in other blood inflammatory markers consisting of C-reactive proteins and matrix-metalloproteinase-2 [49]. Several proinflammatory cytokines, including IL-1 β and IL-15, are associated with an increase in GWI symptom severity [50]. As such, an examination of lipids related to inflammation may provide additional information about the underlying biology of GWI. Since omega-6 AA or omega-3 DHA modulate inflammation and comprise a large proportion of PUFA, we focused on lipids that contain AA and DHA as their fatty acid side chains. Our previous studies have shown that the AA and DHA content within certain PL classes were associated with GWI [17]. We now show that ratios of AA-to-DHA were increased in TG species among male and female veterans with GWI. The ratio of AA-to-DHA was also increased

in PE among male GWI veterans. As AA and DHA compose 50% and 40% of brain PUFAs respectively, sufficient AA is necessary for the growth, repair, and maintenance of neurons and DHA is used in neurotransmission [51]. However, AA metabolites, such as prostaglandins, are known to activate pro-inflammatory pathways whereas DHA metabolites, such as resolvins, contribute to inflammation resolution pathways [24, 52]. In addition, studies have shown that patients with a low DHA/AA ratio, (i.e. a higher AA-to-DHA ratio) had a higher risk of acute coronary syndrome than those with a high DHA/AA ratio, and this was significant for men in particular [53]. These differences in AA and DHA composition could reflect a generalized pro-inflammatory state in veterans with GWI, which would be consistent with reports of markers of chronic, low-grade inflammation being associated with GWI diagnosis [49, 54]. Hence, future studies are needed that interrogate systems-level omic data along with biological parameters of inflammation to identify specific inflammatory pathways that are associated with GWI pathogenesis.

Another indication of pro-inflammatory states in GWI would be the increase in Cer in female GWI veterans, as ceramides are known to stimulate the production of pro-inflammatory cytokines, such as IL-6 and TNF- α , in macrophages and are correlated with IL-6 levels in serum [55, 56]. Production of long-chain ceramides is known to induce apoptosis [57]. The increase in the long chain Cer measured in this study (d32:0–44:2) may therefore indicate increased cell death in female GWI veterans. Ceramide levels are shown to be increased with obesity and metabolic disorders, particularly diabetes. For instance, Boon and colleagues also found that Cer elevation is another marker of the dyslipidemia of type 2 diabetes mellitus and promotes insulin resistance [55]. Others have shown that plasma Cer increases are associated with obesity and may be indicative of lipotoxicity, which may ultimately contribute to the development of diabetes and insulin resistance [58, 59]. These studies suggest a different process in males and females in relation to Cer-mediated metabolic dysfunction that warrants further investigation.

This study provides key information about blood biomarkers associated with adverse metabolic and inflammation-related lipid profiles. The BMI in females was non-significantly lower than in males and therefore did not present as a confounding factor for the differences in lipids seen between the two sex groups. These findings are largely consistent with previous studies showing sex-specific differences in lipid profiles. Another limitation of our study includes unavailability of dietary data and medication data, particularly on the use of statins and omega-3/fish oil supplements. Since statins lower TG levels in blood and our findings suggest increases of

TG in male veterans with GWI, we anticipate that it is unlikely that statin use affected our study results. Similarly, we have previously shown that AA-to-DHA ratios are decreased after DHA supplementation [39]. Since we observed an increase in AA-to-DHA ratios in male GWI veterans, it is similarly unlikely that omega-3 or fish oil supplementation was a confounder in our study. As CE levels were lower in male GWI veterans, measuring free cholesterol would be useful in future studies to determine if either the level of free cholesterol or the rate of cholesterol esterification had decreased. Unfortunately, we could not reliably detect the labelled free cholesterol in our assay but will correct this in future studies. While we have previously examined free fatty acids in a smaller cohort of GW veterans, which suggested that free forms of PUFA were indeed affected in GWI [18], a large study evaluating free fatty acids and their bioactive lipid metabolites will help better elucidate the role of these fatty acids in ongoing inflammatory processes associated with GWI.

Conclusion

This study identifies distinct sex-specific plasma lipid profiles associated with GWI diagnosis, with significantly elevated TG and DG and lowered CE as a distinctive pattern in male GW veterans with GWI. Triglyceride AA-to-DHA ratios were also increased in male GW veterans with GWI. Since these lipid changes are associated with metabolic syndrome and inflammation, our findings suggest veterans with GWI have maladaptive biological processes that involve lipid transport and metabolism. While additional mechanistic work is needed to better elucidate lipid dysfunction in GWI, these blood lipid signatures suggest that focused studies are needed into the role of dyslipidemia in the ongoing pathophysiology of GWI. The current findings suggest that blood lipid monitoring may be beneficial for veterans with GWI. As such, further research is needed to characterize how these lipids and their associated pathways relate to GWI pathology to identify potential biomarkers for tracking general health related outcomes in GWI.

Abbreviations

GWI: Gulf War Illness; GW: Gulf War; TG: Triglycerides; DG: Diglycerides; CE: Cholesterol esters; PL: Phospholipids; SFA: Saturated Fatty Acids; PUFA: Polyunsaturated fatty acids; DHA: Docosahexaenoic acid; AA: Arachidonic acid; ME/CFS: Myalgic encephalomyelitis/chronic fatigue syndrome; RNC: Roskamp Neurology Clinic; GWIC: Gulf War Illness Consortium; CDC: Center for Disease Control; LC-MS: Liquid chromatography–mass spectrometry; ESI: Electrospray ionization; MS: Mass spectrometry; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; SM: Sphingomyelin; Cer: Ceramides; HexCer: Hexosylceramides; QC: Quality control; CV: Coefficients of Variance; MUFA: Monounsaturated fatty acids; ANOVA: Analysis of variance; MLM: Mixed linear modeling; B–H: Benjamini–Hochberg; SD: Standard deviation; VLDL: Very low-density lipoproteins; LDL: Low-density lipoproteins; GOLDN: Genetics of Lipid-Lowering Drugs and Diet Network; BMI: Body mass Index.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-022-03272-3>.

Additional file 1: Figure S1. Individual levels differences in major blood classes between GWI and controls. Bar graphs indicate mean $\pm$ SE and individual values of total lipids (A), degree of unsaturation of each lipid class (B) and AA and DHA content of these lipids (C) that were significantly different between controls and GWI. Black asterisks (*) indicate significant differences between GWI cases and their sex-matched controls ($p < 0.05$).

Additional file 2: Figure S2. Association of blood lipid profiles and the presence of cardiovascular risk factors among the study population. Major lipids were further evaluated to determine the impact of presence of reported cardiovascular risk factors (no = 8 and yes = 32). Heatmap generated from z-scores transformed from individual concentrations. Differences in AA and DHA containing PL and neutral lipids were affected the presence of cardiovascular risk factors. Total lipid content was unaffected by cardiovascular risk factors. Black asterisks (*) indicate significant differences between GWI cases and their sex-matched controls ($p < 0.05$).

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Authors' contributions

SO: Conceptualization, Investigation, Formal Analysis, Writing—Original Draft, Writing—Review and Editing, Visualization, Data Curation AN: Formal Analysis, Writing—Original Draft, Writing—Review and Editing, Visualization. MP: Formal Analysis, Writing—Original Draft, Writing—Review and Editing, Visualization. JEE: Methodology, Formal Analysis, Data Curation, Writing—Review and Editing, NK: Resources, Funding acquisition, Writing—Review and Editing, APK: Resources, Investigation, Writing—Review and Editing, KS: Resources, Funding acquisition, Writing—Review and Editing, MHK: Resources, Writing—Review and Editing, MM: Conceptualization, Writing—Review and Editing, FC: Conceptualization, Methodology, Funding acquisition, Investigation, Resources, Supervision, Project administration, Writing—Review and Editing, LA: Conceptualization, Methodology, Resources, Writing—Original Draft, Writing—Review and Editing, Formal Analysis, Supervision, Funding acquisition, Investigation, Project administration. All authors read and approved the final manuscript.

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Data availability

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Declarations

Ethics approval and consent to participate

An informed consent was collected for each participant and was reviewed by the IRB of each respective site.

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Not applicable.

Competing interests

Not applicable.

Disclaimer

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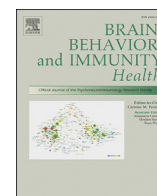
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Short Communication

Association of the tissue microstructural diffusivity and translocator protein PET in Gulf War Illness



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ABSTRACT

About a third of all United States veterans who served in the 1991 Gulf War (GW) report a range of chronic health symptoms including fatigue, neurocognitive symptoms, and musculoskeletal pain. There is growing evidence supporting the detrimental effects of maladaptive neuroimmune reactions in this multi-symptom illness. Indeed, recent studies using positron emission tomography (PET) using the radioligand [¹¹C]PBR28, which binds the neuroinflammation marker 18 kDa translocator protein (TSPO), and diffusion magnetic resonance imaging (dMRI) have independently identified the anterior cingulate (ACC) and midcingulate cortices (MCC) as key regions for differentiating GWI veterans from healthy controls (HC). Here, we used integrated (i.e., simultaneous) PET/MRI imaging techniques, paired with dMRI processing methods (neurite density imaging, NDI, and free-water diffusion tensor model to single-shell high-order dMRI), to directly evaluate the relationship between ACC and MCC microstructural tissue parameters, TSPO signal and clinical parameters in the same cohorts of 10 GWI veterans and 19 HCs.

Within the regions evaluated, TSPO signal elevations were associated with restricted diffusivity in the extracellular compartment, while clinical measures were best explained by neurite density and cellular structure complexity measures. Our study is the first to provide evidence of a relationship between PET and dMRI modalities in GWI and suggests that microstructural changes in the ACC and MCC are correlated to mood symptoms and cognitive performances in GWI veterans.

1. Introduction

Approximately one third of the 700,000 U.S. veterans who served in the 1991 Gulf War (GW) experience an array of chronic health symptoms, characterized by fatigue and sleep problems, pain, neurological, cognitive, and mood symptoms, respiratory complaints, gastrointestinal problems, and skin symptoms, collectively termed Gulf War Illness (GWI) (White et al., 2016). Effects of organophosphate (OP) neurotoxicant exposures, such as pesticides, and sarin/cyclosarin nerve agents that were present in the GW theater, possibly coupled with heightened innate immune responses, may give rise to chronic symptom complaints experienced by GWI veterans (Chao et al., 2011; Steele et al., 2012; Sullivan et al., 2018). Findings from animal studies suggest that the exposure to neurotoxins, stress, and combat-related injuries to the central nervous system (CNS) may induce long-lasting neuroinflammatory responses in

GWI, characterized by dysregulated glial cell activation (Lacagnina et al., 2021; Macht et al., 2019; O'Callaghan et al., 2015). While evidence for neuroinflammation in GWI has until recently been mostly limited to the preclinical literature, in a recent positron emission tomography (PET) study with [¹¹C]PBR28, a second-generation radioligand for the 18 kDa translocator protein (TSPO), our group observed widespread cortical elevations in neuroinflammation. Elevated [¹¹C]PBR28 signal was evident in regions including the anterior and midcingulate cortices (ACC and MCC, respectively), in GWI veterans compared to healthy controls or healthy GW veterans (Alshelh et al., 2020). These abnormal neuro-immune responses appear to trigger downstream macro- and microstructural changes in the brain (Koo et al., 2018). Indeed, diffusion magnetic resonance imaging (dMRI) studies found that microstructural alterations (including the ACC) were associated with elevated levels of peripheral proinflammatory cytokines, and with worse fatigue

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symptoms, in GWI veterans (Cheng et al., 2020).

Despite this converging evidence from separate studies, the relationship between PET neuroinflammatory signals and dMRI measures of microstructural alterations in GWI has never been directly explored in the same individuals.

In this study, we used integrated (i.e., simultaneous) [^{11}C]PBR28 PET/MRI to address this aim. Specifically, we sought to assess if changes in microstructural integrity were related to increased neuroinflammatory signaling in the same brain areas and tissue compartments. We applied two dMRI processing models, neurite density imaging (NDI) and free-water diffusion tensor model (FW-DTI) (Pasternak et al., 2014; Zhang et al., 2012) to single-shell high-order dMRI data collected in GWI veterans (and a healthy control group) from a recent GWI PET study (Alshelhi et al., 2020). Furthermore, we compared subjective clinical outcomes with multi-compartment diffusion measures to investigate the relationships between objective brain imaging markers and self-reported health symptoms in GWI veterans.

2. Materials and methods

2.1. Participants

Brain imaging outcomes from 10 veterans with GWI (2 females, 49.6 ± 3.1 years old [mean $\pm$ SD]) and 19 healthy controls (HC, 11 females, 44.1 ± 13.04 years old) were obtained from an established GWI biorepository and were included for the analysis. GWI case status was defined by the Kansas GWI case criteria, which requires multiple or moderate-to-severe chronic symptoms reported in at least three out of six symptom domains (fatigue, somatic pain, neurological cognitive, gastrointestinal, respiratory, and skin abnormalities) (Steele, 2000). Clinical outcomes including self-reported scales of pain, mood and cognitive testing of sustained attention including the Short-Form McGill Pain Questionnaire (SF-MPQ2), Patient-Reported Outcomes Measurement Information System (Promis-29), Conner's Continuous Performance Test III (CPT3), and -because GWI shares some of the core symptoms reported by patient suffering from fibromyalgia-the 2011 American College of Rheumatology (ACR) Fibromyalgia Diagnostic Criteria survey were also obtained from the repository for analysis (Cella et al., 2010; Conners et al., 2000; Dworkin et al., 2009; Wolfe et al., 2011).

2.2. Procedures

We processed the single-shell, high-order dMRI data (b -value = 3000 s/mm^2 , 60 gradient directions, TR/TE/voxel size: 119 msec/8000 msec/ $2.5 \times 2.5 \times 3 \text{ mm}$) acquired in the GWI PET study by Alshelhi et al. (2020) and shared through the Boston Biorepository and Integrative Network (BBRAIN) for GWI (Keating et al., submitted). Microstructural diffusion indices reporting neurite density (ND), orientation dispersion (OD), and isotropic diffusion (IsoVF) were reconstructed using the NDI model in a similar protocol as described previously (Cheng et al., 2020). FW-DTI derived measures, free-water corrected fractional anisotropy (FW-FA), and free-water corrected mean diffusivity (FW-MD), were extracted using the single shell free-water elimination diffusion tensor model (Pasternak et al., 2014). Reconstructed diffusion maps from NDI and FW-DTI models were then linearly registered to the Montreal Neurological Institute 152 (MNI152) structural template and projected to hemispheric cortical surfaces (Greve et al., 2014). For the PET analysis, standardized uptake value ratio (SUVr) images were generated from data collected over the 60-90 min post-injection [^{11}C]PBR28 PET interval, preprocessed and transformed to MNI152 space, as previously described (Alshelhi et al., 2020; Loggia et al., 2015; Zurcher et al., 2015). The two regions of interest (ROI), ACC and MCC, were structures that displayed significantly elevated [^{11}C]PBR28 PET signal and significant microstructural alterations in GWI veterans compared to healthy controls in previous studies (Alshelhi et al., 2020;

Cheng et al., 2020). Both ROIs were projected to the standard space cortical surfaces using the same method as the reconstructed dMRI maps. Mean dMRI measures and SUVr values were then extracted from these regions. Clinical variables were also obtained as described by Alshelhi et al. (2020).

Partial correlations were performed to assess the relationship between dMRI measures and (1) [^{11}C]PBR28 signal (controlling for sex, age, and TSPO genotype polymorphism, which predicts binding affinity to the PET radioligand) (Owen et al., 2012) and (2) clinical variables (controlling for sex and age). Significant p -values ($p < 0.05$) were reported along with Spearman correlation coefficients (ρ).

3. Results

In the whole group ($N = 29$) analysis, elevated [^{11}C]PBR28 PET signal was significantly correlated with lowered IsoVF and OD in the MCC (IsoVF: $\rho = -0.498$, $p = 0.008$; OD: $\rho = -0.386$, $p = 0.047$) and lowered FW-MD in the ACC ($\rho = -0.382$, $p = 0.049$). The same patterns were also found in MCC IsoVF and ACC FW-MD analysis performed within GWI veterans (Fig. 1). Other multi-compartment diffusion measures did not show significant patterns to [^{11}C]PBR28 PET signals in either ROI.

Table 1 lists the results of partial correlation analyses between clinical variables and multi-compartment diffusion measures in GWI veterans. Positive correlations were evident between (1) CPT3 scores and OD in both ACC (detectability $p = 0.043$, omission errors $p = 0.018$, variability $p = 0.048$) and MCC (detectability $p = 0.032$, omission errors $p = 0.043$, commission errors $p = 0.046$), (2) CPT3 HRT block change test and ND in the ACC ($p = 0.044$), (3) SFMPQ-2 sensory sum and ND in the MCC ($p = 0.027$), and (4) ACR total score and OD in the ACC ($p = 0.016$). Negatively correlations were found between (1) Promis-29 anxiety domain scores and IsoVF in the ACC ($p = 0.042$), (2) CPT3 scores and ND in the MCC (detectability $p = 0.03$, omissions $p = 0.022$), and (3) CPT3 HRT and OD in the MCC ($p = 0.05$).

4. Discussion

Our study revealed significant relationships between the upregulation of the neuroinflammatory marker TSPO, detected by [^{11}C]PBR28 PET signals, and decreased extracellular isotropic diffusivity (IsoVF), reduced cellular complexity (OD), and lowered cellular packing density (FW-MD), in the ACC and MCC of GWI veterans. This study is an extension to our previous analyses, which showed elevated levels of the neuroinflammatory marker TSPO in a widespread set of brain regions in GWI (Alshelhi et al., 2020). The neuroinflammatory marker TSPO is normally expressed at low levels but becomes dramatically upregulated -predominantly in activated glial cells-during neuroinflammatory responses (Rupprecht et al., 2010). TSPO signal elevations observed in diseases such as fibromyalgia and GWI (Albrecht et al., 2019; Alshelhi et al., 2020) suggest that dysregulated glial activation may contribute to the pathophysiology of GWI, as suggested by the preclinical literature. The present study is the first to report dMRI correlates of GWI neuroinflammation. The negative correlations between OD or IsoVF and [^{11}C]PBR28 PET signal in the MCC suggested hindered isotropic diffusivity, which could arise from local glial activation or immune cell infiltration (Yi et al., 2019; Zhang et al., 2012). In fact, decreased OD and IsoVF was shown to reflect chronic stages of microglia-mediate neuroinflammation, which corroborate findings from previous studies (Alshelhi et al., 2020; Yi et al., 2019). We also observed a similar pattern between elevated [^{11}C]PBR28 PET signal and lowered FW-MD in the ACC, which suggested decreased cellular packing density that could be reflecting gliosis or cytoarchitecture disruption (Pasternak et al., 2014). In recent GW studies, biomarkers indicating the presence of neuronal injury and gliosis were detected in GWI veterans 30 years after the war, and in GW rat models, the disruption of oligodendrocyte development and changes in glial morphology were identified as key components in studying the chronic

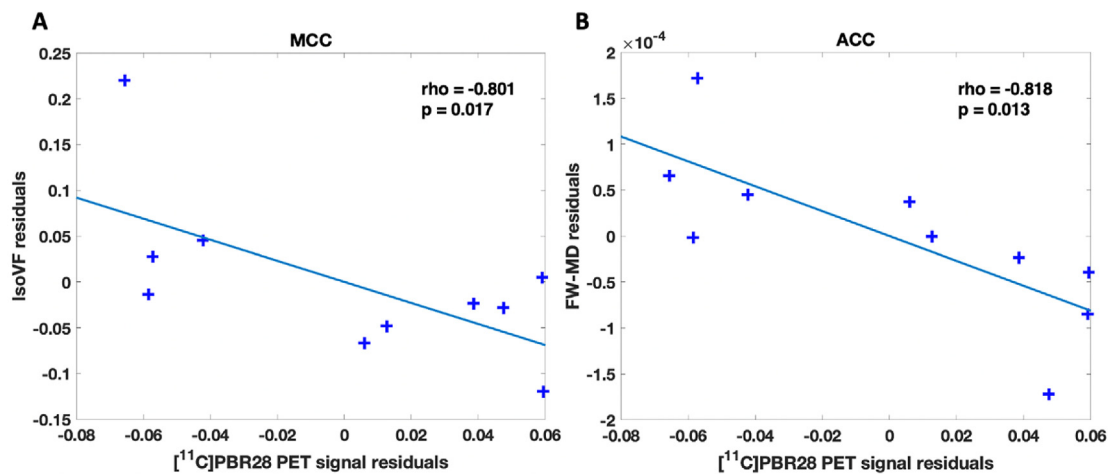


Fig. 1. Multi-compartment diffusion measures correlated with [^{11}C]PBR28 PET signals in the ACC and MCC in GWI veterans. Scatter plots illustrate the negative correlations between the neuroinflammation marker TSPO ([^{11}C]PBR28 PET signal) and (A) the isotropic diffusion measure (IsoVF) in the MCC, or with (B) the free-water corrected mean diffusivity (FW-MD) in the ACC. Results are plotted using TSPO genotype-, sex-, and age-controlled [^{11}C]PBR28 PET signal and age- and sex-controlled diffusion measures. Partial correlation coefficients (ρ) are shown in the plots with p-values. MCC = midcingulate cortex; ACC = anterior cingulate cortex.

Table 1
GWI multi-compartment diffusion measures correlations with clinical variables.

	ACC			MCC	
	ND	IsoVF	OD	ND	OD
Promis29 Raw score:	0.216			0.601	
Anxiety		-0.724*	-0.416		-0.674
SF-MPQ2: Sensory Sum	-0.369	-0.604	-0.111	0.765*	-0.532
Conner's CPT3: Detectability	-0.345	0.593	0.723*	-0.755*	0.751*
Conner's CPT3: Omissions	-0.040	0.647	0.797*	-0.780*	0.722*
Conner's CPT3: Commissions	-0.371	0.525	0.501	-0.656	0.716*
Conner's CPT3: Hit Reaction Time (HRT)	0.272	-0.502	-0.198	0.466	-0.707*
Conner's CPT3: Variability	-0.551	0.423	0.711*	-0.455	0.510
Conner's CPT3: HRT Block Change	0.720*	0.202	-0.327	-0.191	0.174
ACR Total score	-0.146	0.623	0.806*	-0.469	0.354

Partial correlation coefficients are reported, significant ($p < 0.05$) results are bolded. * $p < 0.05$.

neuroinflammatory responses in GWI (Abou-donia et al., 2020; Belgrad et al., 2019). Indeed, our findings on the relationship between [^{11}C]PBR28 signal and dMRI measures confirmed that neuroinflammatory responses may be accompanied by microstructural diffusion alterations in GWI.

Interestingly, while our prior PET analyses didn't reveal statistically significant association with clinical variables, our multi-compartment dMRI measures extracted from the same ACC/MCC regions were correlated with measures of pain, anxiety, and cognitive performance, all symptom domains commonly affected in GWI veterans (Janulewicz et al., 2017; Jeffrey et al., 2019; Sullivan et al., 2018; White et al., 2016). Our results showed that lower IsoVF and OD in the ACC were associated with worse anxiety, while higher ND in the MCC was related to higher pain severity.

GWI veterans often report sensorimotor deficits and memory impairments, often coupled with heightened innate immune responses that give rise to chronic symptom complaints (Chao et al., 2011; Janulewicz et al., 2017; Jeffrey et al., 2019; Sullivan et al., 2018). Furthermore, animal models of GWI show that stress combined with exposure to

chemicals present in the GW theater (pesticides, and sarin/cyclosarin nerve agents) produces GWI symptoms greater than exposure to chemicals alone and causes neuronal cell death in the cingulate cortex (O'Callaghan and Miller, 2019). These changes in the cingulate cortex cause neurobehavioral changes similar to those observed in GWI veterans (Abdullah et al., 2012; Macht et al., 2019). In our study, higher rates of CPT3 detectability, omission and commission errors, and response variabilities were found to correlate with higher OD and lower ND in GWI veterans, whereas slower and varying response rates were associated with lower OD and higher ND. Similar results were reported in other studies, which documented an association between reduced gray and white matter volumes and higher rates of CPT3 omission errors in GW veterans exposed to chemical warfare agents (Chao et al., 2011). Lower ND accompanied with higher OD could reflect decreases in overall volume on the microscopic level and increases in diffusion tortuosity (Colgan et al., 2016), while higher ND has been linked to less efficient information processing capacity (Genç et al., 2018). However, our ability to precisely interpret multi-compartment dMRI measures is limited, particularly as the relationship between microstructural alterations and dMRI measures can vary depending on the etiology of the disease.

In the current study, we demonstrated the presence of changes in microstructural environments of the ACC and MCC, associated neuro-inflammatory signals and behavioral measures, in a small GWI sample. However, the current PET analysis method, using SUVR images from data collected over the 60-90 min post-injection [^{11}C]PBR28 PET interval, was determined from studies on chronic pain and amyotrophic lateral sclerosis, therefore, would require further validation in larger GW veteran cohorts (Loggia et al., 2015; Zürcher et al., 2015). Future studies with a larger GW veteran cohort and post-mortem human studies will be required to shine more light on the neurobiological correlates of neuro-inflammation in GWI.

Declaration of competing interest

None.

Acknowledgments

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A cellular approach to understanding and treating Gulf War Illness

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Abstract

Gulf War Illness (GWI), a disorder suffered by approximately 200,000 veterans of the first Gulf War, was caused by exposure to low-level organophosphate pesticides and nerve agents in combination with battlefield stress. To elucidate the mechanistic basis of the brain-related symptoms of GWI, human-induced pluripotent stem cells (hiPSCs) derived from veterans with or without GWI were differentiated into forebrain glutamatergic neurons and then exposed to a Gulf War (GW) relevant toxicant regimen consisting of a sarin analog and cortisol, a human stress hormone. Elevated levels of total and phosphorylated tau, reduced microtubule acetylation, altered mitochondrial dynamics/transport, and decreased neuronal activity were observed in neurons exposed to the toxicant regimen. Some of the data are consistent with the possibility that some veterans may have been predisposed to acquire GWI. Wistar rats exposed to a similar toxicant regimen showed a mild learning and memory deficit, as well as cell loss and tau pathology selectively in the CA3 region of the hippocampus. These cellular responses offer a mechanistic explanation for the memory loss suffered by veterans with GWI and provide a cell-based model for screening drugs and developing personalized therapies for these veterans.

Keywords Gulf War Illness · Human-induced pluripotent stem cells · Tau · Microtubule · Mitochondria · Neuronal activity · Memory

Introduction

Gulf War Illness (GWI) is a debilitating multi-symptom disease suffered by 30% of the nearly 700,000 United States veterans deployed during the 1991 Gulf War [1–3]. Symptoms include fatigue, musculoskeletal pain, gastrointestinal problems, and alterations in central nervous system (CNS) functioning [4]. CNS dysfunction includes short-term memory problems, poor attention/concentration, reduced information processing speeds, persistent headaches, and mood

and sleep disturbances [5–7]. The etiology of the disease remains unclear, with no effective treatments yet available [8].

Although high-dose organophosphate exposure irreversibly inhibits acetylcholinesterase [9], growing evidence indicates that low-dose organophosphate exposure is responsible for the long-term cognitive deficits in GWI [10, 11], thus implicating novel biological targets. Studies have shown that GWI results from organophosphate pesticides and nerve agents combined with the stress of the battlefield, the latter of which can be mimicked in the laboratory by restraint of an animal's mobility or introduction of a stress hormone [12, 13]. The most prevalent mechanistic hypothesis for GWI is that its symptoms result from a toxicant-induced self-perpetuating state of neuro-inflammation [14, 15], while other studies indicate that microtubule-based abnormalities (including axonal transport deficits) might underlie the disease [16–18]. Rodents exposed to GW-relevant toxicants display neuronal microtubules with fewer associated proteins than normal, leading to reduced microtubule width [19]. This may be due to abnormal phosphorylation (and hence detachment) of

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fibrous microtubule-associated proteins as well as molecular motor proteins [20]. Our own studies have shown that a GW toxicant regimen consisting of a sarin analog and a stress hormone results in reduced microtubule acetylation in cultured rat neurons, as well as deficits in mitochondrial transport and dopamine release, both of which are corrected by pharmacologic restoration of normal microtubule acetylation [21].

Abnormalities in tau, a prominent fibrous microtubule-associated protein in the axon, contribute to many neurological disorders [22]. Aberrantly phosphorylated tau detaches from the microtubule and results in altered microtubule properties [23], while the abnormal tau, both in soluble form and aggregated into intracellular inclusions, produces a variety of toxic effects [24, 25]. Interestingly, auto-antibodies against tau are present at higher levels in peripheral blood serum from veterans with GWI relative to non-veteran controls [26, 27], and low-level organophosphate-based neuroinflammation can also induce tau hyperphosphorylation [28, 29]. Thus, potential tau pathologies in GWI might explain why a limited exposure to neurotoxicants led to a persistent disease condition that was not reversed upon termination of the exposure, and may reconcile mechanistic models based on either neuroinflammation or microtubule abnormalities. A predisposition to tauopathy might also explain a key mystery of GWI, which is why some veterans acquired the disease, while their similarly exposed colleagues did not.

Here, together with a rat model for GWI, we used glutamatergic neurons [30] differentiated from human-induced pluripotent stem cell (hiPSC) lines developed from veterans with or without GWI and banked in a biorepository [31] to delve deeper into the mechanistic basis of the disease. Our results reveal new information on the response of neurons and the nervous system to a GW toxicant regimen, specifically on microtubules, tau, mitochondria, neuronal activity, and memory. Together, our results shine new light on the mechanistic basis of the disease, provide insight into a potential predisposition of some veterans to acquire the disease, and set the stage for the development and testing of new therapies.

Materials and methods

1. hiPSC generation and culture. hiPSC lines were generated from peripheral blood mononuclear cells (PBMCs) isolated from GW veterans with or without GWI, according to the Kansas GWI case definition [4], and banked in the Boston Biorepository, Recruitment & Integrative

Network (BBRAIN) for GWI. The hiPSCs were validated for their pluripotency by multiple methods, including morphology, immunostaining for pluripotency markers (TRA-1–60 and TRA-1–81 positive, and SSEA-1 negative), evaluation of karyotype, and ability to differentiate into the three germ layers. Three-germ layer differentiation was carried out as per the manufacturer's instructions using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems, SC027B), and cells were stained for markers of each germ layer: SOX17 (endoderm), Brachyury (mesoderm), and Otx2 (ectoderm).

All cells were cultured in 5% CO₂ at 37 °C (see supplemental material for more details). Briefly, the hiPSCs were initially cultured on mouse embryonic fibroblast (MEF) feeder cells (Gibco, A34180) for 2–3 passages before transitioning to a feeder-free culture in mTeSR1 (Stem Cell Technologies, 85857) on ES-qualified Matrigel (Corning, 354277) diluted 1:75 in cold DMEM/F12. Before passaging or directed differentiation, hiPSC colonies were selected to remove areas of spontaneous differentiation. hiPSCs at 80% confluency were passaged weekly with Collagenase Type IV (Stem Cell Technologies, 07909) with 10 µM ROCK inhibitor Y-27632 (Tocris, 1254).

2. Neuronal differentiation and GW toxicant treatment. hiPSC lines from three healthy GW veterans (controls) and four veterans with GWI (cases) were differentiated into forebrain glutamatergic neurons by methods adapted with modifications from previously established protocols [32] (see supplemental material for more details). Briefly, hiPSC colonies were dissociated in Accutase (Stem Cell Technologies, 07920) and 3 million hiPSCs were cultured in one well (10,000 cells per microwell) of an AggreWell 800 plate (Stem Cell Technologies, 34811) using the STEMdiff SMADi Neural Induction Kit (Stem Cell Technologies, 08581) supplemented with 10 µM Y-27632 and low-bFGF (4 ng/ml). On day 5, embryoid bodies (EBs) were replated using a cut P-200 pipette tip and cultured in three wells of a 6-well plate coated with ES-Matrigel. The STEMdiff SMADi Neural Induction medium was gradually replaced with neural progenitor cell (NPC) medium (see supplemental material). On day 12, neural rosettes were isolated using the STEMdiff Neural Rosette Selection Reagent (Stem Cell Technologies, 05832), resuspended in NPC medium, and NPCs were plated on Matrigel (Corning, 354234) coated plates. On day 16, NPCs were replated onto the final culture platform pre-coated with 0.01% poly-L-ornithine (Sigma-Aldrich, P4957) and 10 µg/ml laminin (Invitrogen, 23017015). NPCs were detached via Accutase, plated in neural differentiation medium supplemented with 200 µM ascorbic acid (Sigma-Aldrich, A5960),

500 μ M dibutyryl cAMP (Sigma-Aldrich, D0627), and 10 μ M DAPT (Tocris, 2634), and cultured for 6 days before long-term culture in neuronal maturation medium supplemented with ascorbic acid, dibutyryl cAMP, 20 ng/ml BDNF (Peprotech, 450–02), and 20 ng/ml GDNF (Peprotech, 450–10) (see supplemental material for more details). After 3 weeks in neuronal maturation medium, mature neurons were treated with our GW toxicant regimen consisting of 3 days of 2 μ M hydrocortisone (Cortisol) (Sigma-Aldrich, H0888), followed by 2 days of 2 μ M Cortisol plus 200 nM diisopropyl fluorophosphate (DFP) (Sigma-Aldrich, D0879). After 2 days in Cortisol + DFP, one half of the medium was changed, and then, cells were studied 3 days later (8 days after first cortisol exposure).

3. Evaluation of cell viability and plasma membrane integrity in cells exposed to GW toxicants. On day 16 of neuronal differentiation, NPCs were replated at a density of 25,000 cells/well on clear 96 well flat-bottom plates and cultured for 4 weeks followed by treatment with GW toxicants according to the procedure described above, except that DFP was tested at various concentrations, namely 200 nM, 1 μ M, and 2.5 μ M, with all concentrations also including 2 μ M cortisol exposure. Cells treated with our GW toxicant regimen were evaluated for their viability via the MTT assay (Abcam, ab211091) and the lactate dehydrogenase (LDH) assay (Invitrogen, C20300) according to the manufacturers' instructions. The absorbance was read on a Tecan Infinite M200Pro spectral plate reader for optical density with subsequent analyses to calculate percent cytotoxicity. See supplemental material for more detailed methods.

4. Quantitative immunocytochemistry. On day 16 of neuronal differentiation, NPCs were plated at a density of 70,000 cells per well of a 24 well plate on round German glass coverslips (Neuvitro, GG12PRE) and cultured for 4 weeks followed by treatment with the GW toxicant regimen (2 μ M Cortisol plus 200 nM DFP) according to the procedure described above. Cells were fixed in pre-warmed 4% paraformaldehyde (Electron Microscopy Sciences, 19202) in 0.1 M phosphate-buffered saline (PBS) for 10 min at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, X100) diluted in PBS for 10 min followed by blocking with 10% goat serum (Jackson ImmunoResearch, 005–000–121) diluted in PBS for 1 h at room temperature. Primary antibodies were diluted in PBS and incubated with cells overnight at 4 °C. The next day, secondary antibodies (diluted in PBS) were incubated with cells for one hour at room temperature protected from light. Cells were stained with 4,6-diamino-2-phenylindole (DAPI) (1:15,000; Invitrogen, D1306) in PBS for 5 min. Glass coverslips were

mounted on microscope slides using Fluoro-Gel with Tris Buffer (Electron Microscopy Sciences, 17985–10). Primary antibodies used were rabbit anti-TauR1 (1:1000; donation from Nicholas Kanaan, Michigan State University), mouse anti-AT8 (1:500; Thermo Scientific, MN1020), chicken anti-Tbr1 (1:200; Millipore-Sigma, AB2261), mouse anti-NeuN (1:500; Abcam, 104224), rabbit anti-MAP2 (1:500; Millipore-Sigma, AB5622), rabbit anti- β III-tubulin (1:2000; BioLegend, 802001), mouse anti-acetylated tubulin (1:8000; Sigma-Aldrich, T6793), and rat anti-tyrosinated tubulin (1:2000; Millipore-Sigma, MAB1864). Appropriate goat secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 were used at 1:1000 dilutions. Images were acquired on a Zeiss AxioObserver Z1 inverted microscope equipped with a Zeiss 20X/0.8 Plan-Apochromat air objective and AxioCam 506 mono-camera using the Zeiss Zen Blue software. All images from each replicate were acquired with identical exposure settings, did not contain pixel saturation, and showed minimal bleaching during image acquisition. Fluorescence intensity was quantified using the Zeiss Zen Blue software by tracing axon lengths and measuring the average fluorescence intensity per unit length of axon.

5. Extracellular tau ELISA analysis. Cell cultures were treated with 2 μ M Cortisol plus 200 nM DFP, and their supernatant was collected with protease inhibitors (Thermo Scientific, 78430) and phosphatase inhibitors (Thermo Scientific, 78428) and analyzed fresh for extracellular human total tau (Invitrogen, KHB0041) and [pT231] human phosphorylated tau (Invitrogen, KHB8051) according to the manufacturer's instructions. The absorbance was read on a Tecan Infinite M200Pro spectral plate reader for optical density with subsequent analyses to calculate the concentration of extracellular human total tau or [pT231] human phosphorylated tau in each sample. See supplemental material for more detailed methods.

6. Mitochondrial health, dynamics, and transport. On day 16 of neuronal differentiation, NPCs were replated at a density of 35,000 cells on 35 mm round glass bottomed dishes (Cellvis, D35-14–1.5-N) and cultured as described above. After application of the GW toxicant treatment (2 μ M Cortisol plus 200 nM DFP) or vehicle, the cell permeable dye tetramethylrhodamine, ethyl ester (TMRE) (Abcam, ab113852) was added directly into the cell culture medium to a final concentration of 200 nM and incubated at 37 °C for 30 min. After incubation, dishes were washed with FluoroBrite DMEM (Gibco, A1896701) imaging medium pre-warmed to 37 °C, and then, fresh FluoroBrite DMEM was used for live-cell imaging in a heated chamber at 37 °C and 5%

CO₂. Dishes were imaged using a Zeiss AxioObserver Z1 inverted microscope equipped with a Zeiss 63X/1.4 Plan-Apochromat oil objective and a Photometrics Evolve 512 Delta EMCCD camera using the Zeiss Zen Blue software. Images were acquired every 1 s for 2 min at a fixed exposure time across conditions. Mitochondrial transport was analyzed using the MultiKymograph plugin for (Fiji) ImageJ. Mitochondrial fluorescence intensity was analyzed by tracing the circumference of individual mitochondria and dividing the mean arbitrary fluorescence by the area, while length was measured using the longest axis. Fusion and fission events were quantified by counting the number of events per 100 μm of axon per minute.

7. Intracellular HDAC6 activity. HDAC6 activity was measured using a fluorometric kit (Biovision, K466-100) on fresh lysates according to the manufacturer's instructions. The assay utilizes deacetylase activity of HDAC6 toward a synthetic acetylated-peptide substrate resulting in the release of an AFC fluorophore, which can be quantified using a conventional microplate reader. In brief, cells treated with or without the GW toxicant regimen (2 μM Cortisol plus 200 nM DFP) were lysed in HDAC6 lysis buffer for 5 min on ice and centrifuged at $16,000 \times g$ for 10 min at 4 °C, and then, the supernatant was used to quantify protein content via the Pierce BCA protein assay kit (Thermo Scientific, 23225). 3 μl of each sample was mixed with 50 μl of HDAC6 substrate mix and incubated at 37 °C for 30 min, followed by addition of 10 μl of Developer to stop the reaction and incubated at 37 °C for 10 min. Fluorescence was measured on a Tecan spectral plate reader at excitation/emission 380/490 nm in endpoint mode at 37 °C. Manufacturer's instructions were followed to determine the amount of HDAC6 activity in each sample using a standard curve to plot the fluorescence obtained from the sample (ΔRFU) and calculate the HDAC6 activity according to the amount of protein loaded. The results are expressed as U per mg of protein, where U stands for the amount of HDAC6 required to deacetylate 1 pmol of HDAC6 substrate per minute under the assay conditions.

8. Neuronal activity analysis via Multi-Electrode Array (MEA) electrophysiology. On day 16 of neuronal differentiation, NPCs were replated onto Multiwell-MEA plates (Multi Channel Systems MCS GmbH, 24W300/30G-288) pre-coated overnight at room temperature with ~0.07% polyethyleneimine (PEI) (Sigma-Aldrich, 181978) diluted in borate buffer [for 50 ml: 155 mg boric acid (Sigma-Aldrich, B-0252), 237.5 mg borax (Sigma-Aldrich, B-9876), ddH₂O to 50 ml]. Plates were washed with ddH₂O and then thoroughly dried for 1 h before plating cells. Cells were plated at a density

of 50,000 cells per well and differentiated according to the protocol described above for 1 week in neuronal differentiation medium and 1 week in neuronal maturation medium, both with 5 $\mu\text{g}/\text{ml}$ laminin added weekly into the medium. Cells were then cultured in BrainPhys neuronal medium N2-A and SM1 Kit (Stem Cell Technologies, 05793) supplemented with ascorbic acid, dibutyryl cAMP, BDNF, GDNF, and weekly laminin, for 3 weeks before GW toxicant regimen treatment (2 μM Cortisol plus 200 nM DFP) and extracellular electrophysiological recordings. Spontaneous neuronal activity was recorded for 5 min using the Multiwell-Screen software (Multi Channel Systems MCS GmbH). Signals were sampled at 20 kHz and filtered with a 3500 Hz fourth-order low-pass Butterworth filter and a 1 Hz second-order high-pass Butterworth filter. Data analysis was performed using the Multiwell-Analyzer software (Multi Channel Systems MCS GmbH). Spikes were detected when the signal exceeded 6 standard deviations from the baseline noise. Bursts were detected when there were ≥ 4 spikes in 50 ms with 100 ms between bursts, with a maximum interval of 50 ms to start and end the burst. Network bursts were detected when there were six electrodes participating with three of them simultaneously participating. Spike and burst data were exported to Microsoft Excel for quantification. Neuronal activity was quantified for numerous spike, burst, and network burst parameters. An electrode was considered active with a minimum threshold of three spikes per minute. Actively bursting electrodes or active wells with network bursting needed at least one burst or network burst during the 5 min recording to be included for analysis. Neuronal activity was evaluated and confirmed pharmacologically by adding synaptic antagonists into the cell culture media. Activity was recorded before and 5–60 min after addition of the AMPA receptor antagonist CNQX (50 μM ; Sigma-Aldrich, C239), NMDA receptor antagonist AP5 (50 μM ; Sigma-Aldrich, A8054), GABA receptor antagonist Picrotoxin (50 μM ; Sigma-Aldrich, 124–87–8), and sodium channel antagonist tetrodotoxin (TTX) (1 μM ; Hello Bio, HB1034).

9. Extracellular glutamate analysis. Cell cultures were treated with the GW toxicant regimen (2 μM Cortisol plus 200 nM DFP) and their supernatant was collected with protease inhibitors and analyzed fresh for extracellular glutamate using the glutamate assay kit (Sigma-Aldrich, MAK004) according to the manufacturer's instructions. See supplemental material for more detailed methods.

10. Rat Behavioral Studies. Male albino Wistar rats (Envigo RMS, Inc., Indianapolis, IN) approximately 2 months old were housed in pairs in a

temperature-controlled room (25 °C), maintained on a 12 h light/dark cycle with free access to food (Teklad Rodent Diet 8604 pellets, Harlan, Madison, WI) and water. All animal procedures employed during this study were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University and are consistent with AAALAC guidelines. Measures were taken to minimize pain or discomfort in accordance with the Guide for the Care and Use of Laboratory Animals [33]. Efforts were made to minimize the total number of animals used while maintaining statistically valid group numbers. The drug dosing procedures for the *in vivo* experiments in this study were based on Koo et al. [13] where corticosterone (CORT) was administered to rats for several days to simulate physical stress and a single injection of DFP was administered to simulate nerve agent exposure. CORT is the stress hormone in rodents that corresponds to cortisol in humans. In our studies, 0.4 mg/kg CORT was administered in the drinking water (400 mg/L in 1.2% ethanol) for seven consecutive days based on each animal's 24 h water consumption, and DFP 1.5 mg/kg was administered on day seven by intraperitoneal injection. Separate groups of control rats were administered drinking water (1.2% ethanol) for 7 days followed by a saline injection on day 7. Behavioral testing (i.e., day 1 of the water maze hidden platform acquisition trials, and day 1 habituation in the novel object recognition test, see task descriptions below) was initiated 48 h after the DFP or saline control injection.

10a. Morris water maze procedures. Test apparatus—water maze testing was conducted using a modification of procedures that we have published previously [34, 35] in a circular pool (diameter: 180 cm, height 76 cm) made of plastic (Bonar Plastics, Noonan, GA) with the inner surface painted black. The pool was filled to a depth of 35 cm of water (maintained at 25.0 ± 1.0 °C) that covered an invisible (black) 10 cm round platform. The platform was submerged approximately 1 cm below the surface of the water and placed in the center of the northwest quadrant. The pool was located in a large room with diffuse lighting and a black curtain surrounded the pool that hid the experimenter from the test subjects. The curtain fully surrounded the pool with only narrow openings at the north and south quadrants and contained extra-maze geometric images (squares, triangles and circles) hung on the curtain to provide visual spatial cues to the subject. Swimming activity of each rat was monitored via a camera mounted overhead which relayed information including latency to find the platform, total distance traveled, time, and distance spent in each quadrant, to a video tracking system (Noldus EthoVision XT13®).

Hidden platform trials—for hidden platform trials, rats were given three trials per day for five consecutive days to locate and climb onto the hidden platform. A trial was initiated by placing the rat in the water directly facing the pool

wall (i.e., nose approximately 2 cm from the wall) in one of the four quadrants. The daily order of entry into individual quadrants was pseudo-randomized, such that all four quadrants were used across the study. For each trial, the rat was allowed to swim a maximum of 90 s to find the platform. When successful, the rat was allowed a 20 s rest period on the platform. If unsuccessful within the allotted time period, the rat was given a score of 90 s and then physically placed on the platform and also allowed the 20 s rest period. In either case, the rat was given the next trial after an additional 40 min rest period (i.e., intertrial interval = 40.0 min).

Probe trial—After the last hidden platform test on day 5, a 60 s probe trial was performed where the platform was removed from the pool and the “spatial bias” for the previous platform location was assessed [36]. This was accomplished by measuring the percentage of time spent in the previous target quadrant and the total dwell time in the annulus-40, defined as a 40 cm-diameter target zone centered on the previous hidden platform location.

Reversal learning trials—24 days after the DFP injection and 17 days after the completion of the first round of hidden platform and probe trials, the platform was switched to the opposite quadrant (i.e., southwest) in the pool and a second series of “reversal learning” trials was conducted. Other than the location of the hidden platform, these trials were conducted in the same manner as the first round of testing (three trials per day for five consecutive days, plus a probe trial on day five).

10b. Spontaneous novel object recognition (NOR) task. The NOR task used in this study was adapted from [37] and modified from versions published previously in our laboratory [38–40] using 4 h delays between training and test sessions. Briefly, test subjects were acclimated to laboratory conditions (i.e., tail marking, daily handling, and weighing) for at least 3 days prior to experimentation. During experimentation, the animals were transported to the laboratory and acclimated for 30 min prior to initiating the experimental phase; the animals remained in the laboratory for 15 min following completion of the day's testing.

Habituation—The animals were individually placed in a dimly lit (10-lx) training/testing environment (an opaque plastic chamber, 78.7 cm × 39.4 cm × 31.7 cm with bedding on the floor) for 10 min of chamber exploration. The NOR chamber was placed on a table positioned along the wall of the laboratory, and there were no room orienting cues or wall-mounted visual cues (except for the small B/W camera positioned above the NOR chamber). At the beginning of each series of NOR experiments, fresh bedding material was placed in the chamber prior to habituation and allowed to become saturated with animal odors. Animal droppings were removed between experimental sessions; however, the same

bedding remained in the chamber for the remainder of each study (i.e., during training and testing), thus preventing any specific olfactory cues over the course of experimentation.

Training trial—twenty-four hours after the habituation session, the animals were again acclimated to the testing room, then placed in the testing chamber with their nose facing the center of a long wall, and allowed to explore two identical objects for 10 min. The animal's behavior was observed and digitally recorded via a B/W camera located 69 cm above the chamber; the investigator sat quietly 10–15 ft. away from the NOR chamber.

Test trial—four hours after the training trial, two objects, one object identical to training (familiar) object, and a novel object were placed in the chamber, and the animal was allowed to explore both objects for 5 min. Experimental objects to be discriminated were a plastic multicolored Duplo-Lego blocks tower (12 cm in height, 6 cm in width) paired with ceramic conical-shaped green Christmas tree salt/pepper shakers (12 cm in height, 5 cm in diameter); all objects existed in duplicate. The objects were placed 19.3 cm from the walls of the chamber with a distance of 40 cm between the two objects. The role of familiar and novel object as well as chamber position of the object was randomly assigned across subjects and treatments, and objects were cleaned between sessions with a 50% ethanol solution to eliminate olfactory cues. The criteria for the observer to classify an object interaction as exploratory (investigative) behavior were direct interaction with nostrils or head positioning toward the object from a maximum distance of 2 cm. Physically climbing, rearing, and digging around an object was not scored as object exploration. The primary behavioral measure was time (s) spent investigating each object. A discrimination index (d2) was calculated on each test trial and was defined as the difference in time spent exploring the novel and familiar objects divided by the total exploration time for both objects: $d2 \text{ index} = (\text{novel} - \text{familiar}) / (\text{novel} + \text{familiar})$. This measure is considered as an index of recognition memory and takes into account individual differences in the total amount of exploration time. For data inclusion, the rat had to explore each individual object for at least 4 s and spend a minimum of 12 s of total object exploration. Animals were tested only once, and object exploration time was scored live under blind testing methods (i.e., the investigator was unaware of treatment assignment). The test sessions were also digitally recorded for record-keeping purposes.

11. Immunohistochemistry analyses. Rats were anesthetized with isoflurane and euthanized by transcardial perfusion with PBS (pH 7.4) followed by 4% (wt/vol) paraformaldehyde in PBS. Whole brains were post-fixed overnight in 4% paraformaldehyde. Brains were then transferred to a

cryoprotectant of 30% (wt/vol) sucrose and allowed to sink before cryosectioning. Whole 20 μm coronal cryosections were mounted in sequence onto charged microslides by the Histology Core Facility at Augusta University. For tau staining, slides were washed in PBS before incubation in sodium citrate buffer (10 mM tri-sodium citrate dihydrate, 0.05% Tween 20, pH 6.0) at 95 °C for 20 min. After washing in PBS, slides were blocked for 1 h at room temperature in 10% goat serum, 1% bovine serum albumin (Sigma-Aldrich, A7906), 0.5% Triton X-100, and glycine (7.5 mg in 1 ml, Fisher, BP381-5) diluted in PBS. Primary antibodies to tau (see immunocytochemistry above) were diluted in PBS with 10% of the blocking solution without glycine and incubated overnight at 4 °C. Appropriate goat secondary antibodies conjugated to Alexa Fluor 488, 555, and 647 were used at 1:1000 dilutions. Slides were mounted with glass coverslips (Corning, 2975–245) using Fluoro-Gel with Tris Buffer. Images were acquired on a Zeiss AxioObserver Z1 inverted microscope equipped with a Zeiss 20X/0.8 Plan-Apochromat air objective and AxioCam 506 mono-camera using the Zeiss Zen Blue software. All images from each replicate were acquired with identical exposure settings, did not contain pixel saturation, and showed minimal bleaching during image acquisition. Fluorescence intensity was quantified using Fiji (ImageJ) by drawing a box encompassing the CA1 or CA3 regions of the hippocampus, or in the prefrontal cortex, to measure the average fluorescence intensity, followed by dividing by the total number of DAPI-positive cells in the box.

12. Statistics. hiPSC-derived neurons and rat histology: all data analyses were conducted blind and presented as mean $\pm$ SEM. At least three independent repeats were conducted for each experiment. Data were arranged in Microsoft Excel before statistical analyses were performed using Prism 9 (GraphPad). All data sets were tested for normality using Shapiro–Wilk's test, and outliers were excluded using the GraphPad ROUT method (a three-step process that includes a robust nonlinear regression, the residuals were analyzed for outliers, and an ordinary least-squares regression was performed on the remaining data) with a coefficient of $Q = 0.1\%$ set at a strict threshold to remove definitive outliers. The statistical tests used were one-way analysis of variance (ANOVA) followed by the appropriate Tukey, Dunnett, or Šídák post hoc test to account for multiple comparisons (Kruskal–Wallis test followed by Dunn's multiple comparison test for data not normally distributed). An alpha of $p < 0.05$ was considered statistically significant.

Rat behavior statistical analysis was performed using SigmaPlot 11.2 and statistical significance was assessed using an alpha level of 0.05. Student's *t* tests and analysis of variance (with repeated measures when indicated) were used as

appropriate followed by the Student Newman–Keuls test for post hoc analysis after ANOVA comparisons. All results are expressed as the mean ($\pm$ SEM).

Study approval

All human studies involving recruitment and participation of GW veterans were approved by the Institutional Review Board of Boston University. All participants provided written informed consent for use of their blood samples for reprogramming into hiPSCs prior to participation in the study. All patient demographic information was deidentified to protect patient privacy. All animal procedures employed during this study were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University and are consistent with AAALAC guidelines.

Results

Validated pluripotency of veteran-derived hiPSCs. We recently established multiple lines of hiPSCs generated from peripheral blood mononuclear cells (PBMCs) donated by veterans with or without GWI [31] and banked them in the BBRAIN biorepository. Demographic information for the GW veterans whose blood cells were reprogrammed into hiPSCs and used in this study is listed in Table 1. Phase-contrast imaging revealed that the hiPSCs displayed normal morphologies and formed colonies of densely packed cells (Fig. 1 a–c), which also positively stained for the nuclear marker DAPI (Fig. 1 d–f). Pluripotency was validated by three independent methods. First, the hiPSCs positively immunostained for TRA-1–60 and TRA-1–81, which are two surface markers of pluripotent stem cells, but stained negative for SSEA-1, which is not expressed on undifferentiated human stem cells (Fig. 1 g–i). Second, the hiPSCs have a normal karyotype, shown here for a

male (Fig. 1 j). Third, the hiPSCs were able to differentiate into the three germ layers, and cells were positively immunostained for markers of each germ layer: SOX17 (endoderm), Brachyury (mesoderm), and Otx2 (ectoderm) (Fig. 1 k–m).

Forebrain neuronal differentiation of veteran-derived hiPSCs. As shown schematically in Fig. 2a, hiPSCs were differentiated into forebrain glutamatergic neurons which positively immunostained for the neuronal markers MAP2, Tau, NeuN, and the forebrain glutamatergic marker Tbr1 (Fig. 2 b–d). Neuronal differentiation was compared across all the hiPSC lines to determine whether they had similar differentiation capabilities and could thus be compared to each other for experimental analyses. The ratio of the forebrain glutamatergic neuron marker Tbr1 to DAPI-stained nuclei was not significantly different across the lines, $p = 0.0637$ (Fig. 2 e). The neurons all exhibited spontaneous neuronal activity that was recorded on a Multiwell Multielectrode Array (MEA) that detected neuronal spiking and bursting and displayed the spike waveforms (Fig. 2 f–h). No differences in neuronal differentiation were observed at baseline among the lines, indicating that experimental results could be compared across the lines. Because epigenetic memories of the original cells are generally lost during the extensive cloning process as well as the prolonged de-differentiation and re-differentiation procedures during development of hiPSC lines [41], it was necessary to re-expose the hiPSC-derived neurons to the GW-relevant toxicants for our investigations.

Lowest DFP concentration does not impair cell viability and plasma membrane integrity in hiPSC-derived neurons. Previous studies in rodents and primary rat neurons have shown that the stress hormone corticosterone (CORT) exacerbates the effects of organophosphate exposure [12, 13, 21]. Here, we examined in the hiPSC-derived neurons the cytotoxicity of our GW toxicant regimen of the human stress hormone cortisol plus DFP, a sarin surrogate, across multiple concentrations. We exposed neurons to 2 μ M cortisol, a concentration established in our recent studies [21], combined with three concentrations of DFP, 200 nM, 1 μ M, and 2.5 μ M (Fig. 2i, j); previously, the 200 nM DFP concentration was the highest dose tested in our primary rodent neuron cultures [21].

The LDH and MTT assays were used to measure cytotoxicity. LDH is a cytosolic enzyme that is released into the extracellular media upon cell membrane damage. None of the seven lines showed impaired membrane integrity at the 200 nM DFP concentration and only very low levels of cell membrane damage were observed at the

Table 1 Demographic information for GW veterans who donated blood samples for reprogramming

GW veterans with (case) or without GWI	Age	Memory problems
Control 1	58	No
Control 2	69	Yes
Control 3	63	No
Case 1	57	Yes
Case 2	54	No
Case 3	59	Yes
Case 4	54	No

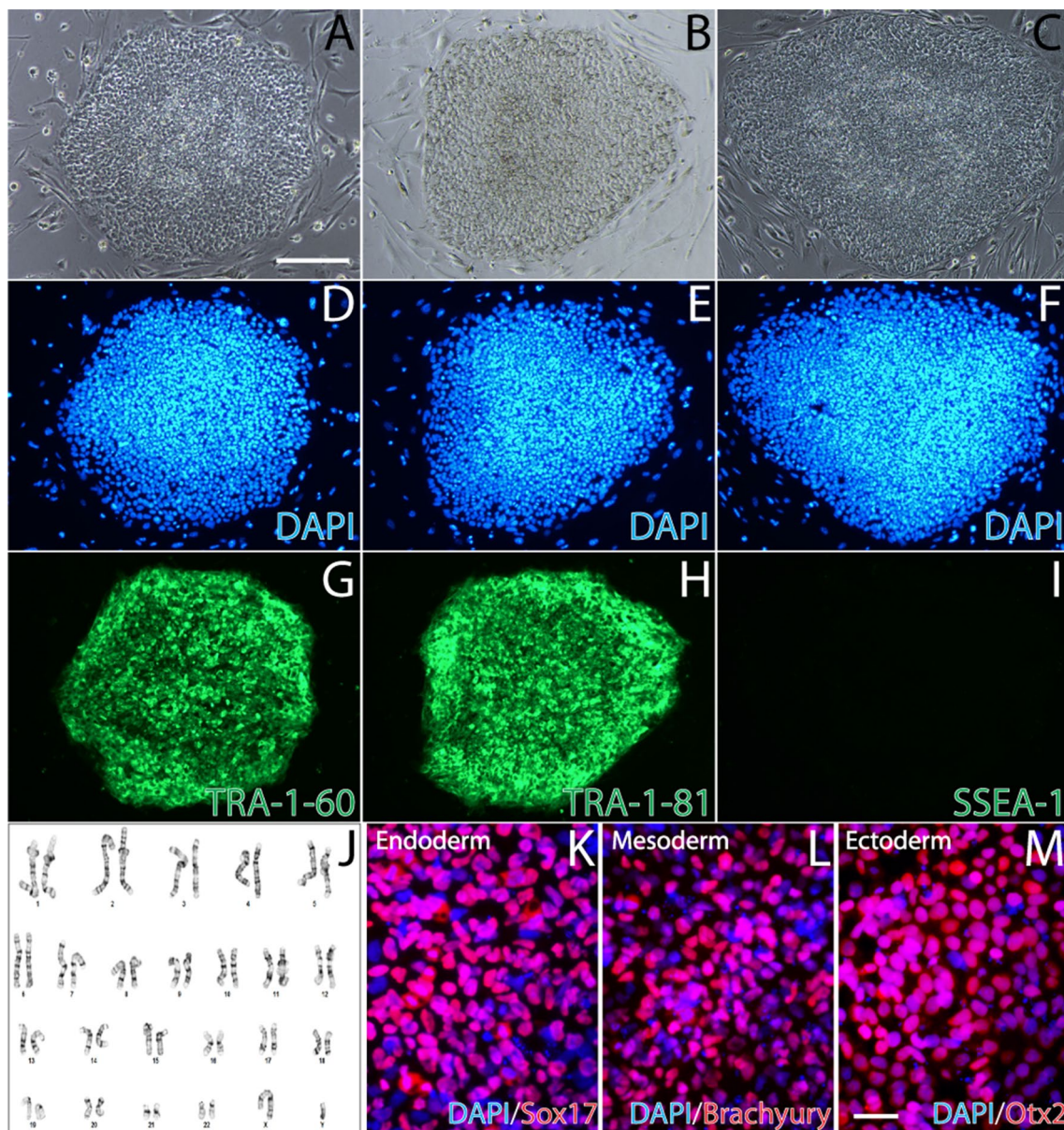


Fig. 1 Pluripotency validation of GW veteran-derived hiPSCs. **a–c** Phase-contrast images of the hiPSC colonies. Scale bar, 100 μ m. **d–f** DAPI staining. **g–i** Immunostaining for the pluripotent stem cell surface markers TRA-1-60 and TRA-1-81, and negative staining for the

differentiation marker SSEA-1. **j** The hiPSCs have a normal (male) karyotype. **k–m** Three-germ-layer differentiation immunostained for the germ layer specific markers Sox17 (endoderm), Brachyury (mesoderm), and Otx2 (ectoderm). Scale bar, 20 μ m

higher 1 μ M and 2.5 μ M DFP concentrations (Fig. 2i). Only two out of seven lines showed significant cell membrane damage at any concentration compared to vehicle control, with significant percent cytotoxicity measured at $4.11 \pm 1.34\%$ ($p = 0.037$) for 1 μ M DFP, and $5.71 \pm 1.18\%$ ($p = 0.008$) and $5.34 \pm 1.25\%$ ($p = 0.013$) for 2.5 μ M DFP

(Fig. 2i). All other lines at all other concentrations were not significantly different from control.

The MTT assay, which is based on the conversion of water soluble MTT to an insoluble formazan product, can be used as a measure of metabolic activity and cell viability;

cells with impaired metabolism will have reduced completion of this reaction, which can be calculated as percent cytotoxicity. Only one out of seven lines showed significant cytotoxicity at the lowest 200 nM DFP concentration ($25.55 \pm 5.82\%$, $p = 0.012$), whereas 6/7 lines showed significant cytotoxicity at the 1 μ M DFP and 2.5 μ M DFP concentrations, ranging from $11.90 \pm 2.94\%$ ($p = 0.015$) cytotoxicity at 1 μ M DFP to $64.47 \pm 1.22\%$ ($p < 0.0001$) cytotoxicity at 2.5 μ M DFP (Fig. 2j).

hiPSC-derived neurons from veterans with GWI are more susceptible to develop tau pathology. For examination of tau phenotypes, hiPSC-derived neurons were cultured for more than 1 month to allow for maturation of neural networks before toxicant exposure. Quantitative immunocytochemistry was performed by tracing axons and cell bodies to measure arbitrary fluorescence units per area. The GW toxicant regimen of cortisol plus DFP increased total tau levels (recognized by the TauR1 antibody) in neurons derived from five of seven GW hiPSC lines, ranging from an increase of 1.30 ± 0.07 ($p = 0.0014$) to 1.74 ± 0.24 ($p = 0.0091$) times relative to vehicle control (Fig. 3a, c). In addition, the toxicants increased the levels of pSer202/pThr205 phosphorylated tau (recognized by the AT8 antibody) in five of seven hiPSC lines, ranging from an increase of 1.26 ± 0.09 ($p = 0.044$) to 1.75 ± 0.21 ($p = 0.014$) times relative to vehicle control (Fig. 3b, d). The increases in total tau and phosphorylated tau levels occurred in all four case lines but in only one out of three control lines. Since active release and uptake of tau have been implicated in the development of tau pathologies [42, 43], and because no overt cell death or plasma membrane damage was identified with 200 nM DFP treatment, we next examined the extracellular tau proteins detected in the media of the hiPSC-neuronal cultures. The neuronal cultures derived from some GW case lines consistently exhibited increased extracellular total tau [in one case line, 1.72 ± 0.03 times relative to vehicle ($p < 0.0013$)] and increased extracellular Thr231 phosphorylated tau [in two case lines, 1.26 ± 0.03 ($p = 0.0003$) and 2.05 ± 0.03 ($p = 0.041$) times relative to vehicle control], whereas none of the control lines showed elevations in extracellular tau (Fig. 3e, f). Taken together, these various results on tau suggest that hiPSC-derived neurons from veterans with GWI may be more susceptible to develop tau pathology than counterpart neurons from their healthy colleagues.

Reduced microtubule acetylation in hiPSC-derived neurons upon GW toxicant exposure. GW toxicant exposure was previously shown to lead to reduced microtubule acetylation in primary rat and hiPSC-derived neurons not derived from veterans [21]. Here, two GW hiPSC-neuronal groups, one control line and one case line, were randomly selected and examined for microtubule acetylation. A consistent reduction in the ratio of microtubule acetylation to

total neuronal β III-tubulin was found in both groups, with levels decreasing to 0.82 ± 0.05 ($p = 0.0274$) and 0.82 ± 0.04 ($p = 0.0122$) times relative to vehicle per line (Fig. 4a, b). There was not a corresponding change in the ratio of tyrosinated tubulin to β III-tubulin, consistent with the toxicant effects being specific to microtubule acetylation.

To further investigate the potential mechanisms that may underlie reductions in microtubule acetylation, we quantified the activity of the histone deacetylase 6 (HDAC6) enzyme, which is the predominant tubulin deacetylase in neurons. We found a corresponding increase in HDAC6 activity in the same two hiPSC-neuronal lines when treated with GW toxicants, with increased levels of 5.36 ± 0.30 ($p < 0.0001$) and 6.44 ± 1.36 ($p < 0.0001$) times relative to vehicle control per line (Fig. 4c).

Altered mitochondrial health, dynamics, and transport in hiPSC-derived neurons exposed to GW toxicants.

Mitochondrial transport defects were previously demonstrated in primary rodent neurons exposed to GW toxicants, and so studies here were limited to two hiPSC lines, one control and one case. TMRE added to cell culture media was taken up by mitochondria with an active membrane potential, which were then imaged live (Fig. 5a). Various parameters relevant to mitochondrial health, dynamics, and transport were then quantified. No significant differences were observed in the percent of mitochondria that were moving vs. stationary, percent anterograde vs retrograde movements, number of reversals, number of stops, time of movement, or instantaneous or average velocity. However, the GW toxicant regimen of cortisol plus DFP reduced mitochondrial fluorescence intensity, which indicates mitochondrial health, but intensity decreased significantly only in the case line to 0.68 ± 0.12 times relative to vehicle control, $p = 0.045$ (Fig. 5a, c). Additionally, a corresponding increase in mitochondrial length and number was observed. Mitochondrial length increased only in the case line to 1.54 ± 0.11 times relative to vehicle ($p < 0.0001$), while the number of mitochondria per 100 μ m of axon increased in the control line to 1.18 ± 0.06 ($p = 0.034$) and in the case line to 1.42 ± 0.09 ($p = 0.0004$) times relative to vehicle per line (Fig. 5d, e). The toxicant regimen also significantly decreased run length, which is a measure of the distance traveled during each transport event, for both hiPSC lines, with length decreasing to 0.74 ± 0.08 ($p = 0.0267$) and 0.73 ± 0.06 ($p = 0.0297$) times relative to vehicle for the control line and case line, respectively (Fig. 5b, f). Finally, GW toxicant regimen exposure increased the number of mitochondrial fusion and fission events per 100 μ m of axon per minute, with an increase in fusion in the case line to 2.98 ± 0.46 times relative to vehicle ($p = 0.0216$), and an increase in fission in the control line to 1.41 ± 0.14 ($p = 0.008$) and in the case line to

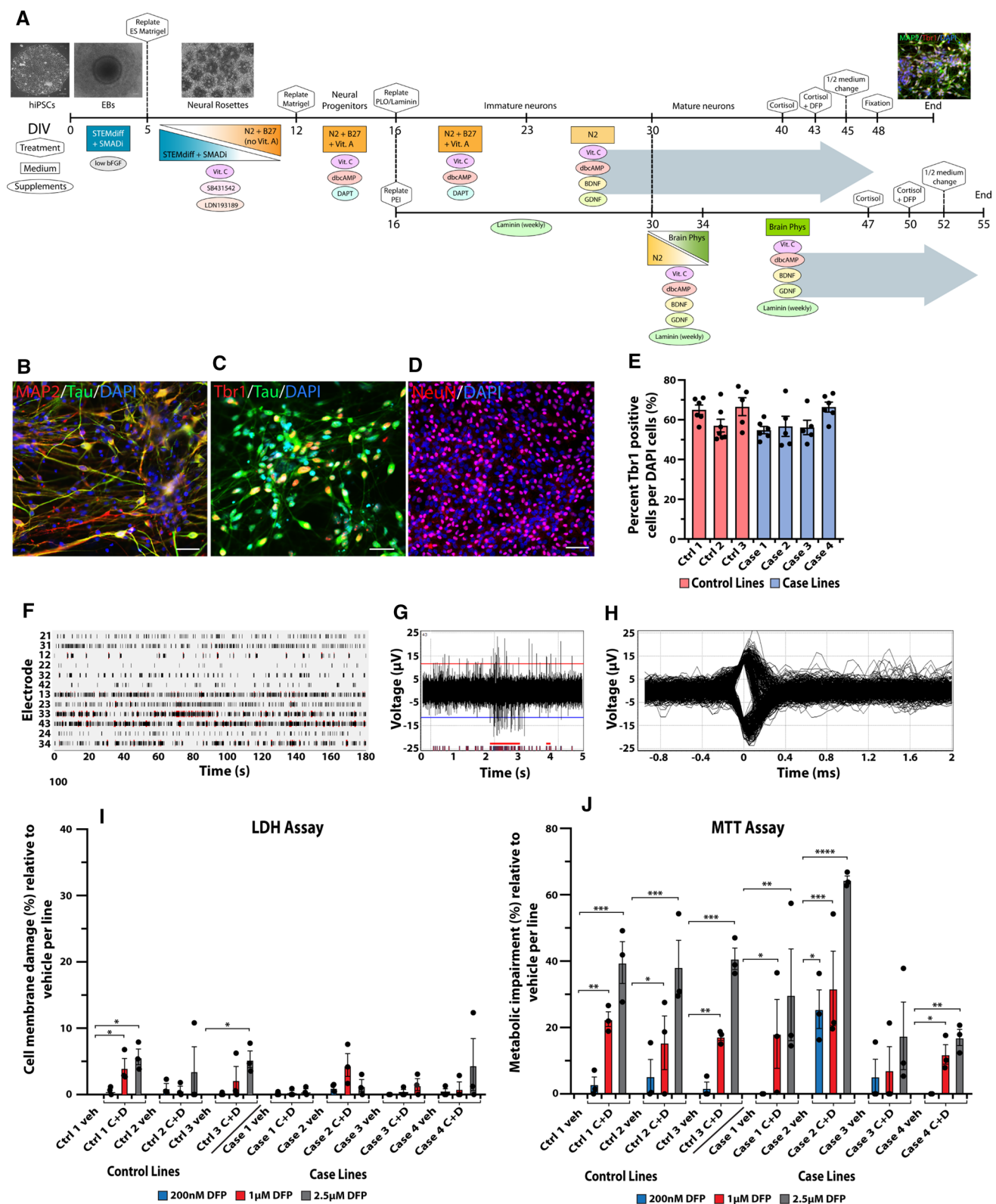


Fig. 2 Validation of forebrain glutamatergic neuronal differentiation from veteran-derived hiPSCs and GW toxicant regimen. **a** Schematic of the neuronal differentiation protocol with phase contrast and immunocytochemical images relevant to each stage. The large gray arrows represent the culture conditions for all experiments (top arrow) except for electrophysiology (bottom arrow). Scale bar for phase contrast images (100 μ m) and for immunofluorescent image (20 μ m). **b–d** Immunocytochemical staining for the neuronal markers MAP2, Tau, NeuN, and the forebrain glutamatergic marker Tbr1. Scale bar, 50 μ m. **e** Bar graph shows quantification of the percent of Tbr1-positive cells per total DAPI-stained cells across the seven lines of hiPSC-derived glutamatergic neurons, analyzed using one-way ANOVA with Tukey's post hoc test. **f–h** Representative images of spontaneous neuronal firing recorded from a Multiwell MultiElectrode Array. **f** Raster plot shows the total activity recorded for all 12 electrodes in a sample well. Black lines indicate detected spikes and red bars indicate detected bursts. **g** Representative spike and burst activity recorded on electrode 43 from F. **h** Spike waveforms recorded on electrode 43 from F. **i, j** All 7 hiPSC lines were differentiated into forebrain glutamatergic neurons and exposed to the GW toxicant regimen of 2 μ M cortisol plus DFP at three different concentrations (200 nM, 1 μ M, and 2.5 μ M). The neurons were evaluated for percent cell membrane damage via the LDH assay (**i**) and the percent metabolic impairment (reduced cell viability) via the MTT assay (**j**). Each line of hiPSC-derived neurons was exposed to vehicle (veh) or cortisol plus DFP (C+D). Empty bars for vehicle represent no cell damage. All data are represented as mean $\pm$ SEM normalized to vehicle per line, and analyzed using one-way ANOVA with Dunnett's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

1.66 ± 0.14 ($p = 0.0416$) times relative to vehicle per line (Fig. 5 g, h). Together, these results suggest that exposure of the hiPSC-derived neurons to the GW toxicant regimen impairs mitochondrial health, dynamics, and transport. The fact that many of the alterations occur only in the case line suggests that these neurons are more susceptible to toxicant exposure than neurons from the control line.

hiPSC-derived neurons exhibit reduced neuronal activity after toxicant exposure. hiPSC-derived neurons from all seven lines were cultured on multielectrode arrays (MEA, Multi Channel Systems) to record how the toxicant regimen might alter neuronal activity. The activity of the hiPSC-derived forebrain glutamatergic neurons was first validated by pharmacological administration into the culture media of antagonists to sodium channels and to NMDA/AMPA/GABA receptors. Spontaneous neuronal activity was recorded before and 5–60 min after addition of the AMPA receptor antagonist CNQX (50 μ M), NMDA receptor antagonist AP5 (50 μ M), GABA receptor antagonist Picrotoxin (50 μ M), and sodium channel antagonist tetrodotoxin (TTX) (1 μ M). Addition of CNQX and AP5 into the culture media reduced network burst frequency, which can be used to measure synchronous network synaptic activity across multiple electrodes, while addition of picrotoxin increased network burst frequency (data not shown). Addition of TTX abolished all neuronal activity (data not shown).

Next, the hiPSC-derived glutamatergic neurons were exposed to the GW toxicant regimen to examine

toxicant-induced impairments in neuronal activity (Fig. 6a, b). Cortisol plus DFP reduced spontaneous neuronal activity for 6 out of 7 hiPSC lines; however, only one control line and one case line showed significant decreases in neuronal firing. The number of active electrodes per well decreased in the control line to 0.40 ± 0.11 ($p = 0.0281$) times relative to vehicle (Fig. 6c). Spike frequency, measured with at least three spikes per electrode per minute, was significantly reduced to 0.46 ± 0.13 ($p < 0.0086$) and 0.46 ± 0.05 ($p < 0.0001$) times relative to vehicle per line for the control and case lines, respectively (Fig. 6d). The GW toxicant regimen also reduced spike bursting, which is another measure of neuronal activity. In the same control and case lines, cortisol plus DFP significantly reduced the number of actively bursting electrodes per well to 0.61 ± 0.10 ($p = 0.044$) and 0.60 ± 0.23 ($p = 0.046$) times relative to vehicle per line, respectively (Fig. 6e). Burst frequency, measured as the number of bursts per electrode per minute, was reduced in two control and two case lines of hiPSC-derived neurons to 0.38 ± 0.17 ($p = 0.0067$), 0.27 ± 0.20 ($p = 0.0322$), 0.43 ± 0.21 ($p = 0.0358$), and 0.25 ± 0.09 ($p = 0.0128$) times relative to vehicle per line, respectively (Fig. 6f). The toxicant regimen reduced burst duration in control line 1 and case line 1 to 0.80 ± 0.08 ($p = 0.047$) and 0.66 ± 0.07 ($p = 0.0015$) times relative to vehicle per line (Fig. 6g), and the number of spikes per burst in the same lines to 0.33 ± 0.08 ($p = 0.0425$) and 0.47 ± 0.04 ($p = 0.0037$) times relative to vehicle per line, respectively (Fig. 6h). We also examined network burst frequency, which measures the synchronous activity of a neuronal network across multiple electrodes at the circuit level. Although network bursts were not detected for every line, the GW toxicant regimen significantly reduced the network burst duration to 0.23 ± 0.03 ($p = 0.0008$) and the number of spikes per network burst to 0.33 ± 0.09 ($p = 0.0465$) times relative to vehicle only in a case line (Fig. 6k, l), suggesting potential disease susceptibility to toxicant exposure in GW veterans with GWI.

In addition to reduced neuronal activity, we collected the cell culture supernatant to measure the amount of extracellular glutamate after toxicant exposure. The GW toxicant regimen reduced the levels of extracellular glutamate in representative control and case lines to 0.65 ± 0.16 ($p = 0.036$) and 0.31 ± 0.02 ($p = 0.005$) times relative to vehicle control for each line (Fig. 6m).

Mild learning and memory deficits identified in adult rats exposed to GW toxicants. To ascertain whether the GW toxicant regimen results in learning and memory deficits in an animal model, Morris water maze tests and spontaneous novel object recognition (NOR) analysis were performed. Figure 7 illustrates the acquisition curves for the

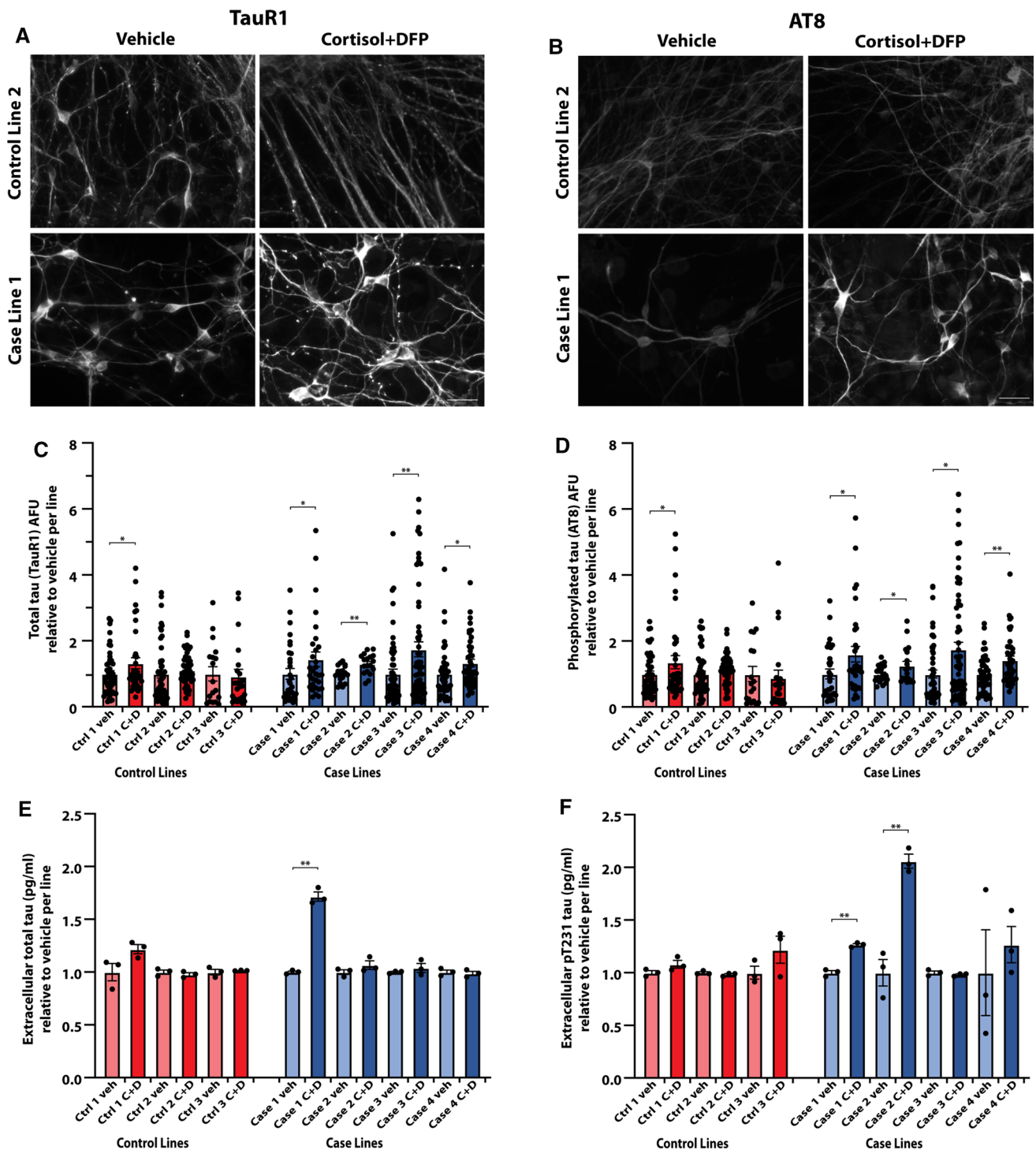
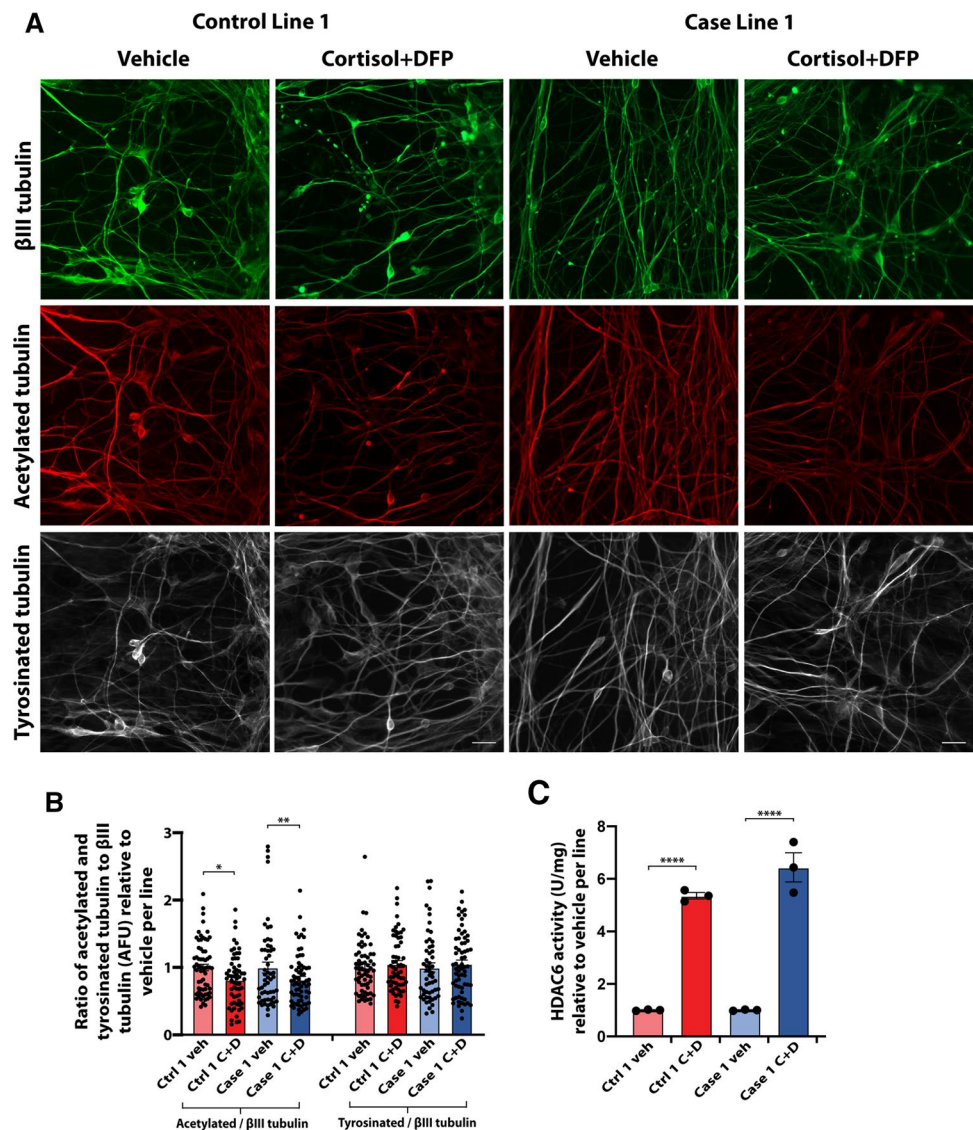


Fig. 3 hiPSC-derived glutamatergic neurons from veterans with GWI are more susceptible to develop tau pathology after toxicant exposure. **a, b** Representative images of hiPSC-derived glutamatergic neurons from one control line and one case line exposed to 2 μ M cortisol plus 200 nM DFP, and immunostained for total tau (TauR1) (**a**) and phosphorylated tau (AT8) (**b**). Scale bar, 50 μ m. **c, d** Graphs show mean arbitrary fluorescence units (AFU) $\pm$ SEM normalized to vehicle per hiPSC line for all seven lines of hiPSC-derived neurons exposed to

vehicle (veh) or cortisol plus DFP (C+D). Analyses were conducted using the Kruskal–Wallis test followed by Dunn’s post hoc test. **e, f** Extracellular cell culture supernatant was collected from all seven lines of hiPSC-derived neurons and measured for total tau or p[T231] phosphorylated tau via ELISA. Graphs show mean $\pm$ SEM normalized to vehicle per line. Analyses were conducted using one-way ANOVA followed by Šidák’s post hoc test. * $p < 0.05$, ** $p < 0.01$

Fig. 4 Reduced microtubule acetylation in hiPSC-derived glutamatergic neurons upon GW toxicant exposure. **a** Representative images of hiPSC-derived glutamatergic neurons from one control line and one case line exposed to 2 μ M cortisol plus 200 nM DFP, and immunostained for β III-tubulin (green), acetylated tubulin (red), and tyrosinated tubulin (white). Scale bar, 20 μ m. **b** Graph shows the ratio of acetylated tubulin and tyrosinated tubulin to β III tubulin expressed as mean arbitrary fluorescence units (AFU) $\pm$ SEM normalized to vehicle per line of hiPSC-derived glutamatergic neurons exposed to vehicle or cortisol + DFP. Analyses were conducted using the Kruskal–Wallis test followed by Dunn’s post hoc test. **c** Quantification of HDAC6 activity from cell lysates of all seven lines of hiPSC-derived glutamatergic neurons exposed to vehicle (veh) or 2 μ M cortisol plus 200 nM DFP (C + D) using an HDAC6 activity assay kit. Bars show mean HDAC6 activity $\pm$ SEM normalized to vehicle per line. Analyses were conducted using one-way ANOVA followed by Šídák’s post hoc test. * p < 0.05, ** p < 0.01, **** p < 0.0001



vehicle and CORT + DFP-treated groups to locate a hidden platform in two phases of water maze testing. For the initial hidden platform trials (acquisition phase), both vehicle control and CORT + DFP-treated rats progressively learned to locate the hidden platform with increasing levels of efficiency over the course of the 5 days of testing as indicated by the decreasing slope of the acquisition curves [main effect of treatment, $F(1, 28) = 0.6$, $p = 0.4$; day, $F(4, 112) = 29.1$, $p < 0.001$; treatment $\times$ day, $F(4, 112) = 0.8$, $p = 0.5$] (Fig. 7a). Moreover, there were no significant differences in performance of the task between the treatment groups. Likewise, in the probe trials after the acquisition phase, there were no significant treatment-related differences in the percentage of

time spent in the previous target quadrant or the time spent in the annulus–40 area (Fig. 7b).

Next, the hidden platform was moved to the opposite quadrant of the pool and a second phase of hidden platform trials (reversal trials) were conducted 24 days after the DFP injection and 17 days after the completion of the first round of hidden platform and probe trials. Again, both vehicle control and CORT + DFP-treated rats progressively learned to locate the hidden platform with increasing levels of efficiency over the course of the 5 days of testing as indicated by the decreasing slope of the reversal learning

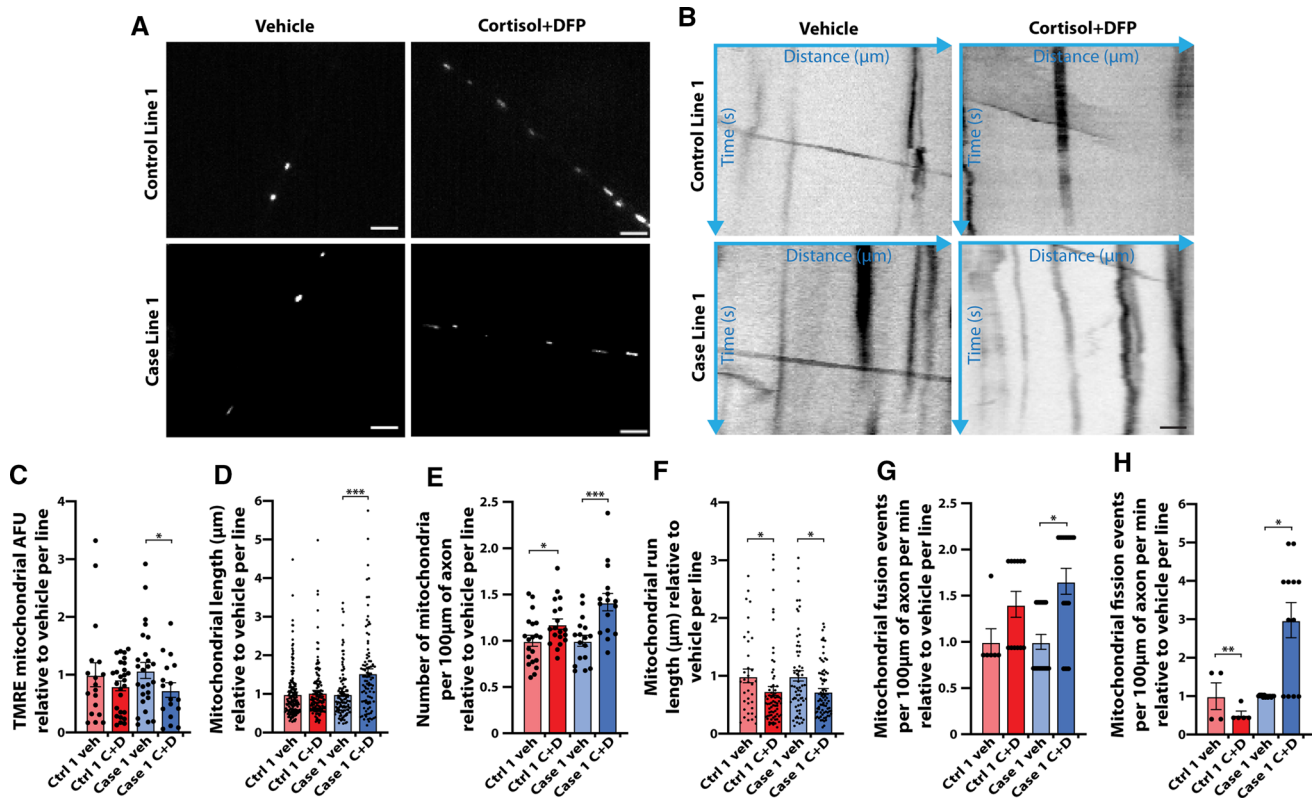


Fig. 5 Altered mitochondrial health, dynamics, and transport in hiPSC-derived glutamatergic neurons exposed to GW toxicants. hiPSC-derived glutamatergic neurons from one control line and one case line exposed to vehicle (veh) or 2 μ M cortisol plus 200 nM DFP (C+D) were incubated with the cell permeable dye TMRE at 200 nM for 30 min to label mitochondria with an active membrane potential. **a** Representative live-cell images of individual mitochondria marked with the mitochondrial dye TMRE. Scale bar, 5 μ m. **b** Representative kymographs depicting mitochondrial distance traveled over time. Scale bar, 10 μ m. **c** Graph shows mean arbitrary TMRE fluorescence

units (AFU) for individual axonal mitochondria in one control line and one case line exposed to vehicle or cortisol+DFP. **d** Bars show mean length for individual axonal mitochondria. **e** Quantification of the average number of mitochondria per 100 μ m of axon. **f** Analysis of mean mitochondrial run length. **g**, **h** Graphs show mean mitochondrial fusion (**g**) and fission (**h**) events per 100 μ m of axon per minute. All data are represented as mean $\pm$ SEM normalized to vehicle per line, and analyzed using the Kruskal–Wallis test followed by Dunn's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

curves [main effect of treatment, $F(1,28)=4.9$, $p < 0.04$; day, $F(4,112)=18.0$, $p < 0.001$; treatment $\times$ day, $F(4,112)=1.5$, $p=0.2$] (Fig. 7c). In this case, there was a modest but statistically significant impairment in performance in the CORT + DFP-treated animals compared to vehicle controls, most notably on day 4 (post hoc comparison, $p=0.02$). In the probe trial tests after the reversal learning trials, CORT + DFP-treated rats also spent significantly less time in the annulus–40 area ($t=2.4$, $p=0.02$) compared to controls (Fig. 7d), but not in the percentage of time spent in the larger target quadrant area.

For the NOR task, all rats met the inclusion criteria of exploring each individual object for at least 4 s and a

minimum of 12 s of total object exploration in both the training (A/A) and testing (A/B) sessions. The effects of CORT + DFP treatment on the mean ($\pm$ SEM) exploration times of the novel and familiar objects in the test (A/B) sessions are illustrated in Fig. 8a, and the calculated discrimination (d_2) ratios (to account for individual variability in exploration times) for each subject are illustrated in Fig. 8b. Statistical analysis revealed the following: main effect of treatment [$F(1,14)=0.03$, $p=0.9$], object type [$F(1,14)=55.7$, $p < 0.001$], and treatment-by-object type interaction [$F(1,14)=2.8$, $p=0.1$]. Post hoc analysis indicated that there was a significant preference for the novel object ($p < 0.01$) versus the familiar object in both treatment

groups (Fig. 8a). For the analysis of the d2 ratios, there was no significant difference in the d2 ratios in the CORT + DFP treatment group versus the vehicle control animals, $p=0.0956$ (Fig. 8b).

GW toxicants induce tau pathology and cell loss in the CA3 region of the hippocampus. After exposure to the CORT + DFP toxicant regimen, rats were sacrificed, and the brains were processed for histological staining. Three regions of the brain involved in learning and memory and executive functioning relevant to the behavioral tasks were examined: the CA1 and CA3 regions of the hippocampus, and the prefrontal cortex (Fig. 9a). In the CA3 region of the hippocampus, rats exposed to CORT + DFP exhibited elevated levels of total tau (recognized by the TauR1 antibody) at 1.40 ± 0.05 times relative to control, $p < 0.0001$ (Fig. 9b), and elevated levels of pSer202/pThr205 phosphorylated tau (recognized by AT8 antibody) at 1.43 ± 0.06 times relative to control, $p < 0.0001$ (Fig. 9c), with fluorescence intensity normalized to the number of nuclei per region quantified. No significant differences in total tau or phosphorylated tau were obtained in the CA1 region of the hippocampus or in the prefrontal cortex. Additionally, the pathological tau phenotypes in the CA3 region of the hippocampus were accompanied by a significant reduction in the total number of cells per $100 \mu\text{m}^2$, decreasing to 0.80 ± 0.03 times relative to control, $p=0.0027$ (Fig. 9d). We did not identify any cellular morphological alterations in the CA1 and CA3 regions of the hippocampus or the prefrontal cortex (data not shown). Together, these results suggest that the CA3 region of the hippocampus may be particularly vulnerable to the GW toxicants, which is consistent with imaging studies carried out in GW veterans [44–46].

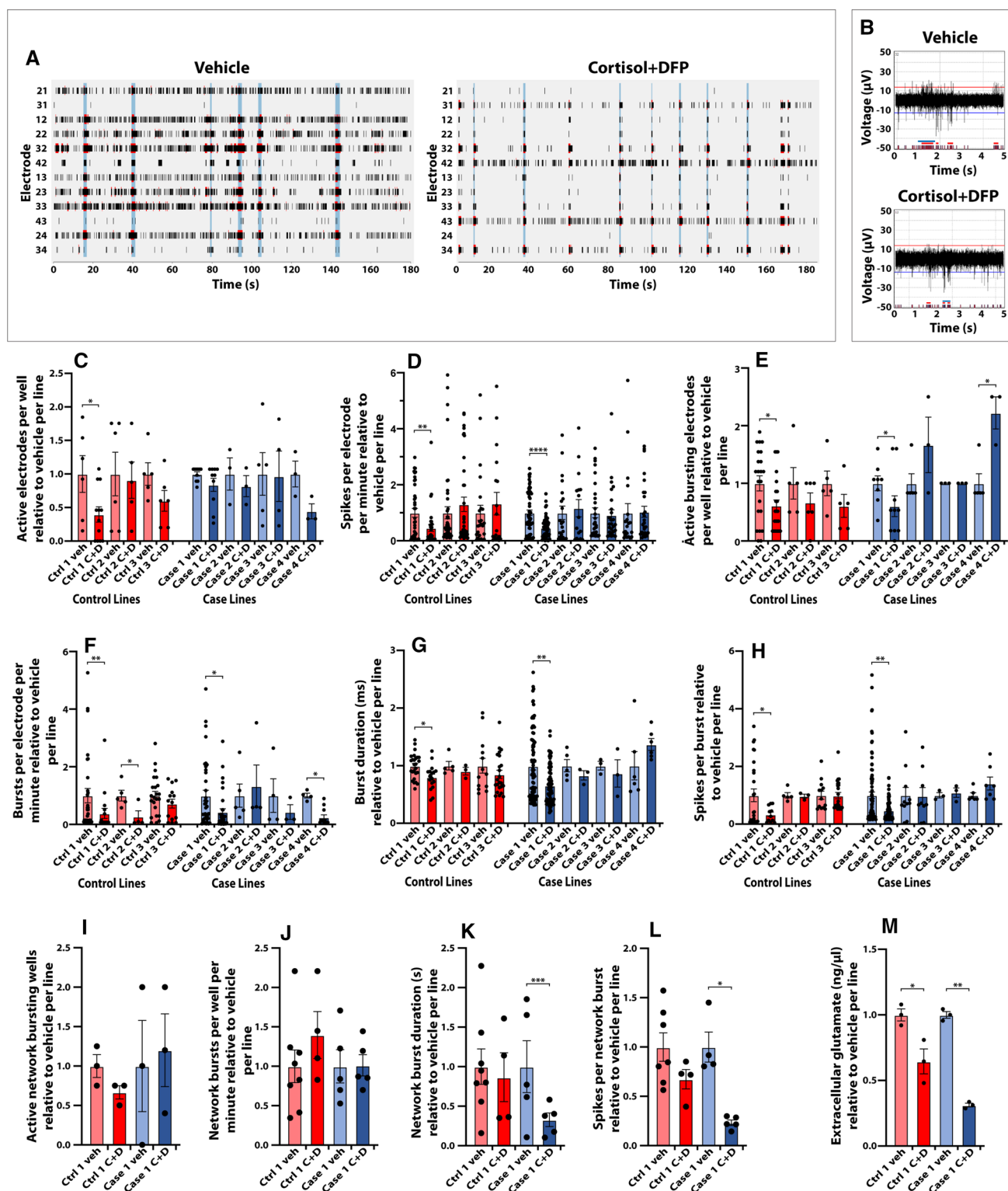
Discussion

The community of researchers and physicians studying GWI in recent years has been funded by the United States Department of Defense to define exactly what the disease is, to elucidate the underlying causes of the disease, to ascertain whether certain veterans are predisposed to develop the disease and why, and most importantly to develop effective therapies for suffering veterans who have been without treatment for decades. While vertebrate animal models remain critical for system-level analyses including behavior, we have advocated for a cellular approach, so that mechanism-based therapies can be developed for the veterans. In addition, there is value in using human cells, because rodents lack significant genomic variation, rendering them unsuitable for tackling the question of why some soldiers

developed the disease, while others did not, despite similar exposures. Although the total number of hiPSC lines derived from the GW veterans included in our current studies limits the statistical power of our conclusions, the present work provides a starting point for experimental studies using these cell lines. Larger genomic studies such as single-nucleotide polymorphism (SNP) investigations carried out on GW veteran-derived non-neural cells and tissues may provide insights into susceptible genetic variants, but the CNS-specific susceptibilities might not be fully revealed.

Our earlier work taking a cellular approach to GWI mainly used primary cultures of rat neurons and our present findings are entirely consistent with those earlier findings on deficits in both microtubule acetylation and mitochondrial transport in response to the GW toxicant regimen [21]. We also observed a reduction in mitochondrial membrane potential, increased mitochondrial length and density in axons, and more frequent mitochondrial fusion and fission. Reduced microtubule acetylation renders microtubules less suitable as substrates for key motor proteins involved in axonal transport, and hence might explain the diminished mitochondrial transport as well as decreased neurotransmitter release. Mitochondria are the primary site of energy production in the cell, and disruptions in mitochondrial health, dynamics, and transport have been implicated in many neurological disorders [47–49]. Studies have shown that organophosphate exposure directly affects many aspects of mitochondrial behavior [50–52], and these alterations might contribute to some of the cognitive symptoms of GWI, such as reduced processing speeds and chronic fatigue [6, 7, 11].

Here, for the first time, we showed that exposure to the GW toxicant regimen resulted in robust and consistent tau pathology in hiPSC-derived neurons, with elevated levels of total tau and phosphorylated tau inside and outside of the neuron. The elevated tau might account for the diminished microtubule acetylation, given that greater tau levels on microtubules correspond to reduced stability as does reduced acetylation. In the rat model, the same tau effects were documented in response to the toxicant regimen only in the CA3 region of the hippocampus, but not in the CA1 region or the prefrontal cortex. The toxicant regimen also resulted in loss of cells in the CA3 region of the hippocampus, but not in the CA1 region or the prefrontal cortex, further highlighting the selective vulnerability of the CA3 region to GW-relevant toxicants. Consistent with our findings, smaller hippocampal CA3 volumes have been identified in GW veterans with GWI via magnetic resonance imaging analyses [44–46]. Taken together with a mild memory deficit in these rats in response to the toxicant regimen, these results implicate tau abnormalities as a potential cause of the memory defects in GWI.



Our behavioral experiments using the Morris Water Maze and the NOR task, both of which have notable translational value for human neuropsychological disorders,

were designed to examine hippocampal-dependent spatial reference memory as well as reversal learning with

Fig. 6 hiPSC-derived glutamatergic neurons exhibit reduced neuronal activity after GW toxicant exposure. Neurons were cultured on 24 well Multiwell-MEA plates with 12 electrodes/well from Multi Channel Systems. Spontaneous extracellular neuronal activity was recorded from all seven hiPSC-neuronal lines exposed to vehicle (veh) or 2 μ M cortisol plus 200 nM DFP (C+D). **a** Representative raster plots show the neuronal activity recorded for all 12 electrodes in a sample well for 3 min. Black lines indicate detected spikes, red lines indicate detected bursts, and vertical blue shadows indicate detected network bursts. **b** Representative spike, burst, and network burst activity for 5 s recorded from hiPSC-derived glutamatergic neurons exposed to vehicle or C+D. **c** Graph shows the average number of active electrodes per well, defined as at least three spikes per electrode per minute. **d** Bars show the mean spike frequency quantified from active electrodes. **e** Graph shows the average number of actively bursting electrodes per well with at least one burst recorded. **f** Bars show the mean burst frequency quantified as the number of bursts per electrode per minute. **g, h** Graphs show the mean burst duration (**g**) and the number of spikes per burst (**h**). **i-l** Network bursts were quantified for one control line and one case line for the average number of wells with network bursts detected (**i**), mean network burst frequency measured as the number of network bursts per well per minute (**j**), network burst duration (**k**), and the number of spikes per network burst (**l**). **m** Cell culture supernatant was collected from two representative lines to measure the levels of extracellular glutamate via a glutamate detection kit. All data are represented as mean $\pm$ SEM normalized to vehicle per line. Analyses were conducted using the Kruskal–Wallis test followed by Dunn's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

cognitive flexibility, and recognition memory, respectively. Moreover, while the hippocampus is considered important for performance of spatial learning tasks, the CA3 region has been specifically described as crucial for both memory acquisition and consolidation in a Morris water maze task in rodents [53]. The exposure produced modest but statistically significant impairments in the performance of reversal learning trials and probe trials (after the reversal learning phase). The deficits in the second phase of water maze testing were suggestive of alterations in cognitive flexibility, which is generally considered a subcomponent of executive function involving the shifting of strategies when the stimulus–response contingency changes [54]. Our results indicate that the behavioral deficit in exposed rats is specific to hippocampal-dependent spatial reference memory, but not recognition memory (tested in the NOR task). Platform re-locating in water maze tasks requires cognitive flexibility, which is suggested to be especially appropriate for extrapolation of learning and memory measures from rodent to human [55]. Correspondingly, deficits in visuospatial processing, learning and memory, and cognitive flexibility/executive function have been consistently identified in veterans with GWI [6, 11].

Various studies have shown that GW-relevant toxicants can impair neurotransmission [21, 56–59] and this disruption may contribute to some of the most prominent cognitive symptoms in GWI, such as reduced processing speeds and poor attention/concentration [7, 11, 60]. We observed

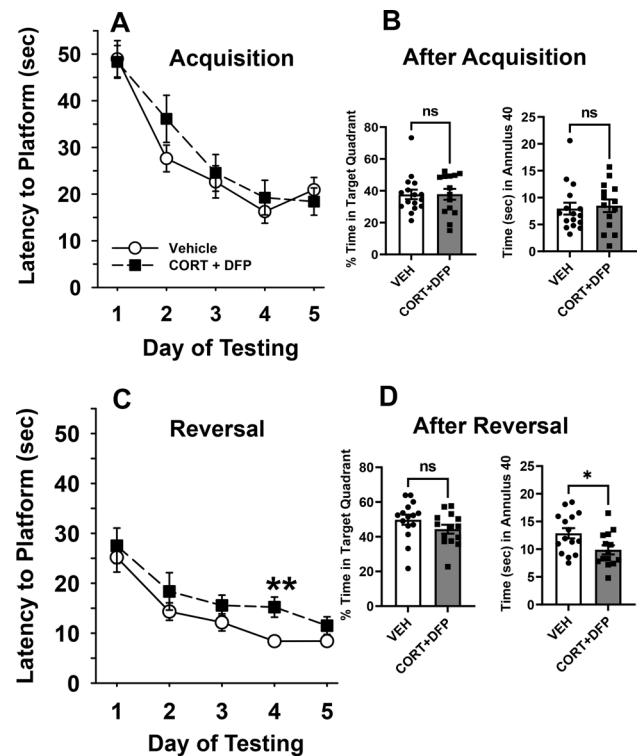


Fig. 7 GW toxicants impair reversal learning and memory in adult rats. Effects of 7 days of exposure to CORT (corticosterone) plus a single injection of DFP 1.5 mg/kg on day seven on a water maze repeated acquisition procedure beginning 48 h after the injection. **a** The graph illustrates the results of the hidden platform tests that were conducted for the first five consecutive days of testing. Each point of the plotted curves represents the mean latency in seconds $\pm$ SEM for each testing day. **b** Effects of CORT + DFP on water maze probe trials. The graph illustrates the results of the probe trial tests that were conducted on day five after the last hidden platform trial in the first phase of testing. Bars represent mean percentage of times spent in the previous target quadrant and the mean times in seconds $\pm$ SEM spent in the annulus 40 with filled circles and squares representing individual rats. **c** The graph illustrates the results of reversal learning trials 24 days after the DFP injection and 17 days after the first phase of water maze testing. In these trials, the hidden platform was moved to a new quadrant location in the pool and 5 additional days of testing occurred. Each point of the plotted curves represents the mean latency in seconds $\pm$ SEM for each testing day. **d** Effects of CORT + DFP on water maze probe trials. The graph illustrates the results of the probe trial tests that were conducted on day five after the last hidden platform trial in the second (reversal learning) phase of testing. Bars represent mean percentage of times spent in the previous target quadrant and the mean times in seconds $\pm$ SEM spent in the annulus 40 with filled circles and squares representing individual rats. * $p = 0.02$, Vehicle vs CORT + DFP. $N = 14$ – 16 . Analyses were conducted using ANOVA (with repeated measures when indicated) followed by the Student–Newman–Keuls post hoc test and Student's t test. ns, not significant

robust electrophysiological impairments in neuronal activity in almost all of the hiPSC lines exposed to the toxicant regimen, including reduced activity at both the individual cell and circuit levels, as well as reduced extracellular release of glutamate. These results are consistent with studies on

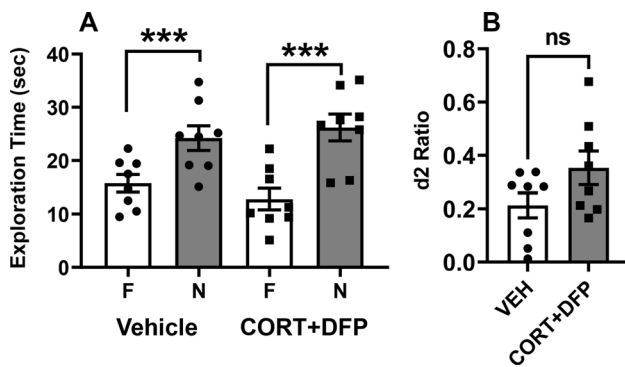
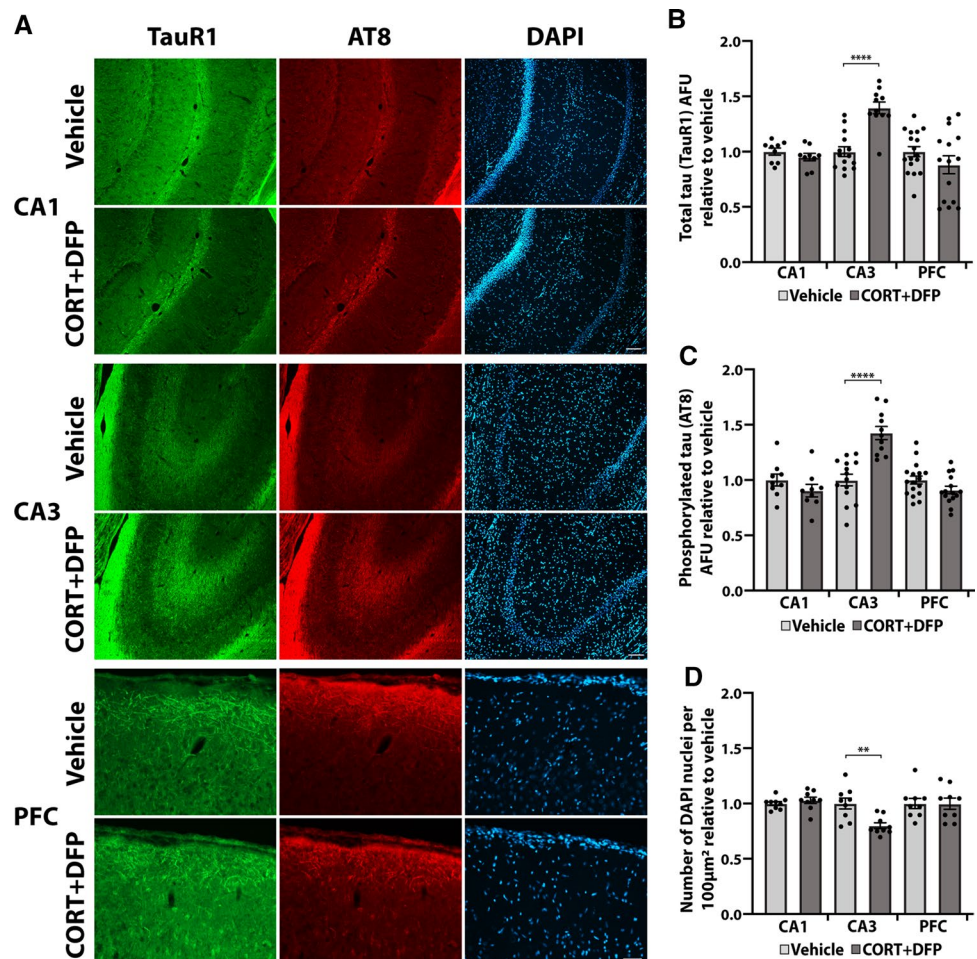


Fig. 8 GW toxicants do not alter object recognition memory in adult rats. Effects of 7 days of exposure to CORT (corticosterone) plus a single injection of DFP 1.5 mg/kg on day 7 on performance of a spontaneous novel object recognition (NOR) procedure 48 h after the injection. **a** Bars on the graph represent the mean ($\pm$ S.E.M.) exploration times of the familiar (F) and novel (N) objects after a 4 h delay. **b** The graph shows scatterplots representing discrimination (d2) ratios with filled circles and squares representing individual rats. d2 ratio = (novel—familiar)/(novel + familiar). *** $p < 0.001$ novel vs familiar object. $N = 8$ for each group. Analyses were conducted using ANOVA followed by the Student–Newman–Keuls post hoc test and Student's t test. ns, not significant

Fig. 9 GW toxicants induce tau pathology and cell loss selectively in the CA3 region of the hippocampus. Rats exposed to 7 days of CORT (corticosterone) plus a single injection of DFP 1.5 mg/kg on day seven were sacrificed 48 h after the injection for histological examination. **a** Representative immunohistochemical images show staining for total tau (TauR1), phosphorylated tau (AT8), and DAPI in the CA1 and CA3 regions of the hippocampus, and in the prefrontal cortex (PFC). Scale bar in CA1 and CA3, 100 μ m; in PFC, 50 μ m. **b, c** Graphs show mean arbitrary fluorescence units (AFU) $\pm$ SEM, relative to the number of nuclei and normalized to vehicle for total tau staining (**b**) and phosphorylated tau staining (**c**) for each brain region. **d** Quantification shows the mean number of DAPI-stained nuclei per 100 μ m² $\pm$ SEM normalized to vehicle per each brain region. Histology was quantified from three mice from each group. Analyses were conducted using one-way ANOVA followed by Šídák's post hoc test. ** $p < 0.01$, **** $p < 0.0001$



animals and on GW veterans that have identified altered connectivity and neurotransmission as important components of GWI [13, 56, 57, 61], as well as with our previous observations on impaired neurotransmitter release in rat neurons exposed to the GW toxicant regimen [21]. We are eager to test whether these effects are due to the alterations in tau and/or microtubules that we have thus far identified.

Throughout our present studies, there were many instances in which the case lines displayed stronger phenotypes in response to the toxicant regimen than the control lines. While the small number of hiPSC lines limits the statistical power with which we can draw conclusions from these observations, we nevertheless find them compelling in light of the often discussed possibility of a predisposition of certain soldiers over others to acquire GWI. Especially compelling is the possibility that a predisposition to tauopathy might be key to which soldiers are most vulnerable. Such considerations may also be relevant to the future of veterans living with GWI, as the potential for tau-based degeneration increases with age. To test for genetic or epigenetic predisposition, we would ideally perform genome-wide association

studies (GWAS) on much larger sample sizes (> 100) to identify genetic variants and SNPs associated with GWI.

In conclusion, the studies presented here represent the first experimental results with GW veteran-based hiPSC lines, which are available to the GWI community through our biorepository. The utility of these lines is multifold and accentuate the importance of a cellular approach to resolving the mechanistic basis of the disease. In addition, hiPSC lines will be instrumental for screening of drugs as well as providing an avenue toward personalized therapy for individual veterans.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00018-021-03942-3>.

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Author contributions PLY conducted all cell culture experiments, acquired and analyzed the data, created the figures, and wrote the manuscript. AP performed the live-cell imaging experiments and analysis. XS conducted the histological experiments and analysis. AN assisted with cell culture and immunocytochemical analysis. RG analyzed the live-cell imaging data. PC and WB performed the animal behavioral studies and analysis. EP conducted the HDAC6 experiments. AVT designed the animal behavioral studies and wrote the behavioral sections of the manuscript. KAS directed the biorepository that stored the hiPSC cell lines, assisted with designing the research studies, and edited the manuscript. PWB and LQ designed the research studies, assisted with data interpretation, and wrote the manuscript.

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Availability of data and materials The data that support the findings discussed here are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have declared that no conflict of interest exists.

Ethical approval All human studies involving recruitment and participation of Gulf War veterans were approved by the Institutional Review Board of Boston University. All animal procedures employed during this study were reviewed and approved by the Institutional Animal Care and Use Committee of Augusta University and are consistent with AAALAC guidelines.

Consent to participate All human participants provided written informed consent for use of their blood samples for reprogramming into hiPSCs prior to participation in the study.

Consent for publication All human participants provided written informed consent for use of their data for publication after all patient demographic information was deidentified to protect patient privacy.

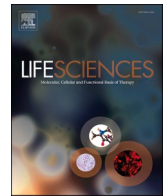
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A common language for Gulf War Illness (GWI) research studies: GWI common data elements

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Abbreviations: BU, Boston University; CDE, Common Data Element; CDMRP, Congressionally Directed Medical Research Programs; CNS, central nervous system; DoD, Department of Defense; GW, Gulf War; GWI, Gulf War Illness; MRS, magnetic resonance spectroscopy; ME/CFS, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome; NINDS, National Institute of Neurological Disorders and Strokes; NSU, Nova Southeastern University; PET, Positron Electron Tomography; QoL, quality of life; RTI, RTI International; VA, United States Department of Veterans Affairs; WRIISC, War Related Illness and Injury Study Center.

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ABSTRACT

Aims: The Gulf War Illness programs (GWI) of the United States Department of Veteran Affairs and the Department of Defense *Congressionally Directed Medical Research Program* collaborated with experts to develop Common Data Elements (CDEs) to standardize and systematically collect, analyze, and share data across the (GWI) research community.

Main methods: A collective working group of GWI advocates, Veterans, clinicians, and researchers convened to provide consensus on instruments, case report forms, and guidelines for GWI research. A similar initiative, supported by the National Institute of Neurologic Disorders and Stroke (NINDS) was completed for a comparative illness, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS), and provided the foundation for this undertaking. The GWI working group divided into two sub-groups (symptoms and systems assessment). Both groups reviewed the applicability of instruments and forms recommended by the NINDS ME/CFS CDE to GWI research within specific domains and selected assessments of deployment exposures. The GWI CDE recommendations were finalized in March 2018 after soliciting public comments.

Key findings: GWI CDE recommendations are organized in 12 domains that include instruments, case report forms, and guidelines. Recommendations were categorized as core (essential), supplemental-highly recommended (essential for specified conditions, study types, or designs), supplemental (commonly collected, but not required), and exploratory (reasonable to use, but require further validation). Recommendations will continually be updated as GWI research progresses.

Significance: The GWI CDEs reflect the consensus recommendations of GWI research community stakeholders and will allow studies to standardize data collection, enhance data quality, and facilitate data sharing.

1. Introduction

The combination of the desire to collect data in clinical trials and research studies that characterize the clinical and biologic symptoms that veterans with Gulf War Illness (GWI) experience and the ability to compare findings from different studies, motivated GWI investigators to develop a common set of assessment tools. GWI is a complex, multi-symptom medical illness of uncertain etiology and limited targeted treatment options. Clinicians and researchers confront the challenge to study this medical condition and to interpret results of independent studies conducted by different research groups at multiple institutions who utilize disparate methodologies and clinical trial designs. This makes cross-study comparisons cumbersome and difficult to replicate. As such, GWI research efforts will benefit from standard data collection methods based on measures that demonstrate evidence for content validation of assessment of veterans with GWI.

For three decades since the 1991 Gulf War, GWI research groups have separately performed epidemiologic, clinical, and basic science studies to gain insight into the underlying mechanisms and heterogeneity of this chronic illness in an effort to discover effective treatments affecting approximately a third of the deployed veterans.[1] The condition negatively affects multiple organ systems and results in a myriad of symptoms that include debilitating fatigue, respiratory issues, musculoskeletal pain, gastrointestinal disturbances, cognitive dysfunction, and dermatologic and neurological complaints. [2,3] Diagnosis of GWI relies on the presence or absence of various criteria that vary by individual and are interpreted differently by clinicians. Treatment is palliative in nature, relying on symptom presentation because the

underlying pathophysiology is elusive. While some progress has been made, the ability to clearly understand these mechanisms has been hindered by the lack of an accepted case definition and the heterogeneity of GWI.

Regardless of the case definition, GWI researchers recognize that applying the same standard measurement methods to research will allow aggregation of research findings. As such, the GWI clinical and research communities sought to identify common definitions employed in clinical research and laboratory studies, to present them in a standard format (instruments, forms, and guidelines), and to provide information for research for clinical and biologic collection.

The foundation of this undertaking was supported by the National Institute of Neurologic Disorders and Stroke (NINDS) for a comparator illness, Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). [4] ME/CFS is phenotypically similar to GWI and presents with an inability to identify markers of disease activity. [5,6] Both illnesses are currently diagnosed and managed based on presenting symptomatology, necessitating objective measures and valid, reliable tools.

The GWI Common Data Element (CDE) initiative was sponsored by the United States Department of Veteran Affairs (VA) and the Department of Defense (DoD) *Congressionally Directed Medical Research Programs (CDMRP)* and aimed to establish consensus on data collection measures across important illness domains to improve the interpretation and applicability of GWI study results. The CDE GWI initiative developed recommendations across survey instruments, case report forms, and laboratory measures to improve data quality, and to increase the efficiency and effectiveness of clinical and laboratory studies.

2. Materials and methods

2.1. GWI CDE development process

Veterans, advocates, clinicians, laboratory scientists, and GWI investigators convened to develop GWI CDEs to establish the GWI CDE working group. The GWI CDE working group was divided into two sub-groups (symptoms and systems assessment), following the similar NINDS ME/CFS CDE initiative. The GWI working group members were tasked to evaluate the tools recommended by the NINDS ME/CFS CDE within specific domains and to review the applicability of the instruments and forms to GWI research.

Through discussion, the working group provided consensus on assessment tools, instruments, case report forms, and guidelines for GWI research studies.

The symptoms assessment working group reviewed six domains: Baseline/covariate information, fatigue, post-exertional malaise (PEM), sleep, pain, and quality of life/functional status/activity/exercise challenge studies. The systems assessment working group reviewed five domains: Neurologic/neuropsychological/neuroimaging, autonomic, endocrine/neuroendocrine, immune, and biomarkers. The working group members evaluated measures in every domain outlined by the ME/CFS CDE guidelines as a foundation for these GWI CDEs (Table 1). Members of both groups also reviewed a domain specific to GWI research: Deployment exposures and GWI risk factors.

The GWI CDE Working Group began meeting by teleconference in February 2018 and assembled in-person during a field-based meeting in March 2018. The meeting was available via webcast to all interested investigators from the VA and CDMRP research programs. Prior to the March 2018 meeting, all members of the GWI working group were asked to review all of the ME/CFS CDEs and evaluate their applicability to GWI research. During the February 2018 teleconferences, participants were assigned to either the symptoms or systems working groups. Each participant was assigned to review two specific domains in detail and to submit their evaluations via a detailed questionnaire in the Research Electronic Data Capture (REDCap) online database system, prior to the in-person, field-based meeting. The GWI CDE administrative team compiled the responses. For each domain, a primary reviewer was tasked with summarizing all submitted comments for an assigned domain, presenting the key issues, and leading the discussion during the in-person field-based meeting. Regular working group meetings were held via teleconference to review and classify tools within each domain until recommendations for instruments, case report forms, and guideline documents were finalized.

2.2. GWI CDE development classification

The working group classified assessment tools based on their applicability to GWI research. Determinations were made whether to recommend a psychometric instrument as a “Core” data element to establish the presence or absence of a key domain in all GWI studies, and to select specific data elements that should be standardized or reported

Table 1
Gulf War Illness common data elements domains by assigned group.

Symptoms Assessment Working Group	Systems Assessment Working Group
Baseline/covariate Information	Neurologic/neuropsychological/ neuro imaging
Fatigue	Autonomic
Post-Exertional Malaise (PEM)	Endocrine/Neuroendocrine
Sleep	Immune;
Pain	Biomarker
Quality of Life/Functional Status/Activity/ Exercise Challenge Studies	
Deployment Exposures and GWI Risk Factors Questionnaire	

Reference: VA/DoD Gulf War Illness (GWI) Common Data Elements, 2/2021.

in detail for replication. The best available tools were selected, despite differences across studies to account for the ceiling and floor effects of self-reported measures of GWI-related symptoms. In many domains, information regarding the reliability and validity of specific measures in GWI studies was limited due to the vast variability of measures employed by the research community. Consistent with the NINDS ME/CFS CDE terminology and approach, each tool was classified as, Core, Supplemental-Highly Recommended, Supplemental, or Exploratory (Table 2).

3. Results

The completed GWI CDE document was posted for public comment on the DoD CDMRP website in January 2019. Comments received during public review were brought to the GWI CDE working group oversight committee for review. The final version was posted in March 2019 under the GWI research section of the DoD CDMRP website (https://cdmrp.army.mil/gwirp/research_highlights/19gwi_cde_initiative_highlight.aspx).

These GWI Common Data Elements (CDEs) standardize data collection for clinical assessment, laboratory, and imaging research studies. The GWI CDE initiative recommends the GWI case definitions (Centers of Disease Control and Prevention (CDC) and Kansas GWI case definitions) and the Veteran Short Form 36 item Health survey (VR-36) as “Core” data elements for use in GWI research (Table 3). The case definition of GWI is essential and is recommended to be included as part of the research study’s inclusion criteria.

The general health survey, the Veteran Short Form 36 item Health survey (VR-36), is recommended as a “Core” data element in all GWI human subject investigations. This instrument is relatively short, containing 36-self-report questions. The VR-36 is currently the most widely used instrument to measure quality of life (QoL) in GWI and is used as a clinical assessment in the intake of GWI patients at the Veteran’s Administration (VA) War Related Illness and Injury Study Centers (WRIICs). The physical and vitality subscales are often used as functional measures, and some use alternate item weightings to (de) emphasize the contributions of selected domains. It is also important to use the sub-scales of the VR-36 (physical component summary (PCS) and mental component summary (MCS) scores) to capture emotional well-being and not to focus entirely on physical aspects of health. There is a need for empirical evidence regarding sensitivity to change within the GWI population for many of these measures. In the absence of additional data, the VR-36 is recommended as the “Core” instrument.

The GWI CDE initiative employed expert consensus to select GWI CDEs to recommend tools for future studies involving Veterans diagnosed with GWI. The list of specific instruments, case report forms, and guidelines can be found on the CDMRP website. GWI researchers have a choice of valid, reliable tools that will assist in the comparability of variables across studies and will facilitate meta-data analyses. A

Table 2
GWI common data elements data standards classification [4].

Rating	Definition
Essential	Core CDE: Collects essential information, applicable to any study which span across all disease and therapeutic areas, or that are specific to one disease area. Supplemental-Highly Recommended CDE: Essential to collect, based on certain conditions or study types in GWI clinical research studies, depending upon study type or design.
Additional	Supplemental CDE: Commonly collected in clinical research studies, but relevance depends upon the study design (i.e., clinical trial, cohort study, etc.) or type of research involved. Exploratory CDE: Requires further validation but may fill current gaps in the CDEs and/or substitute for an existing CDE once validation is complete. The instruments are reasonable to use with the understanding that limited study has been done for veterans with GWI.

Reference: VA/DoD Gulf War Illness (GWI) Common Data Elements, 2/2021.

Table 3

Classification of GWI Common Data Elements: Core.

GWI Common Data Elements Instrument name	Classification
CDC Case Definition of GWI [7]	Core
Kansas Modified Case Definition of GWI [2]	Core
Veterans RAND Short Form 36 item Health survey (VR-36) [8]	Core
• Physical Component Summary (PCS) Score	
• Mental Component Summary (MCS) Score	

Reference: VA/DoD Gulf War Illness (GWI) Common Data Elements Version, 2/2021.

summary of the classification of instruments by domain is included in Table 4. For each domain, the number of recommended tools is listed. The baseline/covariate information contains the most measures and forms, which can be selected by GWI researchers to gain an initial understanding of the symptoms experienced by patients or research study participants with GWI. The GWI CDE working group classified tools as “supplemental-highly recommended” when the study focuses on a certain domain and its objectives are to assess the entire spectrum of GWI symptoms experienced by Veterans. In many cases, tools are classified as “supplemental,” when they have not been used extensively in GWI research but demonstrate promising opportunities for future explorations.

4. Discussion

The GWI CDE initiative employed expert consensus to select GWI CDEs to guide instrument recommendations and standardize GWI research efforts, which will be used to guide future studies involving Veterans with GWI and improve study comparisons and replication. These leading experts are sensitive to GWI symptoms and outcomes. In the process of selecting instrument recommendations within specific domains, thoughtful consideration was employed to ensure effective utilization, consideration of instrument advances, and future directions. The working group selected a few “Core” measures, including the GWI case definitions (CDC and Kansas case definitions) and a general health survey (VR-36).

Table 4

Number of measurement tools and level of classification by CDE domain and area.

GWI CDEs domain and area	Supplemental-highly recommended	Supplemental	Exploratory
Baseline/covariate information	10	–	–
Post Exertional Malaise	1	7	–
Fatigue	1	6	–
Sleep	2	–	8
Pain	1	3	6
Quality of Life	–	2	5
Exercise Challenge	–	2	2
Functional Status	2	–	1
Neurological	1	–	–
Neuropsychological	8	8	–
Neuroimaging	8	–	–
Autonomic	1	–	7
Endocrine/Neuroendocrine	3	2	–
Immune	4	2	–
Biomarkers	3	–	2
Deployment Exposures and GWI Risk Factors	1	–	–

Note: Measurement Tools include survey instruments, case report forms, and guidelines.

Reference: VA/DoD Gulf War Illness (GWI) Common Data Elements Version, 2/2021.

4.1. Selection of additional “Core” CDEs

While some recommended tools have yet to be rated in terms of reliability and validity in GWI research, GWI investigators recognize that certain measures are useful in gauging clinical symptoms, which are significant to GWI Veterans. The consensus of the GWI CDE working group was that “Core” instruments should be identified to assess certain domains significant to GWI, including fatigue and pain. Additional research involving Veterans with GWI is needed before the selection of specific recommendations of additional instruments can be made. However, this is an ongoing process as potential newer and more sensitive measures are tested and established in the field.

4.2. Utilization of CDEs

The GWI CDE working group recommends that GWI researchers use these CDEs to standardize clinical and biologic data tools. These leading experts are sensitive to GWI symptoms and outcomes, as well as the research participant burden. When initially designing the research assessment platform, researchers should review the twelve CDE domains, incorporate the “Core” data elements (Kansas and CDC case definitions and VR 36), and select additional domain measures that match the study’s objectives. Prior to implementation, researchers are encouraged to consider the participant burden with respect to the number tasks, which may affect the participants’ level of fatigue, influence cognition, and, ultimately, affect the completion of all tasks outlined in the research study platform. The focus is on the order the research participant performs the surveys and tests, such that the assessments that are most germane to the study’s objectives are completed early in the research encounter.

4.3. Presence or absence of operational definition of a domain

Discussions by the GWI CDE working group focused on the degree to which a data element met the needs of the GWI research community and if it had been previously published in the literature and found to be sensitive to GWI case status. In addition, they also considered how a measure could be interpreted in the context of several gaps in the literature. For some domains that are important in diagnosing GWI, like post-exertional malaise (PEM), there is a lack of consensus among experts for an operational definition, and few questionnaires have been designed to measure this symptom. This presents challenges and opportunities for GWI clinicians and researchers.

In contrast to PEM, questionnaires are often used to diagnose and treat fatigue and assess the level of severity, and the level of functioning. Even subdomains are well-defined in GWI research in terms of general fatigue, physical fatigue, mental fatigue (cognitive difficulties), post-exertional fatigue, and fluctuating fatigue. Although there is limited evidence for content validation in GWI research, GWI clinicians and researchers often use the Multidimensional Fatigue Inventory (MFI) to gain a better understanding of the debilitating fatigue experienced by veterans with GWI.

4.4. Domains as outcome measures

For many GWI studies, assessment tools represented in the ME/CFS CDEs are commonly sufficient to address the needs of studies when the domain is not a primary outcome variable. Throughout the CDEs, these instruments were deemed to be “supplemental” and recommended for selection depending on the goals of a study. Specific instruments may be more relevant to dimensions examined in a given study and should be considered on a per-study basis. In some cases, a study may focus on the presence or absence of a domain, but other studies may prefer a measure that possesses sensitivity to change to detect day-to-day fluctuations in GWI symptom severity, which is essential to understand the time-course of the domain.

4.5. GWI CDE departures from ME/CFS CDE

The GWI CDE working group departed from some decisions recommended by the ME/CFS CDE committee and chose to draft new versions of published questionnaires in an attempt to derive more appropriate measures that will need validation in GWI research efforts. Case report forms that were not considered to be appropriate for GWI were replaced with new forms and classified as exploratory measures. For example, neuroimaging case report forms for positron electron tomography (PET) and magnetic resonance spectroscopy (MRS) scans have been updated to reflect neuroinflammatory and oxidative stress measures, which are important in understanding GWI pathophysiology. [9] [10].

Given that NINDS supported the development of CDEs for multiple diseases, in addition to ME/CFS, the GWI CDE working group modified some domain sub-categories by adding additional categories relevant to GWI and adopted CDEs created by other NINDS CDE working groups. For example, the endocrine/neuroendocrine sub-group recommended a separate dietary survey and dietary supplements use form adopted from the mitochondrial and gastrointestinal (GI) disease CDEs to better address the assessment of GWI. (See supplemental materials).

An additional domain was developed by the GWI CDE working group to assess deployment exposures and GWI risk factors. When assessing this domain, GWI clinicians and researchers are encouraged to survey patients or study participants about their experiences rather than on their direct exposures. This orientation was selected both because of the potential for recall bias on reporting prior exposures and to improve the recall of past events. This survey was designed to assist with future gene-environment and genetic susceptibility biomarker and treatment trial studies.

4.6. Limitations and future directions

The approach taken for choosing common data elements for GWI was to focus on measures which had been used previously in other studies and found to be sensitive to particular aspects of the disorder. However, this approach has clear limitations that should be noted. There may be newer tests or clinical measures that are in fact more sensitive to GWI but have not been included in multiple publications to date and were therefore not chosen for use in the current CDE recommendations. That is the reason that the GWI Common Data Elements platform is considered to be a dynamic resource that will evolve based on the results of evidenced-based GWI investigations that employ and validate these measures, allowing comparability across research studies. All updates to the GWI CDEs will undergo a thorough review process including approval by the GWI CDE Working Group Oversight Committee prior to implementation. This committee is comprised of up to ten individuals who are clinicians, laboratory researchers, investigators, veterans, and GWI advocates. The committee may include representatives from the U. S. Veterans Affairs Office of Research and Development, and the U.S. Department of Defense CDMRP GWI research programs. The leadership of these two GWI programs continues to participate in every step of the CDE development process and notes the use of CDEs in the study design of future investigations of Veterans with GWI. Benefits of CDEs will advance scientific knowledge in the understanding of the etiology and treatment of GWI to assist Veterans.

Interested researchers and clinicians are welcome to send comments or suggestions for new CDEs to the committee at any time. As two large, funded efforts currently underway involve evidence-based outcome trials, it is anticipated that these Consortia, Gulf War Illness Clinical Trials and Interventions (GWICTIC) and Boston, Biorepository, Recruitment, and Integrated Network for GWI (BBRAIN), will share study results within the next two years. The GWI CDE Working Group Oversight Committee will review the sensitivity and reliability of measures used in these and other GWI investigations to inform the recommended measures presented in the next CDE platform.

5. Conclusion

GWI research data can be systematically collected, analyzed, and shared across research studies by using common data elements that represent the predominant symptoms experienced by Veterans with GWI. Investigators of clinical and laboratory studies are encouraged to incorporate the GWI CDEs Core instruments into their research designs to enhance data quality and promote meta-analyses. By recommending valid and reliable clinical and biologic assessment tools for use in GWI clinical trial designs and laboratory investigations, a better understanding of GWI disease pathology and accompanying targeted treatment approaches may be realized in the future.

Author contributions

DC, NK, KS, and RM contributed to the conception of the article. DC wrote the initial manuscript. DC, NK, KS, and RM contributed to the manuscript revision, read, and approved the submitted version. The GWI CDE Working Group members contributed to the supplementary material. We also acknowledge Beth Gilbert for formal review.

Declaration of competing interest

The authors declare no financial or other conflicts of interests.

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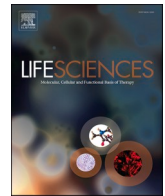
Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2021.119818>.

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Boston biorepository, recruitment and integrative network (BBRAIN): A resource for the Gulf War Illness scientific community

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ABSTRACT

Aims: Gulf War Illness (GWI), a chronic debilitating disorder characterized by fatigue, joint pain, cognitive, gastrointestinal, respiratory, and skin problems, is currently diagnosed by self-reported symptoms. The Boston Biorepository, Recruitment, and Integrative Network (BBRAIN) is the collaborative effort of expert Gulf War Illness (GWI) researchers who are creating objective diagnostic and pathobiological markers and recommend common data elements for GWI research.

Main methods: BBRAIN is recruiting 300 GWI cases and 200 GW veteran controls for the prospective study. Key data and biological samples from prior GWI studies are being merged and combined into retrospective datasets.

Abbreviations: BBRAIN, Boston Biorepository, Recruitment and Integrative Network for Gulf War Illness; GW, Gulf War; GWI, Gulf War Illness; GWIC, Gulf War Illness Consortium; VA, Veterans Affairs.

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They will be made available for data mining by the BBRAIN network and the GWI research community. Prospective questionnaire data include general health and chronic symptoms, demographics, measures of pain, fatigue, medical conditions, deployment and exposure histories. Available repository biospecimens include blood, plasma, serum, saliva, stool, urine, human induced pluripotent stem cells and cerebrospinal fluid.

Key findings: To date, multiple datasets have been merged and combined from 15 participating study sites. These data and samples have been collated and an online request form for repository requests as well as recommended common data elements have been created. Data and biospecimen sample requests are reviewed by the BBRAIN steering committee members for approval as they are received.

Significance: The BBRAIN repository network serves as a much needed resource for GWI researchers to utilize for identification and validation of objective diagnostic and pathobiological markers of the illness.

1. Introduction

Gulf War Illness (GWI) is a debilitating, chronic, multi-symptom disorder affecting nearly one-third of veterans in the 1991 Gulf War (GW) [1,2]. The illness is characterized by debilitating fatigue, chronic pain, cognitive dysfunction, headaches, respiratory problems, and gastrointestinal disturbances [3–5]. Veterans suffering from GWI can experience significant impairment in their daily activities and quality of life. Despite promising recent research in correlating biomarkers to GWI symptoms, GWI primarily remains diagnosed by self-report. The study of potential diagnostic biomarkers to date has not been supported by larger sample sizes and has not been validated in other cohorts [6–11]. Basing diagnosis on self-reported symptoms makes treatment development and access to care for GW veterans persistently difficult. There is a critical need for an objective diagnostic test for GWI to alleviate GW veterans' difficulties with obtaining service-related benefits and validation of their symptoms and for use as primary outcome measures for treatment trials.

“A biorepository is an entity that receives, processes, stores, and/or disseminates biospecimens, their derivatives, and relevant data, as needed [12]. It encompasses the physical location and the full range of activities associated with its operation [12].” A recent review by Garcia et al., showed that rare disease biobanks have the ability to identify and validate genetic and omics biomarkers as well as inform treatment development strategies for these rare disorders [13]. However, it was also noted that many of these repositories lacked the corresponding clinical outcomes data needed to make the biomarker samples most useful for correlation with the disease symptoms.

Therefore, the need is clear for a biorepository network of freely sharing biospecimens with corresponding comprehensive clinical outcomes data in the field of GWI research. There is also a need for retrospective data mining from prior studies that are hard to replicate (i.e., cerebrospinal fluid, PET₂ and MRI brain imaging outcomes). These clinical outcomes that are common (common data elements) among the different prior studies provide power to document differences that might not emerge in the smaller sample cohorts. These common data elements are also needed to ensure comparability of study results, particularly for treatment trial efficacy testing [14,15].

The Boston Biorepository, Recruitment and Integrative Network (BBRAIN) for GWI was designed to serve as a resource for the GWI research community to hasten biomarker discovery and validate prior results in a well-characterized cohort of GW veterans. The BBRAIN study was built upon and incorporates the already existing Boston GWI consortium [16]. The GWI consortium brought together leading experts from different fields into the GWI research community. Since its conception, the GWI consortium has established an extensive multi-site data set with cognitive measures, brain imaging, health symptom data, and biorepository blood and saliva specimens for several hundred GW veterans. GWIC has greatly expanded the field's ability to explore and identify specific ‘objective’ biomarkers and ‘personalized’ treatment strategies for veterans with GWI by utilizing a small biorepository shared with the GWI research community, resulting in 20 additional federally funded studies. This led to 34 biomarker publications of lipidomic, proteomic, epigenetic, genetic susceptibility, mitochondrial,

CNS autoantibodies, and tau markers in clinical and preclinical translational studies [7,11,17–43]. It also resulted in funding to establish two additional consortia, including BBRAIN and the Gulf War Illness Clinical Trials Consortium [14]. Since its inception in 2018, BBRAIN has built upon this existing infrastructure at Boston University and 14 other participating sample and data resource sites to establish a much-needed resource for the GWI research community that is available for data and sample sharing.

2. Methods: BBRAIN structure

2.1. Leadership

The lead site of BBRAIN is at Boston University School of Public Health and makes up the network coordinating center. The network coordinating center staff members have diverse expertise in neuropsychology, brain imaging, exposure assessment, data management, statistical programming and study operations. These skills are integral for maintaining a multi-site biorepository and promoting collaboration within the GWI research field.

2.2. Participating sites

The BBRAIN collaboration brings together leading investigators from 15 institutions to support participant recruitment, administrative activities, data management and biostatistics, and biorepository and biomarker evaluation. The BBRAIN collaboration is composed of the network coordinating center, steering committee, retrospective resource sites and four prospective resource sites (Fig. 1). The four resource sites where prospective subject recruitment is taking place include Boston University School of Public Health, Miami VA Medical Center and Nova Southeastern University, Bronx VA Medical Center and the San Francisco VA Medical Center.

2.2.1. Steering committee

Oversight of the BBRAIN is coordinated by a Steering Committee made up of the BBRAIN PI, the resource site PIs, the network coordinating directors, and the consumer advocate. The Steering Committee monitors research site performance and determines individual study performance. The Steering Committee is also responsible for establishing standard operating procedures, utilizing ISBER Best Practices for Biorepositories, and utilizing BUSPH criteria templates for Data Use Agreements across sites and institutions sharing samples and data [12]. Researchers interested in obtaining BBRAIN samples can apply to the Steering Committee. The group will decide on the appropriateness (i.e., for GWI research and not redundant with ongoing research) and priority of sharing samples on a case-by-case basis.

2.2.2. Network coordinating center

The network coordinating center for the biorepository is responsible for overseeing IRB protocol and regulatory submissions and approvals, establishing standard protocols across all sites, and conducting data management and monitoring while ensuring study participant confidentiality. The center is led by the BBRAIN study PI at BUSPH and

supported by faculty and administrative staff at BUSPH. The network coordinating center provides support and coordination for prospective data collection of demographic surveys, cognitive test data, serum, plasma, saliva, stool, and urine samples from 500 GW veteran study participants. Additionally, the network coordinating center serves as a gatekeeper for requests for repository site sharing and coordinates approvals with the steering committee members in consultation with the biorepository contributors. A virtual biorepository is established using laboratory software LDMS (Frontier and/or FreezerPro for resource sites). Resource sites send newly obtained biospecimens and data to laboratory storage facilities at Nova Southeastern University and Boston University Medical Campus (BUMC) as the prospective repository is being created.

2.2.2.1. Subject Confidentiality. As in all human subject research, protecting subject confidentiality is imperative. For retrospective data, all samples and data are de-identified. For prospective data collection, participants' contact information is kept in a study-specific electronic capture web-based platform on a secure server, including multiple password protection layers that are only accessible by approved study team members. All source documents are kept in a locked cabinet, and all data is behind password-protected and encrypted devices. Samples that are shipped are labeled with a unique identifier code for tracking purposes within the biorepository. Participants are made aware of the confidentiality measure taken at the time of the phone screener and again during the consent process and are consented to share their coded study samples and data for the repository and other future GWI related studies. All data and samples that are shared from the repository are coded with no individual identifiers.

2.2.3. Retrospective resource sites

One of the BBRAIN's primary objectives is to establish a retrospective biorepository network by data mining from existing BBRAIN collaborators' stored specimens, cognitive data, and brain imaging data from study participants who have consented to share these data and samples for future studies in a de-identified manner. Retrospective resource sites have already provided some stored specimens from prior studies with GW veterans cataloged and made available for within Network and outside of Network investigators. Common data element datasets from these prior studies are also being created to improve power for new

analyses. Currently, available samples include blood serum ($n = 1100$), plasma ($n = 1100$), peripheral blood mononuclear cells (PBMCs $n = 600$), DNA ($n = 600$), human-induced pluripotent stem cells ($n = 9$), cerebrospinal fluid ($n = 150$), cognitive data ($n = 400$), brain imaging data (see Fig. 3 range $n = 50$ -280) and corresponding demographic/survey data from retrospective resource sites, including University of Alabama at Birmingham, San Francisco VA, Harvard / Beth Israel Deaconess Medical Center (BIDMC), Georgetown University, Boston University and Drexel University (Fig. 2) [38]. Brain imaging data includes MRI volumetric and diffusion tensor imaging, MR spectroscopy, functional MRI and positron emission tomography (PET) imaging with peripheral benzodiazepine receptor [^{11}C]-PBR28 and fluorodeoxyglucose 18F-FDG tracers [7,10,11,44,45]. Although some of these samples have been stored and processed differently, these details will be made available to the requesting research investigators to meet their study needs during a study consultation. In addition, preclinical animal retrospective data and tissue samples are also available for sharing upon request from the CDC/NIOSH and Roskamp Institute GWI animal models. As previously mentioned, data collection for the biorepository is coordinated and quality checked by the network coordinating center.

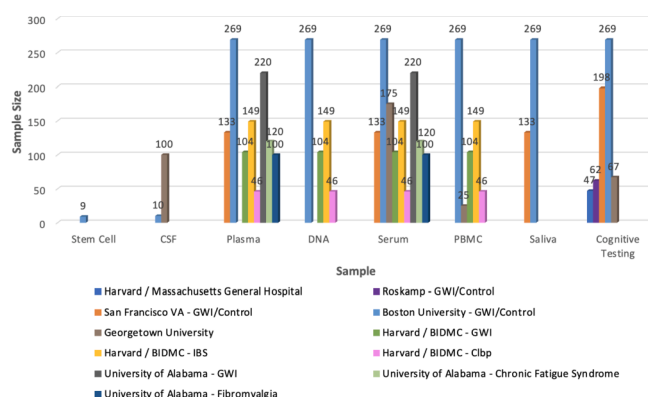


Fig. 2. Retrospective BBRAIN Samples and Data Repository.

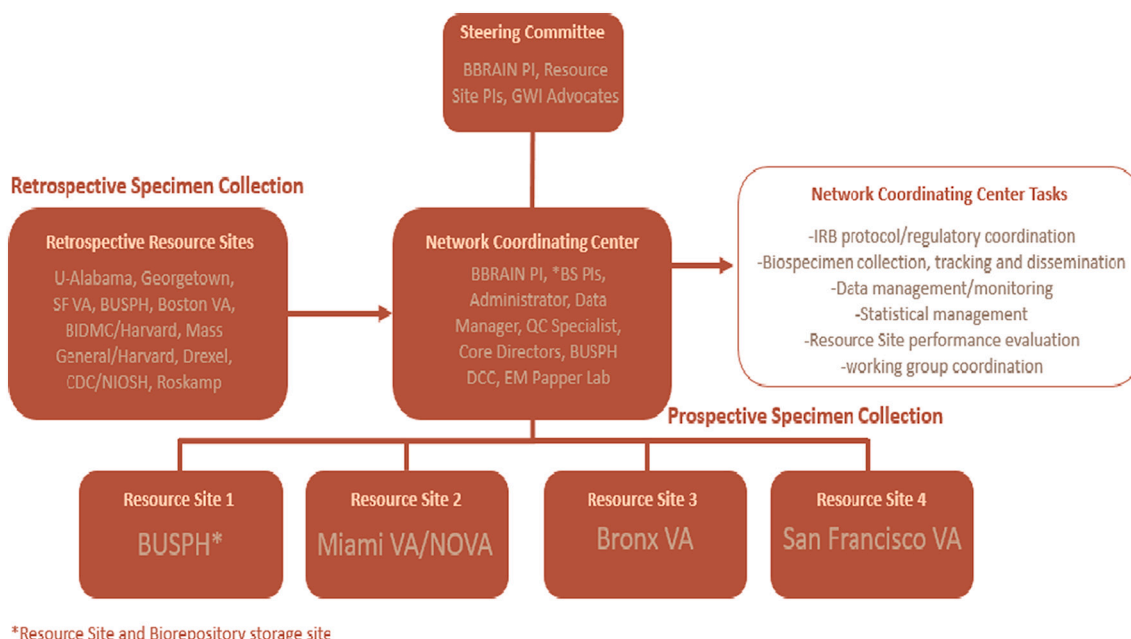


Fig. 1. BBRAIN organizational structure.

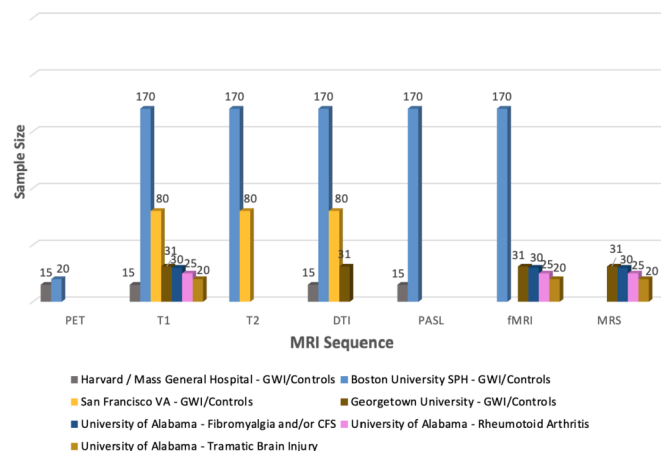


Fig. 3. BBRAIN MRI and PET Imaging Repository.

2.2.4. Prospective resource sites

Subject recruitment for the prospective study is conducted at four prospective sites comprising of Boston University School of Public Health (BUSPH), Miami VA Medical Center / Nova Southeastern University (NSU), Bronx VA Medical Center and San Francisco VA Medical Center (Fig. 1). These sites were chosen due to their access to established GW veteran cohorts and important prior research contributions. Data from the prospective resource sites is being added to the BBRAIN biorepository as subject recruitment accrues.

3. Prospective study methods

3.1. Participants

This case-control study is recruiting 500 GW veterans encompassing 300 GWI cases and 200 GW veteran controls. GWI cases are determined by the Kansas GWI criteria and the four recruitment sites are oversampling women veterans from their prior cohorts [4]. Although not exclusion criteria, smoking history, medication use, and other demographic and health outcomes are being collected and available to requesting investigators.

3.2. Inclusion/exclusion criteria

Study eligibility includes deployment to the Persian Gulf in the 1990–1991 Gulf War without any medical exclusions required for participation. To meet case criteria, the individual needs to endorse symptoms in three of six health symptom domains: pain, fatigue, neurological/cognitive/mood, skin, gastrointestinal, and respiratory [4]. If an individual does not meet the Kansas criteria and has no exclusionary conditions, they are categorized as a control. The criteria for prospective study participants is determined by using the Kansas GWI case definition [4]. Veterans are excluded from being considered GWI cases or controls for the Kansas criteria if they report being diagnosed by a physician with medical or psychiatric conditions that would otherwise account for their symptoms or interfere with their ability to report their symptoms. The Kansas exclusion criteria encompass conditions such as diabetes, heart disease other than hypertension, stroke, lupus, multiple sclerosis, cancer, liver disease, chronic infection, or serious brain injury. Veterans are also excluded if they report being diagnosed with schizophrenia or bipolar disorder or if they have been hospitalized in the past 5 years for alcohol/drug dependence, depression, or post-traumatic stress disorder (PTSD). Potential participants are screened by telephone to determine whether they meet inclusionary or exclusionary criteria for study participation [4]. Additionally, during the phone screen eligible participants are categorized as a case or control based on the Kansas GWI case criteria [4]. Although Kansas criteria are primarily used for comparing study outcomes, the CDC chronic multi-symptom illness case criteria are also obtained for all study participants [5]. These criteria include symptoms in two out of three symptom domains including fatigue, mood-cognition and pain [5].

3.3. Methods

The study protocol for the prospective resource site clinical case-control study consists of five components:

- 1) Neuropsychological testing: Measures from a previously validated assessment of cognitive function and common data elements in GW veterans are included to assess cognitive outcomes [15,35,46–48]. The neuropsychological test battery assesses the functional domains of attention and executive abilities, psychomotor function,

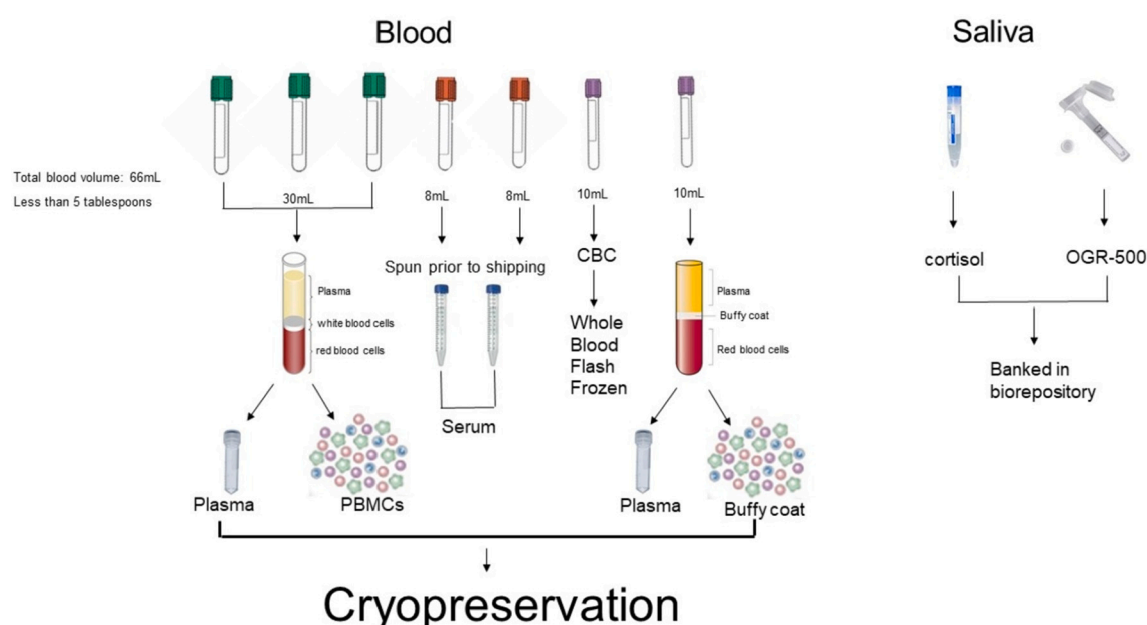


Fig. 4. Blood and saliva aliquot scheme.

visuospatial skills, memory, general intellectual abilities and mood. The battery includes tests shown to have high specificity and sensitivity for detecting changes in neuropsychological functions between veterans with and without GWI and which were recommended to be used across studies as common data elements [35,47].

- 2) Surveys: The set of surveys administered were all included in the GWI common data elements and collect data on health symptoms, neurotoxicant exposures, mood and quality of life. Clinical assessments include the Pittsburgh Sleep Quality Index (PSQI), Visual Analog Scale (VAS) for pain, Kansas Gulf War and Health Questionnaire, Multidimensional Fatigue Inventory (MFI-20) questionnaire, MOS Short Form 36-veteran version (SF-36V), and Profile of Mood States (POMS), as well as the medical conditions checklist. Additional surveys that were also included in the exposure assessment common data elements include the Kansas Gulf War Experiences and Exposures Questionnaire and the Structured Neurotoxicant Assessment Checklist (SNAC) [49–54].
- 3) Blood draw: Approximately 79 mL of blood are drawn from the participant for local lab clinical testing, immune biomarker lab testing, and biorepository storage. Fasting blood samples are collected by venipuncture by a trained phlebotomist in the morning. A small amount of blood is analyzed at the local labs for complete blood count, lipid panels, thyroid stimulating hormone, antinuclear antibodies, and rheumatoid factor. The remaining blood samples for each participant are shipped to Nova Southeastern University for sample processing and storage in the biorepository. Blood samples are analyzed to measure plasma cytokine levels, complete blood count, and RNA extraction is performed from PBMCs collected. PBMCs isolated from sodium heparin and EDTA tubes are stored in liquid nitrogen for cryopreservation. RNA isolated from PBMCs is aliquoted and stored at -80 °C. All data from these analyses will be made available as part of the biorepository. Whole blood and blood derivatives such as serum, heparin and EDTA plasma are prepared at various volume aliquots and frozen at -80 °C for storage.
- 4) Saliva sample: Approximately 6 mL of saliva are collected throughout the study visit. These samples are collected at four different time points: after the participant consent form is signed, after the blood draw and physical exam, after completion of surveys, and at the end of the visit. Three of the collections are performed when the participant is in a fasting state. These samples are used to measure salivary cortisol levels and are stored in a -80 °C freezer. An OGR-600 tube for saliva sample collection is also performed upon fasting for DNA analysis and stored at room temperature before shipping to EM Papper Lab at NOVA Southeastern University. All saliva samples are batch shipped to Nova Southeastern University for planned assessment, cortisol analyses and DNA extraction. The remaining saliva samples are also aliquoted down and frozen for biorepository sample requests. Cortisol and DNA will be made available in the biorepository.

3.3.1. Sample processing methods

Blood samples are shipped on the day of collection at room temperature overnight to the EM Papper laboratory at Nova Southeastern University. As previously mentioned, saliva samples are batch shipped on dry ice, from storage at each prospective site's local storage location. Samples received by the biorepository laboratory at Nova Southeastern University are processed within 2 h of delivery. This includes an immediate quality check of the condition of sample tubes, the outer and inner packaging, and the associated chain of custody that accompanies the sample. Acceptable samples are then accessioned by the biorepository team and entered into the Laboratory Data Management System. This allows for the tracking of barcoded aliquots from each phase of the process, including storage conditions and requirements, and will allow expeditious processing of sample requests. Primary tubes are processed to isolate the blood derivatives: plasma, serum, PBMCs, buffy

coat, and red blood cell pellets. Aliquots will be created per the aliquot scheme (see Fig. 4) and tracked for their temperature and location. Each of these blood derivative type are stored at its optimal temperature per standard protocol for the EM Papper laboratory at Nova Southeastern University. Specific blood processing methods are listed below.

3.3.1.1. Separation of plasma from the cellular fraction. Whole blood samples are processed as described below to obtain a buffy coat fraction and plasma for cryopreservation. In the area designated for processing blood, the whole blood (collected in tubes containing an anticoagulant such as ethylene-diaminetetraacetic acid-EDTA or Heparin) is fractionated by centrifuging at 2000 x g for 10 min at room temperature. This separates the blood into three visible layers (Fig. 4). The upper layer, the plasma layer, is generally clear or pale yellow in color. The second layer is a narrow grayish white interface band representing the “buffy coat” or leukocyte fraction. The third or bottom layer is dark red and consists of the erythrocytes or red blood cells. Using an appropriate disposable transfer pipette, the plasma layer is aspirated off down to approximately 1 mm from the buffy coat layer taking care not to disturb the leukocyte or buffy coat layer. All plasma is expelled from the pipette into a plasma collection tubes. Recovered plasma is aliquoted and placed into labeled cryovials. The barcoded cryovials are placed in appropriate storage units for long-term storage in -80 °C freezers at the EM Papper laboratory at Nova Southeastern University.

3.3.1.2. Recovery of white blood cells. After removing the plasma layer, removal of buffy coat is performed. To isolate PBMCs, sufficient quantity of PBS is added to bring blood back to its original whole blood volume and mixed gently to continue PBMC processing. A transfer pipette is used to transfer all of the blood into a 15 mL tube containing 3 mL Ficoll-Hypaque solution. The tube is centrifuged (without a brake) at 2250 x g for 25 min at 25 °C. Using a sterile serological or transfer pipet, all cells are collected at the cloudy white interface taking care not to aspirate any more separation medium solution than necessary. The collected cells are transferred from one conical centrifuge tube to a single corresponding, pre-labeled, sterile conical centrifuge tube. After centrifugation of the wash step, cells are resuspended in 10 mL of PBS for cell count and viability using the Beckman Coulter ViCell Counter. For this study, 5×10^6 cells/mL per vial are aliquoted in final freezing solution of 70% RPMI 1640 with 20% fetal bovine serum and 10% dimethyl sulfoxide (DMSO) as a cryoprotectant added. The cryovials are placed in the appropriate storage units at -80 °C for short-term storage. For long-term storage, cells stored in freezing vials are transferred into the liquid nitrogen cryopreservation tanks and their location is mapped and recorded.

3.3.1.3. Separation of serum from blood samples. Blood is drawn into BD Vacutainer® SST™ Venous blood collection tube with separator gel. This tube is spun down prior to shipping to the EM Papper laboratory. Serum sample above the gel separator is collected and stored in bar-coded cryovials at various aliquot volumes at -80 °C for long term storage.

Quality Assurance and Assessment protocols take place before, during and after the samples are isolated from primary tubes and placed into the biorepository. Samples for all four prospective resource sites are processed with the same protocols.

3.3.2. Common issues of aliquot size

Biorepositories face a lack of predictability in future research direction, limited resources in space and maintenance manpower, as well as new technology innovations, which makes planning a biorepository difficult. It is imperative to create a versatile sample aliquot scheme in order to combat these challenges. However, repositories commonly will need to be flexible with remaining sample subsets should circumstances change, while avoiding “freeze thaw cycles” that are potentially damaging to certain proteins in serum or plasma. While large aliquots of

2 mL or greater can reduce maintenance costs and space requirements, small aliquots are the versatile option that allow for greater flexibility when fulfilling sample requests. This laboratory takes a different approach, setting up large numbers of small aliquots (0.5 and 0.25 mL) while still creating a small number of large aliquots (1.0 mL) for longer term storage (Fig. 4; Table 1).

5) Home specimen and data collection

Home collection by study participants includes urine, stool samples, and Fitbit data collection. Specific methods and details are listed below.

3.3.2.1. Fitbit data. Each participant is asked to wear a study-provided Fitbit activity monitor for 7 consecutive days. On the eighth day, participants are expected to extract their sleep quality and heart rate variability data and upload the results to the network coordinating center through a secure link. An instructional manual is provided to the participant for proper data extraction. All Fitbit data are made available for sharing in the repository.

3.3.2.2. Urine samples. Urine sample kits are provided to the participant with comprehensible instructions during the in-person study visit to complete at home and mail back in prepared pre-paid packaging. Participants are expected to collect at least 20 mL urine during their first morning void. Urine samples from all study sites are overnight shipped with an ice pack to maintain cold chain transport to the Boston University Medical Campus for aliquoting and storage. Once received, the urine samples are aliquoted, frozen and stored in the repository for later urinalysis, sharing and sample requests (Table 1).

3.3.2.3. Stool samples. Stool sample kits are provided to the participant during the in-person visit with clear instructions to complete the at home stool sample kit and mail it back in a prepared pre-paid packaging. Participants are expected to collect two tubes of stool. Stool samples from all study sites are overnight shipped to Boston University Medical Center for storage. Samples are stable for 15 days at room temperature and very well preserved over a long period of time at -4°C . Samples will be stored at the Boston University Medical Center Laboratory and available for collaborative research and for requests through the repository.

4. Data sharing

The BBRAIN biorepository has a responsibility to provide access to samples for pilot work and other initiatives to further the field. This is accomplished by a three-stage approval process for any data or samples that exist as part of this project and/or the biorepository. This process is streamlined through a web-based data request platform created by the network coordinating center. The first steps involve a potential investigator contacting the project PI to determine validity of an idea. Once initial conversations are considered positive, the potential investigator submits a proposal, along with a sample or data request form, to the

Steering Committee. This committee then assesses the proposal for feasibility (samples must be available, technology/assays must have a reasonable chance of success), duplicative effort (avoids overlap from funded portion of the grant or previously approved requests), and sample volume requested (protects the integrity of the aliquot scheme, i. e. requesting 1 mL when 200uL would suffice). Finally, the request is reviewed by the Steering Committee for further consideration and final approval or denial. There is no cost for the sharing of samples from the repository except the cost of shipping the samples to the approved requestors.

4.1. How to request samples and data

As previously described, the BBRAIN Repository Network provides samples and data from newly collected whole blood, RNA, DNA, plasma, serum, saliva, stool, and urine samples for 500 GW veterans (300 GWI cases, 200 controls) in addition to demographic surveys and cognitive test data. In addition, a repository of previously collected demographic and health survey, clinical (cognitive testing, MRI data) and preclinical data (animal tissue) has been compiled from the 15 participating GWI investigators and made available to the BBRAIN repository for data mining and sharing. 9 lines of hiPSCs collected from 5 GW-veterans with GWI and 4 from those who did not develop GWI are available upon request. As previously outlined, the Network Coordinating Center organizes approvals with the steering committee members in consultation with the biorepository contributor sites. The site for requesting prospective and retrospective biospecimens, brain imaging and other health symptom or cognitive data is available at https://wwwapp.bumc.bu.edu/BEDAC_BBRAINRetro.

5. Discussion

The BBRAIN is the first GWI repository network designed to gather and store samples, provide new data and to mine data from prior studies of difficult to obtain samples (CSF, PET, MRI imaging). In doing so, BBRAIN has grown, and continues to shape and fill the need for an easily accessible biospecimen repository in the field of GWI research. An additional primary objective of this biorepository network has been to determine minimal data elements using a common data platform from retrospective studies, thus creating centralized resource websites for BBRAIN researchers and other interested GWI researchers seeking to obtain repository samples and data for analyses. The task has been completed, with common data elements for symptom and system domains now identified. To date, BBRAIN has published recommended common data elements for neuropsychological and other outcomes [15,47]. Importantly, the BBRAIN prospective sample and data collection is consistent with the same elements of the common data recommendations and will therefore provide additional validation of nearly all of the common data elements.

BBRAIN will build upon the initial progress of the Boston GWIC biorepository. It has been stated that the success of a biorepository is not in how many samples are collected but in how many samples are shared

Table 1
BBRAIN blood and saliva aliquot and storage procedures.

Sample type	Tube type/color	# tubes	Tube volume (mL)	Total volume (mL)	Product	Expected product volume - biobank	Aliquot scheme	Test
Blood draw	(3) Green Na Heparin	3	10	30	Blood, PBMC, Plasma	60 × 10 ⁶ PBMC; 12 mL Plasma	PL2: 4 × 1 mL, 4 × .5 mL, 24 × .25 mL; CEL: 12 × 5 × 10 ⁶ /mL	NKCC, NPY, Nanostring
	(2) Red Tiger Top SST Tube	1	8	16	Serum	3 mL	2 × 0.5 mL; 8 × 0.25 mL	Hormone specific TBD
	(2) Purple EDTA	2	10	20	Blood, Serum	6 mL	4 × .5 mL; 16 × .25 mL	CBC, Flow, Cytokines
Saliva	(1) OGR-500	1	2	2	Saliva	2 mL	N/A	SNP, TBD
	(4) Salimetrics' Cryovials	4	2	8	Saliva	8 mL	2 × 1 mL; 12 × .5 mL	Salivary Cortisol, TBD

that lead to important new results [55–58]. To date, BBRAIN has shared samples with five research investigators including cerebrospinal fluid, serum and plasma samples, PET brain imaging, diffusion MRI brain imaging data and cognitive outcomes [59]. Building on this strong foundation, BBRAIN aims to provide the infrastructure, scientific expertise, biological specimens and collaborative nature to vastly speed up objective biomarker discovery and treatments for ailing veterans with GWI. The robust infrastructure of the BBRAIN repository network will serve as a key resource for the GWI research field.

6. Conclusion

BBRAIN, built from a strong foundation of collaboration and need for a biorepository in the community of GWI research, aspires to provide the scientific resources to identify more definitive biomarkers for GWI. The team approach of sharing samples will lead to faster identification of diagnostic tests for GWI and targeted personalized medicine treatments for ill veterans. For veterans who have remained ill for over 30 years, the importance of quickly identifying diagnostic tests and effective treatments for GWI cannot be overstated.

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Article

Neuroimaging Markers for Studying Gulf-War Illness: Single-Subject Level Analytical Method Based on Machine Learning

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Abstract: Gulf War illness (GWI) refers to the multitude of chronic health symptoms, spanning from fatigue, musculoskeletal pain, and neurological complaints to respiratory, gastrointestinal, and dermatologic symptoms experienced by about 250,000 GW veterans who served in the 1991 Gulf War (GW). Longitudinal studies showed that the severity of these symptoms often remain unchanged even years after the GW, and these veterans with GWI continue to have poorer general health and increased chronic medical conditions than their non-deployed counterparts. For better management and treatment of this condition, there is an urgent need for developing objective biomarkers that can help with simple and accurate diagnosis of GWI. In this study, we applied multiple neuroimaging techniques, including T1-weighted magnetic resonance imaging (T1W-MRI), diffusion tensor imaging (DTI), and novel neurite density imaging (NDI) to perform both a group-level statistical comparison and a single-subject level machine learning (ML) analysis to identify diagnostic imaging features of GWI. Our results supported NDI as the most sensitive in defining GWI characteristics. In particular, our classifier trained with white matter NDI features achieved an accuracy of 90% and F-score of 0.941 for classifying GWI cases from controls after the cross-validation. These results are consistent with our previous study which suggests that NDI measures are sensitive to the microstructural and macrostructural changes in the brain of veterans with GWI, which can be valuable for designing better diagnosis method and treatment efficacy studies.

Keywords: Gulf War illness; MRI; objective biomarker; machine learning; Kansas case criteria; diffusion; grey matter; neurite density imaging

1. Introduction

Gulf War illness (GWI) refers to the variety of chronic symptoms experienced by about 250,000 United States veterans who served in the 1991 Gulf War (GW) [1]. According to the Kansas

case criteria, symptoms of GWI fall into six categories: fatigue (fatigue and sleep problems), pain (joint and muscle), neurological (cognitive, mood, headache, and dizziness), respiratory (persistent cough and wheezing), gastrointestinal (diarrhea and nausea), and skin (rashes and other) problems. Exposure to neurotoxicant chemicals (organophosphate pesticides and sarin) during the war and other central nervous system (CNS) damage, such as mild traumatic brain injury (mTBI), are thought to have caused an innate immune over-response in the CNS, resulting in the development of these chronic GWI symptoms [2–7]). In order to meet the Kansas criteria for GWI, veterans must display chronic symptoms in at least three of the six categories, without presenting concurrent psychiatric and medical disorders [8]. However, accurate diagnoses of GWI remained challenging due to the heterogeneous clinical presentation of this condition, as well as the level of subjectivity associated with self-reported symptoms and neurotoxicant exposure history [8–10]. To improve management and treatment of GWI, there is an urgent need for defining sensitive and objective biomarkers of the disorder.

Previous neuroimaging studies demonstrated distinct changes within brains of veterans with GWI, which may underlie physiological symptoms. For example, T1W-MRI studies showed that GW veterans with exposure to the neurotoxicant chemical sarin exhibit reduced gray matter (GM) and white matter (WM) volumes, as well as reductions in hippocampal subfield volumes when compared to non-exposed veterans [11,12]. More recent studies using diffusion tensor imaging (DTI) have shown greater hippocampal mean diffusivity (MD) and increased axial diffusivity (AD) in the WM of sarin and cyclosarin exposed GW veterans, which are correlated to fatigue, pain, or hyperalgesia, and may serve as a potential biomarker for GWI [13–15]. We have previously applied a novel MRI diffusion processing method, neurite density imaging (NDI), on high-order diffusion MRI to demonstrate that the NDI measure can successfully identify and validate different levels of neurological abnormalities in veterans with GWI from the Boston Gulf War Illness Consortium cohort [16].

ML algorithms have been applied to study a wide range of neurological disorders, including Alzheimer's disease, Parkinson's disease, and traumatic brain injury [17,18]. These studies have reported promising results for identifying diagnostic biomarkers [19,20]. The ML approach has strengths on exploiting features from different domains (i.e., neuropsychological, genetic and neuroimaging) and providing further insights on the potential interactions between different markers for classifying illness [21]. For the current study, we aimed to expand our previous work (on NDI) to cross-compare different types of neuroimaging markers (T1W-MRI, DTI and NDI) to determine whether these measures are useful for single subject-level classification of GWI cases vs. controls. Specifically, we incorporated the machine learning (ML) framework to search out key imaging features valuable for defining GWI. Computerized models were then trained based on the selected features and tested for classifying veterans with GWI.

2. Methods

2.1. Participants

In this study, we included brain imaging data of 119 GW veterans from Boston University Gulf War Illness Consortium (GWIC) (Table 1). GWIC is a multi-site study designed to identify the etiology and potential biomarkers of GWI. The inclusion criterion was deployment to the GW between August 1990 and July 1991. The exclusion criteria included having a diagnosis of chronic medical illnesses that could otherwise account for the symptoms experienced by GW veterans, including autoimmune, CNS, or major psychiatric disorders that could affect the brain and immune functions (e.g., epilepsy, stroke, severe head injury, etc.). Each participant completed an assessment protocol of health surveys, a neuropsychological test battery, brain imaging, and collection of blood and saliva samples [2]. In this study, we utilized brain imaging outcomes to study GWI. All participants provided written informed consent to participate in the study. This study was reviewed and approved by the Boston University institutional review board.

Table 1. Subject Characteristics.

BU Subjects	GW Control	GW Case
N	21	98
Age (years)	54.06	52.46
Gender (F/M)	3/18	20/78

Gulf War Illness Criteria and Symptom Surveys

GW case status was defined from the Kansas GWI case definition, which requires multiple or moderate-to-severe chronic symptoms in at least three of six statistically defined symptom domains: fatigue/sleep problems, somatic pain, neurological cognitive/mood symptoms, gastrointestinal symptoms, respiratory symptoms, and skin abnormalities [8]. GWIC participants not meeting Kansas GWI or exclusionary criteria were considered controls. Veterans were excluded from being considered GWI cases, for purposes of the research study, if they reported being diagnosed by a physician with medical or psychiatric conditions that would account for their symptoms or interfere with their ability to report their symptoms. GWIC subjects were administered a general demographic information and medical conditions questionnaire and the Kansas Gulf War and health questionnaire for assessing symptoms [8,10]. Additional validated health symptom surveys were completed by study participants and included the multidimensional fatigue inventory (MFI-20), McGill pain inventory and the Pittsburgh sleep quality index (PSQI) where higher scores suggested worse conditions [22–24].

2.2. Image Acquisition

All veterans were scanned on an Achieva 3T whole-body MRI scanner (Philips Healthcare, Best, The Netherlands) at the Center of Biomedical Imaging, Boston University school of Medicine. T1W-MRI were obtained using an MPRAGE sequence developed by the Alzheimer's disease neuroimaging initiative (ADNI) (Repetition time (TR) = 6.8 ms, Echo time (TE) = 3.1 ms, flip angle = 9°, slice thickness = 1.2 mm, 170 slices, Field of view (FOV) = 250 mm, matrix = 256 × 256) (accessible from <http://adni.loni.usc.edu/>). Diffusion MRI data were obtained using 124 gradient directions utilizing parallel imaging on a 16-channel parallel head coil (70 slices, TR = 13,214 ms, TE = 55 ms, with a matrix size of 128 × 128 yielding a resolution of 2.0 × 2.0 × 2.0 mm³, no slice gap). Multi-shell diffusion encodings with b-values 1000, 2000 and 3000 s/mm² were acquired with a single-shot echo planar imaging (EPI) sequence, and 6 b = 0 s/mm² field maps were collected in addition to distortion corrections built into the scanner.

2.3. Image Processing and Anatomical Defining

Structural T1W-MRI scans were analyzed with the Freesurfer package (version 6.0) to generate anatomical regions of interest (ROI) for assessing GM morphometric measures, and to provide GM anatomical co-registration references for diffusion images [25]. A total of 78 ROIs defined in the average template space were co-registered to each subject's cortical surface by applying nonlinear co-registration parameters. All results were visually inspected for artifacts or incomplete segmentation. Fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), and radial diffusivity (RD) maps were created using tract-based spatial statistics (TBSS), part of FSL package that projects all subjects' diffusion tensor imaging (DTI) data onto a mean tract skeleton [26]. A total of 20 major WM tracts were defined using the Johns Hopkins University (JHU) white-matter tractography atlas provided in the FSL package, the same template was also used for special normalization and linear co-registration of diffusion MRIs [27,28].

2.4. High-Order Diffusion Processing

Microstructural diffusion measures were reconstructed from multi-shell diffusion MRI images containing 3 b-value encodings using the NDI model [16]. Two parameters, neurite density (ND)

index and orientation dispersion (OD) index were extracted from the NDI model. In brief, ND is a fraction of tissue composed of neurites which include axons and dendrites, and OD provides the spatial configuration of the neurite structures based on the composite pattern of intra- and extracellular diffusivity [29]. For WM NDI measures, all subjects' NDI data were registered to a common space based on nonlinear transformation and projected to the WM tract skeleton. Next the major WM tract ROIs were then applied to the skeletonized WM NDI maps to extract ROI-wise NDI measures [26]. For the GM diffusivity assessment, diffusion modeling parameters were determined by voxel wise iterative parameter selection method. We used the maximum likelihood estimation of model fitting error to define the optimal intrinsic free diffusivity parameters [30]. The optimal parameters were used to reconstruct the GM NDI maps and then merged into the 78 GM ROIs to extract ROI-wise NDI measures [30,31].

2.5. T1-Weighted MRI Measures

From the Freesurfer cortical reconstruction process of T1W-MRI, we extracted six measures per subject, including cortical thickness, cortical surface area, cortical volume (cVolume), subcortical GM volume (scVolume), WM volume, curvature (curv). Specifically, cortical thickness, surface area, volume, and curvature are extracted from 62 ROIs based on Desikan–Killiany–Tourville (DKT) atlas, while subcortical ROIs are defined by Freesurfer built-in atlas [31,32].

2.6. Statistical Analysis

From the data processing steps, we generated in total 14 types of imaging measures: 4 NDI, 4 DTI, and 6 T1-weighted morphometric measures. For each type of imaging measure, we conducted statistical comparisons of GWI cases vs. controls using linear regression models adjusting for age and sex, and then corrected for multiple comparison using false discovery rate (FDR) [33]. We reported *t*-values and FDR-corrected *p*-values (FDR-*p*), significant features are defined as FDR-*p* < 0.05.

2.7. Machine Learning Classification

Imaging measures described in the previous sections are used as pre-defined features for training ML classification models. Age- and sex-related confounds were removed from the raw data before training the model. This step is achieved by estimating the effects of age and sex on imaging measures using a linear regression model that is similar to a method applied in an early study [19]. For building the classifier for each imaging measure we adapted a reinforcement learning algorithm with artificial bee colony algorithm for feature selection (BSO: bee swarm optimization), and the K nearest neighbors (KNN) algorithm for classification training and performance evaluation [34,35].

2.7.1. Feature Space Selection and Classifier Training

As mentioned previously, some specific neuroimaging markers (i.e., NDI measures) may be more sensitive for detecting the subtle neurological changes occurring in GWI cases [16]. For training the classifiers, each type of imaging measures (i.e., measurement domains) serves as prior information that will allow us to set up specific feature space for potentially better ML outcomes. Within each feature space, reinforcement learning-based BSO (QBSO) was used to perform iterative search of the subset of features that provides the best classification performance on the training dataset (more details described in QBSO Tuning). Through QBSO, a final subset of features (final solution) was selected to build a final classifier. Final classifiers trained on each feature space were then tested on the validation dataset (see more details in Ensemble Approach).

QBSO Tuning

This feature selection concept combines the BSO and reinforcement learning (specifically Q-learning) to upgrade simple local search to a more adaptive and efficient search for the final

solution [34,35]. Previous study has shown that this hybrid method outperforms other well-known ML algorithms for feature selection [35]. More specifically, the BSO method mimics the foraging behavior of natural bees by performing iterative local search for an optimized solution [36].

From the predefined feature space explained earlier, the initial solution is randomly generated. Then, BSO randomly modifies the initial solution to multiple different secondary solutions, where each will be assigned to a bee (an agent) to perform local search to find local optimum (based on k-fold cross-validation accuracy). In this local search stage, each bee refers to a series of experiments obtained in previous steps to make a decision to do further search in the current search space, and this local search will continue until no further improvement of accuracy occurs. When the bee reaches this point, each bee's search history is shared to other bees and used for the diversification of searching process.

In the diversification process, the most distant solution will be selected based on the shared information. During this process, the role of reinforcement learning is to allow the agent learn through an interactive environment by trial and error. As the result, the QBSO method will search for a solution (i.e., resulting feature list) that maximizes the reward through multiple iterations. In each iteration, KNN runs on the candidate features (one of the secondary solutions) selected from the bee and tested for 5 iterations of 5-fold cross-validation on the training dataset. We used an average accuracy measure from the 5-fold cross-validation for estimating the reward. Finally, the search process will terminate based on the pre-defined parameters. To set up the optimal parameters, we used a grid-search strategy that is empirically searching the parameters resulting in the highest classification accuracy for the training dataset. The final parameters used in this experiment are listed as follows: flip: 20, max. chance: 9, nBees: 30.

Ensemble Approach

Per each feature space (i.e., one type of imaging measure), QBSO produces a subset of final features that provides the highest average accuracy from the iterative search. QBSO is repeated 5 times in total to generate 5 final solution candidates for a single training dataset. Per each solution, we built 3 different classifiers- KNN, support vector machine, and random forest classifiers. The training dataset was further split into 2 parts (i.e., training and testing) and used to train each classifier. Then the weighted majority voting was used to ensemble those 15 classifiers (i.e., 3 classifiers from each solution) to make a final prediction on the validation dataset. The following weight function was used: $W_i = P_i / (1 - P_i)$, P_i : performance of i -th classifier, $i = [1:15]$.

2.7.2. Comparing Classification with Different Imaging Measures

As mentioned previously, each type of imaging measures was used to set up distinct candidate feature space for training the classifiers. The resulting 14 different classifiers (4 NDI, 4 DTI, and 6 T1W-MRI morphometric measures) were evaluated based on their classification performances. For the benchmark testing, the entire dataset was initially divided into a training dataset and a validation dataset based on a 5-fold partitioning. We took one fold as a validation dataset and used the remaining 4-fold data for performing the QBSO training framework (Section 2.7.1). This process was repeated 5 times as training/validation datasets rotate among the 5 folds (by taking each fold as the validation dataset in each iteration). For the classification performance comparison, we reported performance measures (averaged from 5 iterations after validation) of accuracy, sensitivity, specificity, and F-score. We included F-score as a more representative performance measure for the imbalanced case and control groups [37]. In addition to the average accuracy, we included the standard deviation (SD) of accuracy, as an estimate of variations between iterations, and the highest accuracy value for the top three classifiers.

3. Results

3.1. Group-Level Statistical Comparison and Key Imaging Features

Statistical analysis of NDI measures showed significant differences between GWI cases and controls in both WM tracts and GM ROIs (FDR- $p < 0.05$) (Figure 1). The full result can be found in Table S1. All major WM tracts showed significant decreases in ND and OD for GWI cases compared to controls (Figure 1A). The greatest significant group differences between GWI cases and controls were seen in the bilateral corticospinal tract (CST, $t = -3.119$ FDR- $p = 0.017$ (left), $t = -3.129$, FDR- $p = 0.017$ (right)) and the bilateral anterior thalamic radiations (ATR, $t = -2.891$, FDR- $p = 0.017$ (left), $t = -2.808$, FDR- $p = 0.017$ (right)) for WM ND, and in the bilateral cingulum cingulate gyrus bundle (CCG, $t = -4.041$ FDR- $p = 0.002$ (left), $t = -3.384$, FDR- $p = 0.007$) for WM OD. Both ND and OD showed decreased patterns (FDR- $p < 0.05$) for most GM ROIs as well (Figure 1B). The greatest significant group differences between GWI cases and controls were seen in the left isthmus of cingulate gyrus ($t = -3.319$, FDR- $p = 0.036$) and the bilateral thalamus proper ($t = -3.168$, FDR- $p = 0.036$ (left), $t = -3.015$, FDR- $p = 0.036$) for GM ND, and in the bilateral caudal anterior cingulate gyrus ($t = -3.262$, FDR- $p = 0.016$ (left), $t = -3.182$, FDR- $p = 0.016$ (right)), the bilateral posterior cingulate gyrus ($t = -3.832$, FDR- $p = 0.016$ (left), $t = -2.461$, FDR- $p = 0.03$ (right)), the bilateral amygdala ($t = -3.593$, FDR- $p = 0.016$ (left), $t = -3.516$, FDR- $p = 0.016$ (right)) and the bilateral putamen ($t = -3.228$, FDR- $p = 0.016$ (left), $t = -3.134$, FDR- $p = 0.016$ (right)) for GM OD. The full list of statistically significant imaging features can be found in Table S1.

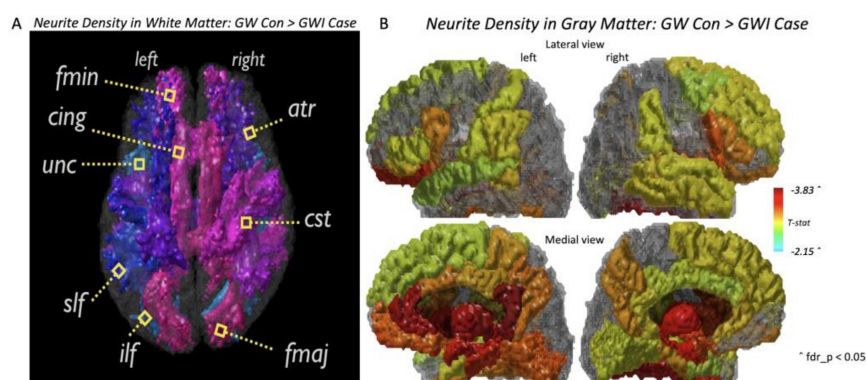


Figure 1. Gulf War illness (GWI) cases vs. Gulf War (GW) control group comparisons of gray matter (GM) and white matter (WM) neurite density imaging (NDI) measures and summary of significant regions. (A) 3D tract representation of significant WM ND differences between GWI case and control groups. (B) 3D region of interest (ROI) representation of significant GM ND differences between GWI case and control groups. Color bar corresponds to the magnitude of t-value, red indicates greater difference between groups, and vice versa. Fmaj = corpus callosum forceps major, Fmin = corpus callosum forceps minor, atr = anterior thalamic radiations, cst = corticospinal tract, cing = cingulum cingulate gyrus bundle, ilf = inferior longitudinal fasciculus, slf = superior longitudinal fasciculus, unc = uncinatus fasciculus.

3.2. Machine Learning Classification Performance

As shown in Figure 2 and Table 2, the best classifier for GWI cases vs. control we had is trained using the WM OD measures, which achieved F-score of 0.941, an accuracy of 90% (SD: 0.063, highest accuracy: 91.7%), sensitivity of 95%, and specificity of 65%. The specific features include the left CST, the corpus callosum forceps minor (fminor), the left inferior fronto-occipital fasciculus (IFOF), the left inferior longitudinal fasciculus (ILF), the left superior longitudinal fasciculus (SLF), and the left superior longitudinal fasciculus temporal (SLFT). All features were statistically significant based on group-level analysis (Figure 1A, Table S1). The second-best classifier is trained using the GM ND measures, which achieved F-score of 0.922, an accuracy of 86.7% (SD: 0.054, highest accuracy:

91.7%), sensitivity of 96%, and specificity of 40%. The specific features used by this GM ND classifier include both cortical and subcortical structures of the limbic system, including the bilateral caudal anterior cingulate gyri (Table 2). The third best classifier was trained using the WM ND measures, which achieved F-score of 0.914, an accuracy of 85% (SD: 0.048, highest accuracy: 91.7%), sensitivity of 96%, and specificity of 30%. For this classifier, the specific features included the bilateral anterior thalamic radiations (ATR), the bilateral IFOF, the bilateral ILF, the left SLF, the right SLFT and the Fminor (Table 2). The full list of imaging features used by the top three classifiers can be found in Table 2 and the full list of classifier performances can be found in Table S2.

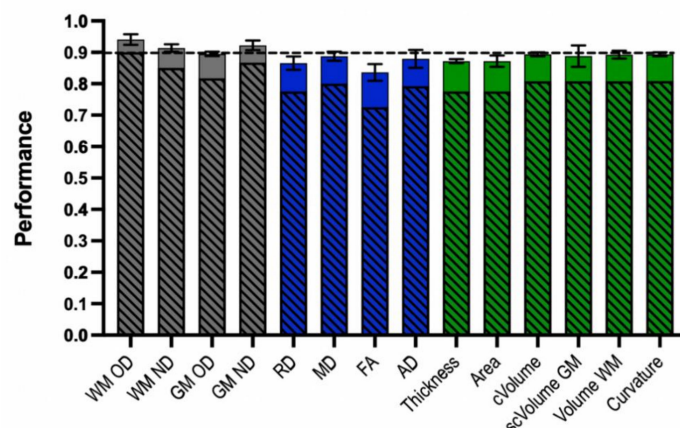


Figure 2. Classification performances of all classifiers. Each bar represents the performance (solid-colored bar: average F-score, shaded area: average accuracy) of each type of classifier trained on one imaging measure, data is presented as mean $\pm$ SEM after cross-validation. Grey-colored bars: NDI measure-based classifiers. Blue-colored bars: diffusion tensor imaging (DTI) measure-based classifiers. Green-colored bars: T1-weighted structural MRI (T1W-MRI) measure-based classifiers. WM OD = white matter orientation dispersion, WM ND = white matter neurite density, GM OD = grey matter orientation dispersion, GM ND = grey matter neurite density, RD = radial diffusivity, MD = mean diffusivity, FA = fractional anisotropy, AD = axial diffusivity, thickness = cortical thickness, area = cortical surface area, cVolume = cortical volume, scVolume = subcortical GM volume, volume WM = white matter volume.

Table 2. Summary of classification performances and feature characteristics.

Measure	ACC	SEN	SPE	F-Score	Key Features	
WM OD	90%	95%	65%	0.941	L CST ** L IFOF ** L ILF ** L SLF ** L SLFT ** Fminor **	
GM ND	86.7%	96%	40%	0.922	L caudal anterior cingulate * L cuneus L inferior temporal L paracentral * L posterior cingulate * L thalamus proper *	R caudal anterior cingulate R lingual R pars orbitalis R amygdala * R putamen *
WM ND	85%	96%	30%	0.914	L ATR * L IFOF * L ILF * L SLF * Fminor *	R ATR * R IFOF * R ILF * R SLFT *

ACC: accuracy, SEN: sensitivity, SPE: specificity, F-score: F1 score, WM OD: white matter orientation dispersion index, GM ND: gray matter neurite density index, WM ND: white matter neurite density index, L: left hemisphere, R: right hemisphere, CST: corticospinal tract, IFOF: inferior fronto-occipital fasciculus, ILF: inferior longitudinal fasciculus, SLF: superior longitudinal fasciculus, SLFT: superior longitudinal fasciculus temporal, Fminor: corpus callosum forceps minor, ATR: anterior thalamic radiation. *: FDR- $p < 0.05$ in group-level statistical comparison. **: FDR- $p < 0.01$ in group-level statistical comparison.

4. Discussion

In this study, we used various neuroimaging techniques (NDI, DTI, structural T1W-MRI) to identify important features that may help to differentiate between veterans with GWI and control veterans. These features were selected through two different analytical frameworks: (1) group-level statistical analysis, and (2) single subject-level ML classification models. From our group-level, univariate analysis, we identified important imaging features, especially from WM and GM NDI and T1W-MRI regional volumetric measures, which showed high contrasts between veterans with GWI and control veterans. From the multivariate classification results, we could additionally identify unique imaging features that are important for making single-subject level inferences regardless of its relevance to the group differences.

The results from the group-level statistical analysis showed that NDI measures are the most sensitive marker for detecting GWI pathology than other types of neuroimaging measures. For WM NDI measures, all major tracts showed significant decreases for veterans with GWI compared to control veterans (Figure 1A). The greatest significant group differences were seen in the bilateral CST for WM ND and bilateral CCG bundle for WM OD (Table S1). The roles of these tracts in many essential physical and neuropsychological functions have been well described by previous literatures. For instance, earlier studies showed that disruption of the CST WM integrity was associated with motor impairment that occurs in the early stages of many neurological conditions such as Huntington's Disease and Multiple Sclerosis [38,39]. Similarly, disruption of CCG has been associated with impaired executive functioning, pain, memory deficits, and has been a main target for conditions including major depression, schizophrenia, post-traumatic stress disorder (PTSD), and autism spectrum disorder [40]. Changes in these tracts captured by our WM NDI results may also be important to understand specific symptoms such as muscle pain, fatigue, and depression observed in GWI.

From the ML framework, we confirmed that WM OD, GM ND, and WM ND measures were the sources of the top three classifiers (based on average accuracy) (Figure 2, Table 2). The classifier trained using the WM OD measure showed the best performance and consistently reporting six features: the left CST, IFOF, ILF, SLF, SLFT, and the Fminor (Table 2). Due to the completely imbalanced distribution of the data used in this study, performance on classifying controls were more challenging in QBSO and this calls better ideas on handling this issue. For example, synthetic oversampling method such as the synthetic minority oversampling technique (SMOTE) may help addressing this issue [41]. Additionally, in this type of imbalanced sample, assessing the F1-score might serve as a more realistic measure of the classification performance [37]. Although we used average accuracy measure for comparing classifiers, WM OD showed a high F-score (0.941), showing that our proposed ML framework is providing reasonable performance at least in this sample. Compared to the NDI classifiers, the classifiers from DTI measures or T1W-MRI measures all had lower classification performance than NDI measures (Table S2). These results suggest that (1) NDI measures are important imaging markers for defining GWI, and (2) the features defined from ML framework provides distinct information from the group-level statistics on describing GWI. While several features from the group-level statistics may present with overlapping patterns to ML classifiers, there are also unique features reported by ML classifiers but not captured in the group-level analysis framework.

Both our findings on group-level statistics and single subject-level classification model demonstrated the importance of NDI measures for defining GWI. Moreover, considering the other ML methods tested on mild or preclinical stage illness, such as mild cognitive impairment staying with ~78% accuracy levels, the classification performance obtained from NDI QBSO is impressive and brings more attention into the complex diffusion imaging measures for studying preclinical stage or mildly progressive illness [42]. In the current study, we not only identified widespread statistically significant NDI features through group-level analysis, but also demonstrated that WM OD measures trained a better classifier compared to other imaging measures. This is consistent with our previous studies on NDI showing that this technique is sensitive to microstructural and macrostructural brain alterations and useful for detecting neurological abnormalities in GW veterans [16]. Our result also

corroborated with our previous findings that showed a higher sensitivity for the novel NDI measures compared to the common DTI measures (e.g., FA, MD, etc.). As we suggested before, this might be due to the higher specificity of NDI for detecting changes in different tissue components [16]. We previously found that there is a strong correlation between alterations in GM ND measure and worse self-reported fatigue and sleep symptoms, and with upregulated levels of proinflammatory cytokines TNFRI and TNFRII [16]. However, based on our current findings, GM ND measures provided slightly lower classification performance than WM OD and ND measures in this study. In addition, while classifier trained on WM OD resulted in nearly identical final solutions across five iterations of validation, GM measures resulted in more variabilities in the selected feature solutions. This might be due to the differences in dimensional size between WM and GM feature space. GM measures have more numbers of features (more complexity in the feature space) to be searched out during the QBSO process than WM measures, and thereby requiring more delicate optimization process especially in this not-a-large dataset problem. Although further investigations based on larger dataset is key to address the issue, this may also indicate that WM OD measures can be better markers for simply classifying veterans with GWI from control veterans, while GM ND can be a sensitive marker to specific symptom domains. Our results also support the diagnostic value of these NDI markers for clinical applications.

Altogether, these results suggest that the microstructural changes measured by NDI may be attributed to GM and WM deficits following chronic neuroinflammation. In line with this finding, other studies have shown that chronic neuroinflammation related to GWI symptoms may be a result of both morphological and functional changes that occurred in glial cells. For instance, a study using a rat model of GWI showed that exposure to the chemical agent, diisopropyl fluorophosphate (DFP: a sarin surrogate), was associated with fewer numbers of both mature and dividing oligodendrocytes in the prefrontal cortex, which in turn interrupted the neuron-glial interactions [43]. DFP injection also induced neuroinflammation and neurodegeneration in multiple brain regions, which is associated with impaired contextual fear learning in these rats [44]. Similarly, mice exposed to DFP demonstrated epigenetic changes to genes related to the immune and neuronal systems and altered proportions of myelinating oligodendrocytes in the frontal cortex, which led to disrupted synaptic connectivity and WM alterations in GWI [45]. A recent in-vivo positron emission tomography study corroborated these findings and reported elevated levels of translocator protein (TSPO), a protein upregulated in activated microglia and astrocytes, in veterans with GWI compared to control veterans [46]. This elevation pattern was observed in many areas including the precuneus, prefrontal, primary motor, and somatosensory cortices [46]. Considering this evidence, our current findings further support the importance of novel NDI measures for detecting microstructural changes in the brain following chronic neuroinflammation in GWI.

Besides NDI measures, some T1W-MRI measures also demonstrated good performances for classifying veterans with GWI vs. control veterans. Among classifiers trained using T1W-MRI measures, the cortical volume, subcortical volume, WM volume, and mean curvature models achieved 80.8% accuracy, and highlighted key features in the frontal and temporal regions (Table S2). The results on the group-level statistical analysis also showed reduced volumes of frontal regions among veterans with GWI (Table S1). GM atrophy has been well studied as a hallmark for various neuropsychological disorders. Previous studies showed that reduced total cortical and regional frontal lobe volumes are associated with poor subjective sleep quality and increased self-reported frequency of hearing chemical alarm among GW veterans [12,47].

For DTI measures, the best performance was demonstrated by the MD classifier with an accuracy of 80% and F-score of 0.887 (Table S2). There is evidence that DTI measures may correlate with GWI symptom severity. An early study on GWI veterans showed that fatigue, pain, and hyperalgesia are associated with increased AD in the right IFOF [15]. Another study showed that changes in frontal-limbic WM connectivity, as indicated by reduced MD and increased FA in the right cingulate bundle, was associated with higher PTSD symptom severity score among a sample of 20 GW veterans [48]. In addition, GW veterans who had been exposed to chemical agents have increased

AD throughout many regions of the brain including the temporal stem, cingulum bundle, IFOF, etc., compared to unexposed veterans [13]. Through our results, we found that while T1W-MRI and DTI measures are less significant based on group-level statistical analysis, a subset of the regional measures may still explain key components of GWI symptoms.

In this study, we showed that neuroimaging markers help to identify GWI. Nevertheless, we are expecting that the current approach can be improved in several aspects. One of the limitations of the current work is the imbalanced sample size, where the number of case subjects greatly exceeded the control subjects for building the classification model. This issue is reflected by the higher sensitivity and lower specificity for all the classifiers. To better handle this issue, we are planning to employ an oversampling method on the minority group to balance the samples. In our follow up work, we will also expand our analysis to a larger GW cohort including more control veterans recruited from other sites. Another important future direction is to test if the combination of multiple imaging measures, or combination of imaging and clinical measures (e.g., cognitive scores, inflammatory profiles, etc.) can improve the classification performance. This multivariate approach will be useful for identifying important features from large datasets. In conclusion, our current work provided the first evidence that novel NDI measures are not only useful for defining GWI based on the conventional group-level statistical comparisons, but also constitute key features for building single-subject level ML models for automated diagnostic classification. The features that are highlighted by our analysis suggest neurological changes underlying GWI pathology and support neuroinflammation as a potential target for therapeutic interventions.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2076-3425/10/11/884/s1>, Table S1: List of key imaging features based on group-level statistical comparison, Table S2: The classification performance for all classifiers.

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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

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 studies¹.

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

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) – (Wechsler, 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 (HVLTR)* – (Brandt and Benedict, 2001)
Brief Visual Memory Test (BVM)* – (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.

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.

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;

¹https://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 Reitan-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 Cancellation 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 ($d = -2.53$)

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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

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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 <i>d</i> 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

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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 BVM-T-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.

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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 than 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 BVM-T-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 GWs: 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 <i>after</i> 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 HVT 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 neurotoxins 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 (≥ 48) and those with low scores (≤ 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 adrenocorticotrophic 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.

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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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