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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. We have recruited 75 new study participants to date and have published six collaborative publications.					
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1. INTRODUCTION:

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. This has already resulted in five publications from our collaborating institutions.

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 3 is inclusive of Tasks 1-7 above. The statement of work for year 3 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 2, 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.

<u>Summary of Clinical Assessments and Tests Collected from 4 Clinical Study Sites</u>												
	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 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.

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 prepare for the clinical studies kick-off once all institutional approvals were obtained. A significant amount of time and effort was devoted to obtaining all required study materials and to developing centralized web-based data collection forms for the consortium studies. Smaller working group meetings were held during the past year to plan for the study and to deal with the recruitment stopping due to COVID-19. Table 3 lists these planning meetings 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 HRPO approval, and we are in the process of finalizing and cataloging samples to be shared with the consortium for analysis and catalog. To date, four requests have been approved for sharing of imaging data and CSF samples through our online request forms. The consortium website and sample/data request form are now online at the links below:

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

Table 3. BBRAIN Monthly Planning and EAB Meetings

Date	Type of Meeting	Discussion Items
11-18-2021	Monthly Web-meeting	Prospective and Retrospective site updates
12-16-2021	Monthly Web-meeting	Prospective and Retrospective site updates
01-27-2021	Monthly Web-meeting	Prospective and Retrospective site updates
03-10-2020	Monthly Web-meeting	Prospective and Retrospective site updates
04-28-2021	Monthly Web-meeting	Prospective and Retrospective site updates
05-26-2021	Monthly Web-meeting	Prospective and Retrospective site updates
06-30-2021	Monthly Web-meeting	Prospective and Retrospective site updates
07-28-2021	Monthly Web-meeting	Prospective and Retrospective site updates
08-25-2021	Monthly Web-meeting	Prospective and Retrospective site updates
09-28-2021	Monthly Web-meeting	Prospective and Retrospective site updates

Table 4. BBRAIN Network Working Groups

Working Group	Tasks	Members
Data Management Service Group	Assist with QC issues, data cleaning, data management and sharing, website management.	Joe Palmisano, DCC Consortium PI, co-PIs
Statistics Service Group	Perform analyses and provides statistical planning and advice for study investigators and research site PIs.	Timothy Heeren, Joe Palmisano, Consortium PI/co-PIs
Translational Working Group	Forum for Intellectual property and material (IP) issues, translation of results into papers, abstracts, new grant submissions and how clinical 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 Kregel, 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

Task 2a. Obtain MRI, MRS, DTI, PET Imaging data from retrospective resource (RR) sites and send to BUMC for cataloging, 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 and PET imaging scans were transferred electronically in either extended DICOM or par/rec format to the Center for Biomedical Imaging at Boston University School of Medicine. Data from the PET and MRI were combined from the Mass General datasets and recently published in Cheng et al., 2021 (see appendix).

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. 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 most study sites and the demographic, health symptom and cognitive outcome data have now been obtained from several RR sites. Data variables names, REDCap shells, and data dictionary were obtained from retrospective sites to create common variable names for specimens that are in the process of being catalogued for sharing.

GWIC demographic, health symptom and cognitive data from 269 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. Common data elements (CDEs) have been determined, and has now been published in Cohen et al., 2021 (see appendix).

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 (CSF). Collaborative CSF studies are currently underway.

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

- Expanded capabilities to provide partially differentiated neurospheres
- Expanded technical know how into organoid technology
- Plans to provide Dr. James Cai of Texas A&M with hiPSC lines if his proposal is funded by the DOD. If funded, this study will use single-cell RNA sequencing (scRNAseq) to map transcriptomic landscapes in neurons derived from induced pluripotent stem cells (iPSCs) of symptomatic and nonsymptomatic Gulf War Veterans. We will use machine learning (ML) approaches to infer single-cell gene regulatory networks (scGRNs) from scRNAseq data for neuronal samples to identify genes implicated in neuropathological development associated with GWI and gene expression programs underlying GWI predisposition. A better understanding of these mechanisms will shed new light on evidence-based strategies of clinical intervention.
- Drs. Baas and Qiang have submitted DOD proposals using the hiPSC lines that would involve collaborations with Dr. James O’Callaghan of the CDC and Dr. Ashok Shetty of Texas A&M.
- Additional requests from Drexel University investigators would also utilize hiPSC cells and brain tissue from veterans with GWI from the VA Brain, bank repository in collaboration with Dr. Christopher Brady.

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

Post-processing and cataloguing of images has begun for sites that have already shared their imaging data and will continue into the coming year. To date, Dr. Koo has collaborated with Dr. Loggia at Mass General Hospital and published on outcomes from the PET PBR28 ligand outcomes now combined with MRI microstructural integrity data (Cheng et al., 2021).

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 is being conducted on brain scans that are post-processed and added to the machine learning data analysis computational pipeline led by Dr Koo. This has resulted in one publication to date (Guan et al., 2020).

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

As previously described, monthly web meeting and working group meetings were ongoing during the past year to track progress on the planned clinical studies. In addition, extensive training was conducted with any new site research

assistants as the cognitive battery and other parts of the protocol were updated due to COVID-19 safety issues.

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 will contain 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. All project-specific applications (e.g., tracking, data entry and upload) is 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 that BEDAC staff have finalized the study participant telephone recruitment screening tool and constructed an electronic participant screening tool using REDCap software. BEDAC staff also finalized data collection forms for study personnel use and built an electronic data capture system using REDCap web-based software. A customized participant tracking and appointment log system was also constructed with separate secure login for each study site. BEDAC also worked with consortium research investigators to design a customized bio-specimen tracking and inventory system stored in REDCap and LDMS, and conducted in-person trainings with all clinical research staff for web-based screening, tracking, and data collection tools. The cleaned data will be converted into analytic datasets for the centralized statistical programming and data analysis. The sample request electronic form and approval process has also now been created and is shown below:

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. The BBRAIN protocol has also now been published by Keating et al., 2021 (see appendix).

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. All neuropsychological testing materials and survey instruments are routinely inventoried and restocked for the four clinical 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 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, RECRUIT AND ASSESSMENT OF GW VETERANS FROM FOUR RESOURCE SITES (MONTHS 9 - 36)

After a hiatus due to COVID-19, we resumed participant enrollment in February 2021. Our prospective study site at the Miami VA was first to receive approval to resume in February. In March, the Bronx VA and San Francisco VA received approval to resume their study

visits. Shortly following, Boston University lifted their COVID-19 restrictions in April allowing for participants from the state of Massachusetts. Since resuming enrollment 75 participants have completed the study protocol. At the Boston site, geographical restrictions have lifted and the number of study visits per week have increased. This will continue until we reach our target enrollment.

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, 236 participants have been screened. Of the 236, 161 are eligible (138 GWI Cases and 23 healthy controls) and 75 have been completed to date (58 cases, 12 controls). Please see recruitment data tables below.

BBRAIN: Screening and Enrollment Summary
October 18, 2021

	Total	Boston	Bronx	Miami	San Francisco
Number of Subjects Contacted	257	122	18	32	85
Number of Subjects Screened	246 (95.7%)	118 (96.7%)	18 (100.0%)	32 (100.0%)	78 (91.8%)
Number of Subjects Eligible	170 (69.1%)	95 (80.5%)	14 (77.8%)	22 (68.8%)	39 (50.0%)
Number of Subjects with Appointments Made	86 (50.6%)	47 (49.5%)	8 (57.1%)	16 (72.7%)	15 (38.5%)
Number of Subjects Assessed	75 (87.2%)	36 (76.6%)	8 (100.0%)	16 (100.0%)	15 (100.0%)

	Total		Boston		Bronx		Miami		San Francisco	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Number of Subjects Screened	213	29	103	14	15	2	29	1	66	12
Number of Subjects Eligible	146	24	82	13	12	2	21	1	31	8
Number of Subjects Assessed	63	12	30	6	6	2	15	1	12	3

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

To date, we have received informed consent from 75 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.

Seventy-five 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 reduce COVID-19 risks, we have 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. 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 list the updated complete survey and cognitive data that will be collected at the prospective sites. We have added a survey to collect COVID-19 vaccination status data. To date, 75 participants have completed the study protocol.

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 will 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 120 samples total and are continuing 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 will maintain the tracking database for the Fitbit sleep, heart rate variability and blood pressure data collection and transfer. The database will reside 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 will be automatically backed up on a nightly basis. Fitbits capable of collecting heart rate variability and sleep metrics have been purchased and shared with the recruitment sites to give to participants. The BEDAC team has created a secure website for participants to upload their Fitbit data to promote higher compliance with obtaining Fitbit data. This has been working well to date.

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

Neuropsychological protocols have been collected for 75 study participants to date and study staff at all sites have been trained for all test administration and standardized scoring procedures at the Boston Administrative Core now that slight modifications have been made to increase the safety of the cognitive assessments. As study protocols are obtained and data is collected, quality control procedures will 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.

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

Seventy-five blood and saliva samples have been sent to NOVA Southeastern University

for this study. The analysis of cytokine, chemokine and cortisol measurements will include testing for neuroendocrine and immune alterations and for hypothalamic pituitary adrenal axis abnormalities. Specifically, blood samples will be sent to NOVA Southeastern University for analysis of proinflammatory cytokine and chemokines and RNA analysis. Multiplex Quansys ELISA system will be used with an existing cytokine platform created by Dr. Klimas' research laboratory. Dr. Klimas will measure 16 cytokines in plasma. Saliva samples will be analyzed for DNA and cortisol measurements. See Keating et al., 2021 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 resumed subject recruitment in February and have completed 75 subjects to date. Samples from these 75 subjects were sent to the NOVA (NSU) and BUMC laboratories for processing and storage and study protocols would 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 worked closely with Dr. Sullivan on the logistics of this project in weekly meetings to initiate the project and ensure the best approach. Reagents needed, such as blood collection tubes, saliva kits, and shipping kits were 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 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. See Keating et al., 2021 for further details.

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 75 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 75 participants and will continue this protocol for the next 430 participants.

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

Enough samples have been collected to extract DNA and RNA from saliva samples in batches. These analyses are being prepared to be performed at the NOVA labs.

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

Enough samples have been collected to run batched assays for cortisol and cytokine results. These analyses are being prepared to be run at the NOVA labs.

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

We have gotten four requests for samples/data to date that have been sent to our steering committee for approval. These four requests have been approved and we have shared brain imaging data and will soon share CSF samples with Boston University, Roskamp investigators and Baylor Medical College investigators.

Task 6f. Ship samples to requesting investigators

We have shared brain imaging data and have shipped CSF and blood samples to the requesting investigators who have been approved to date through the online BBRAIN sample request form.

Major Task 7: Merge Data and Perform interim Data Analyses (Months 6-24)

Data merging has been performed and cleaned and made ready for interim data analyses.

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

Retrospective data sets are currently being merged for neuroimaging, cognitive, demographic and health symptom outcomes by the BEDAC data management team and interim data analysis will be performed shortly on these outcomes.

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 compiled from the prior clinical datasets that have been shared and 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). The additional CDEs have also now been published in Cohen et al., 2021 (see appendix).

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

Study results and preparation of abstracts for meeting presentations are discussed during monthly web meetings and will continue during the upcoming year.

7d. Annual reports of progress will be written

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

OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT

- o **Research Staff Training:** personnel who will be using the web systems for either direct data capture, participant tracking, or viewing reports will be trained to ensure uniformity of procedures, 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 including Behavioral Neuroscience and Environmental Health have also been 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.
- o **Participation in conferences, workshops, and seminars:** BBRAIN researchers will attend conferences, workshops and seminars to present research findings.
 - o On October 3rd 2019, Drs. Sullivan and Klimas presented a talk titled, ‘Military Veteran Biorepositories’ where they explained available biorepositories for military research and discussed plans for BBRAIN sharing of samples for military veteran studies as part of the National Academy of Sciences (NAS) workshop on Gulf War Respiratory Health Workshop on Military Burn Pits in Washington, DC. This Committee will determine next steps for burn pit studies for Iraq war veterans and for GW veterans with respiratory problems. The NAS report has now been published and included GWIC and BBRAIN studies in their report.
 - o On October 11-13th 2019, Drs. Sullivan and Klimas were invited keynote speakers at the American Academy of Environmental Medicine (AAEM) annual meeting in Louisville, KY. Dr. Sullivan’s talk was titled ‘Neuropathology and Toxicology of Gulf War Illness and Fatigue-related Disorders’ and Dr. Klimas’ presentation was titled, ‘Can We "Reboot" Human Homeostasis to Cure Chronic Illness? What We Are Learning from Gulf War Illness and ME.’
 - o On February 28th, 2020, Drs. Sullivan and Klimas were invited keynote speakers and presented a talk title: “Moving Knowledge to Treatment” at the State of the Science: Gulf War Illness conference in Ft. Lauderdale, FL.
 - o On August 18, 2020, Dr. Sullivan was invited to present the BBRAIN network at the GWI State of the Science 30th Anniversary of the Gulf War, Operation Desert Shield virtual meeting that was co-led by CDMRP and VA.
- o **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. Another BBRAIN story is planned for Veterans Day 2021.

- o **Plan to do during the next reporting period to accomplish the goals:** In the next reporting period, we plan to:
 - o Continue to obtain Biorepository data for analysis and catalogue available biospecimens for sharing from Repository Resource Sites.
 - o Continue to obtain imaging data from retrospective sites and perform post-processing of and machine learning analysis with combined common data elements.
 - o Continue recruitment at the four prospective sites now that sites have gotten approval to resume during the COVID pandemic. We will continue to process, store and manage samples for a total of 430 new participants. We also plan to merge data and perform interim data analyses.
 - o We believe that our media stories and working with new web advertising companies will catch us up on subject recruitment by the end of year 4 barring further delays from the COVID pandemic.

4. IMPACT

- o **Impact on the development of the principal discipline(s) of the project:**
 - *"Nothing to Report."*
- o **Impact on other disciplines:**
 - o 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.
- o **Impact on technology transfer:**

- o 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.
- o Drs. Baas and Qiang have been actively collaborating and sharing hiPSC samples and technical skills
- o Dr. Brady at the VA GW Brain bank and biorepository will now also share brain tissue within the GWI research community.
- o Dr. Koo is actively creating machine learning outcomes for objective diagnostic markers of GWI based on brain imaging outcomes that will be made publically available in the near future and could be updated for other similar disorders.
- o **Impact on society beyond science and technology:**
 - o 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:

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

Nothing to Report

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

Nothing to Report

Changes that had a significant impact on expenditures

We obtained a 1 year for cost extension to complete the subject recruitment at the four study sites. We believe this will allow to complete our project goals for recruitment of samples and data for the repository.

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

Nothing to Report

6. PRODUCTS:

- o **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 in addition to neuropsychological CDE recommendations. We have also published two publications on PET and MRI imaging outcomes.

- 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>
- 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. Logging, 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.
- o 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>
- o Chia-Hsin Cheng, Zeynab Alshelh, Yi Guan, Kimberly Sullivan, Marco L. Loggia, Bang-Bon Koo. Association of the tissue microstructural diffusivity and translocator protein PET in Gulf War Illness. *Brain, Behavior, & Immunity - Health* 18 (2021) 100364. Epub: link: <https://doi.org/10.1016/j.bbih.2021.100364>
- o 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; PMCID: PMC7699718.
- o 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 Sep 27. doi: 10.1007/s00018-021-03942-3.*

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

Nothing to Report.

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

Presentations:

- o K. Sullivan. Military Biorepositories. National Academy of Science (NAS) Workshop on Gulf War Respiratory Health Committee (Invited Speaker), Washington, DC, October 3, 2019.
- o K. Sullivan. Military Occupational Health and Toxicology. American Academy of Environmental Medicine annual meeting. (Invited keynote speaker), Louisville, KY, October 12, 2019.

- o 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.
- o 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.
- o **Website(s) or other Internet site(s)**
 - <http://sites.bu.edu/bbrain/>
 - https://wwwapp.bumc.bu.edu/BEDAC_BBrainRetro
- o **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.
- o **Inventions, patent applications, and/or licenses**

Nothing to Report.
- o **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- o **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	Project Role	<i>Co-Investigator, PI of Miami Site</i>

Klimas	Researcher Identifier (e.g. ORCID ID):	https://orcid.org/0000-0003-1459-3268
	Nearest person month worked:	12
	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:	12
	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:	12
	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>

- o **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.

- o **What other organizations were involved as partners?**

- o **Organization Name:** Harvard University and New England School of Acupuncture
- o **Location of Organization:** Boston, Massachusetts
- o **Partner's contribution to the project (identify one or more)**
 - o **Financial support:** *Nothing to Report*
 - o **In-kind support:** *Nothing to Report*
 - o **Facilities:** *Nothing to Report*

- o **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.
- o **Personnel exchanges:** *Nothing to Report*
- o **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. Kregel 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-Ghezala, 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 GWI. 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

o COLLABORATIVE AWARDS:

Nothing to report

o QUAD CHARTS:

9. APPENDICES:

Appendix:

Please see attached publication:

- 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>
- Chia-Hsin Cheng, Zeynab Alshelh, Yi Guan, Kimberly Sullivan, Marco L. Loggia, Bang-Bon Koo. Association of the tissue microstructural diffusivity and translocator protein PET in Gulf War Illness. *Brain, Behavior, & Immunity - Health* 18 (2021) 100364. Epub: link: <https://doi.org/10.1016/j.bbih.2021.100364>
- 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.
- 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; PMCID: PMC7699718.
- 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 Sep 27. doi: 10.1007/s00018-021-03942-3.*



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.

2.2.2.3. 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.

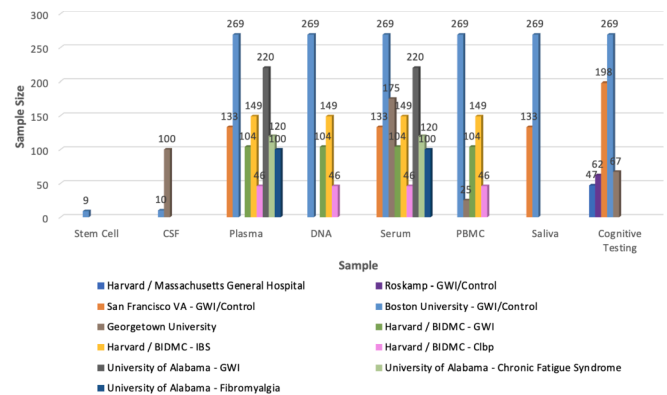


Fig. 2. Retrospective BBRAIN Samples and Data Repository.

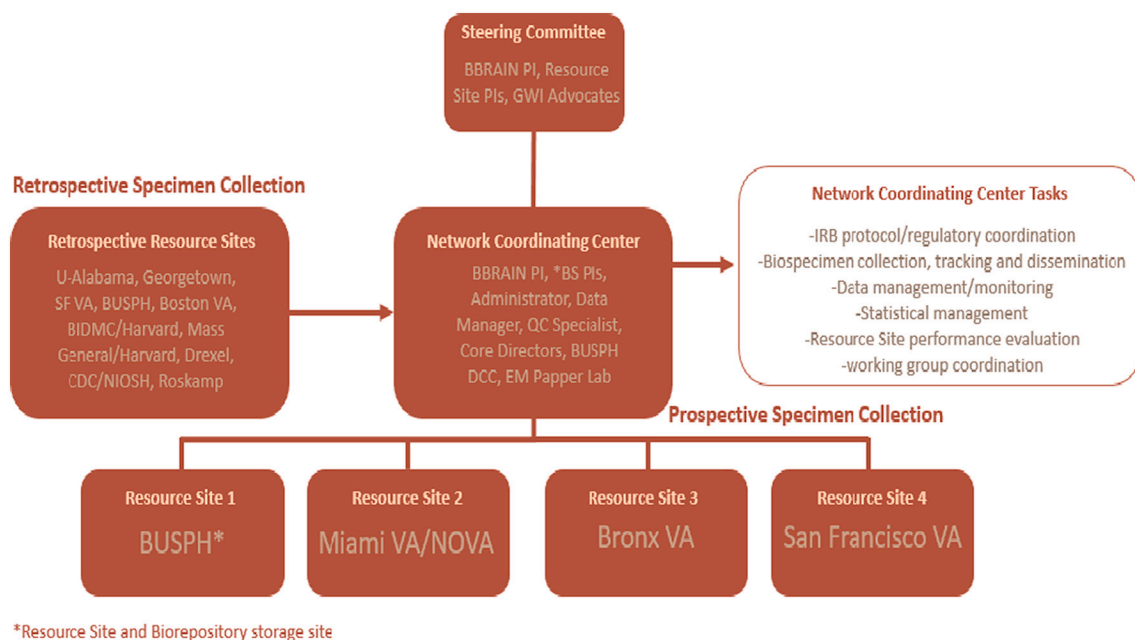


Fig. 1. BBRAIN organizational structure.

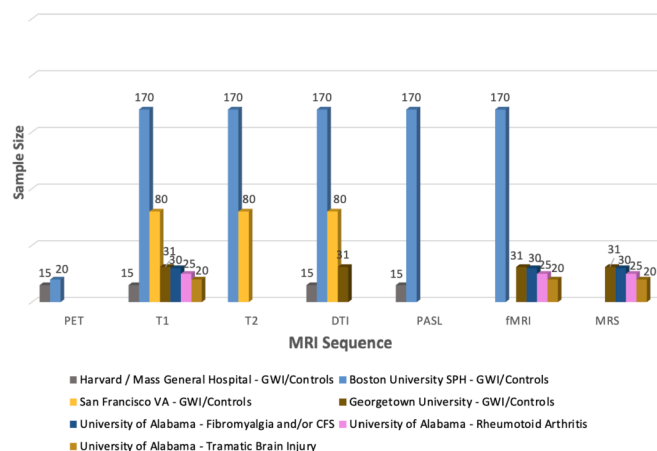


Fig. 3. BBRAIN MRI and PET Imaging Repository.

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 over-sampling 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,

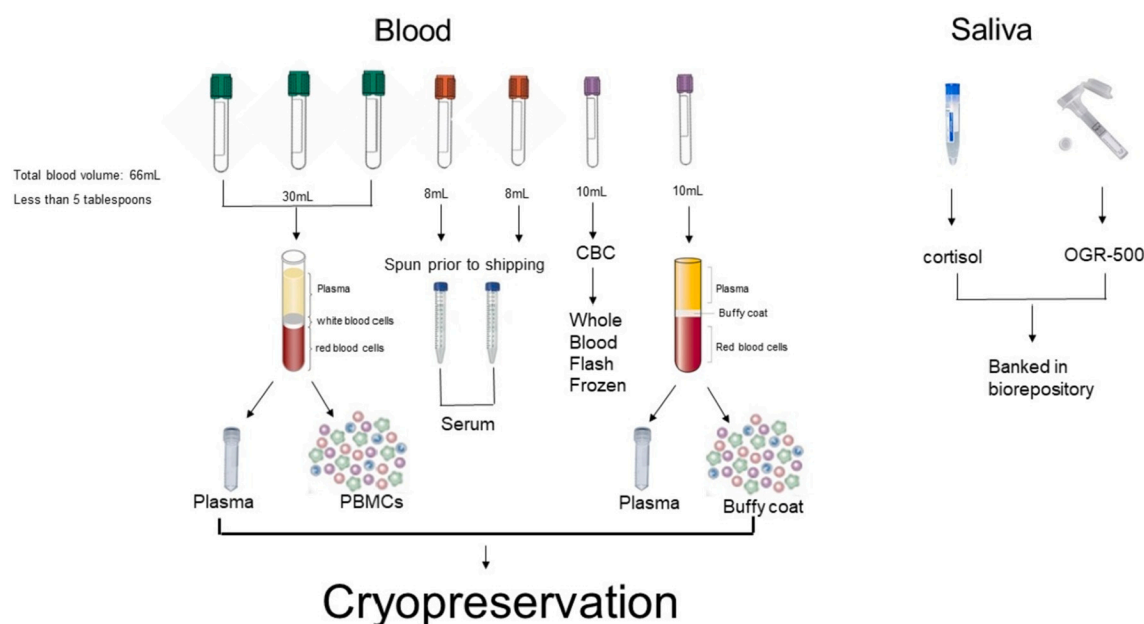


Fig. 4. Blood and saliva aliquot scheme.

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.

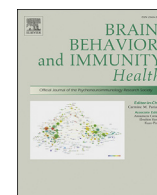
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- [59] Chia-Hsin Cheng Zeynab Alshelhi Yi Guan Kimberly A Sullivan L Marco B-BK Loggia Association of the tissue microstructural diffusivity and translocator protein PET in Gulf War Illness Brain Behav. Immun. Submitted.



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 neurotoxicants, 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

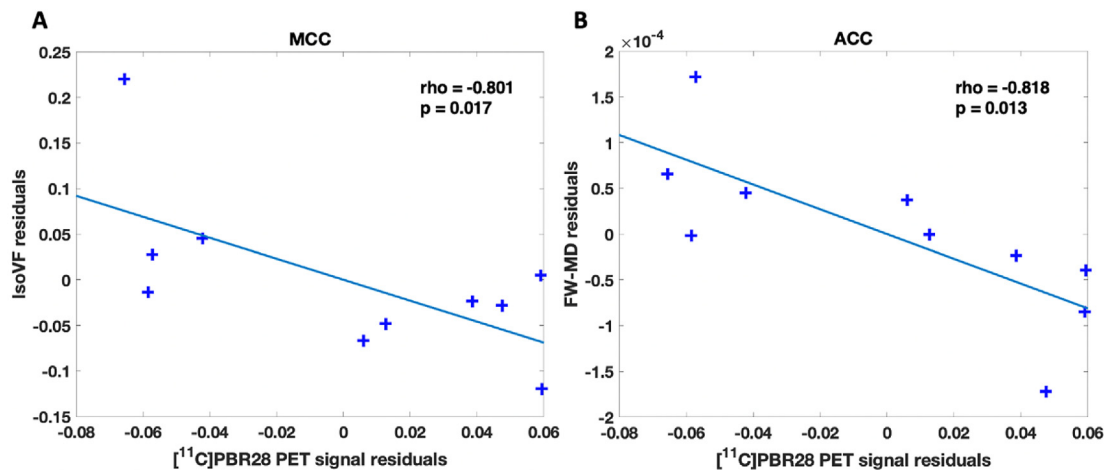


Fig. 1. Multi-compartment diffusion measures correlated with [^{11}C]PBR28 PET signals in the ACC and MCC in GWI veterans. Scatter plots illustrate the negative correlations between the neuroinflammation marker TSPO ([^{11}C]PBR28 PET signal) and (A) the isotropic diffusion measure (IsoVF) in the MCC, 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					-0.674
SF-MPQ2: Sensory	-0.369	-0.604	-0.111	0.765*	-0.532
Sum					
Conner's CPT3:	-0.345	0.593	0.723*	-0.755*	0.751*
Detectability					
Conner's CPT3:	-0.040	0.647	0.797*	-0.780*	0.722*
Omissions					
Conner's CPT3:	-0.371	0.525	0.501	-0.656	0.716*
Commissions					
Conner's CPT3: Hit	0.272	-0.502	-0.198	0.466	-0.707*
Reaction Time					
(HRT)					
Conner's CPT3:	-0.551	0.423	0.711*	-0.455	0.510
Variability					
Conner's CPT3: HRT	0.720*	0.202	-0.327	-0.191	0.174
Block Change					
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 neuroinflammatory 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; Zurcher 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 neuroinflammation in GWI.

Declaration of competing interest

None.

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Journal Pre-proof

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

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A Common Language for Gulf War Illness (GWI) Research Studies: GWI Common Data Elements

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Keywords: Gulf War Illness; Common Data Element, GWI, VA CDMRP, DoD, Veterans

Abbreviations:

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

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.

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

Keywords GWI, CDE, GW, GWV, CDMRP, VA, DoD

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 veteran.[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.

Materials and Methods

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.

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

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.

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 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).

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

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 Injury and Illness 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.

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] Physical Component Summary (PCS) Score Mental Component Summary (MCS) Score	Core

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

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 CDRMP website. GWI researchers have a choice of valid, reliable tools that will assist in comparability of variables across studies and will facilitate meta-data analyses. A 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.

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	6
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

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).

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.

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.

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, assess the level of severity, and 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.

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.

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.

Limitations and Future Directions: The approach taken for choosing common data elements for GWI was those 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 why the GWI Common Data Elements platform is a dynamic resource that will evolve based on the results of evidence-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 Veterans Affairs Research and Development, and 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.

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.

Declaration of competing interest

The authors declare no financial or other conflicts of interests.

Author Contributions

DC, NK, KS, and RM contributed to the conception of the article. DC wrote the initial manuscript. All authors contributed to the manuscript revision, read, and approved the submitted version. We also acknowledge Beth Gilbert for formal review.

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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.

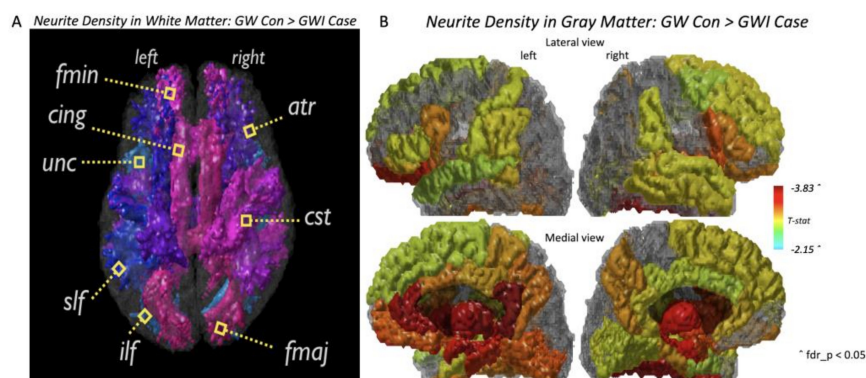


Figure 1. Gulf War illness (GWI) cases vs. Gulf War (GW) control group comparisons of gray matter (GM) and white matter (WM) neurite density imaging (NDI) measures and summary of significant regions. (A) 3D tract representation of significant WM ND differences between GWI case and control groups. (B) 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 = uncinate 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.

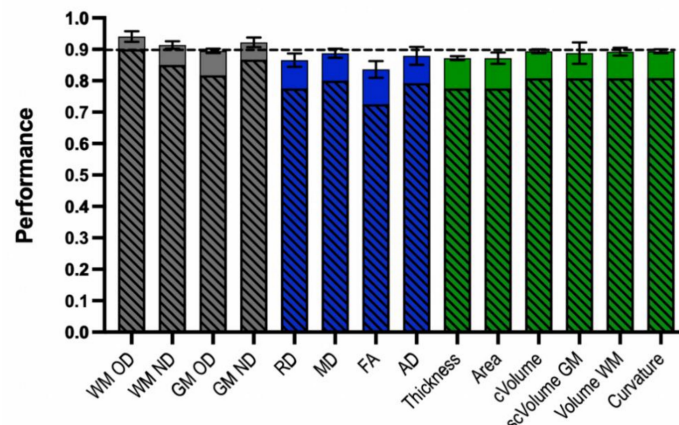


Figure 2. Classification performances of all classifiers. Each bar represents the performance (solid-colored bar: average F-score, shaded area: average accuracy) of each type of classifier trained on one imaging measure, data is presented as mean $\pm$ SEM after cross-validation. Grey-colored bars: NDI 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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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

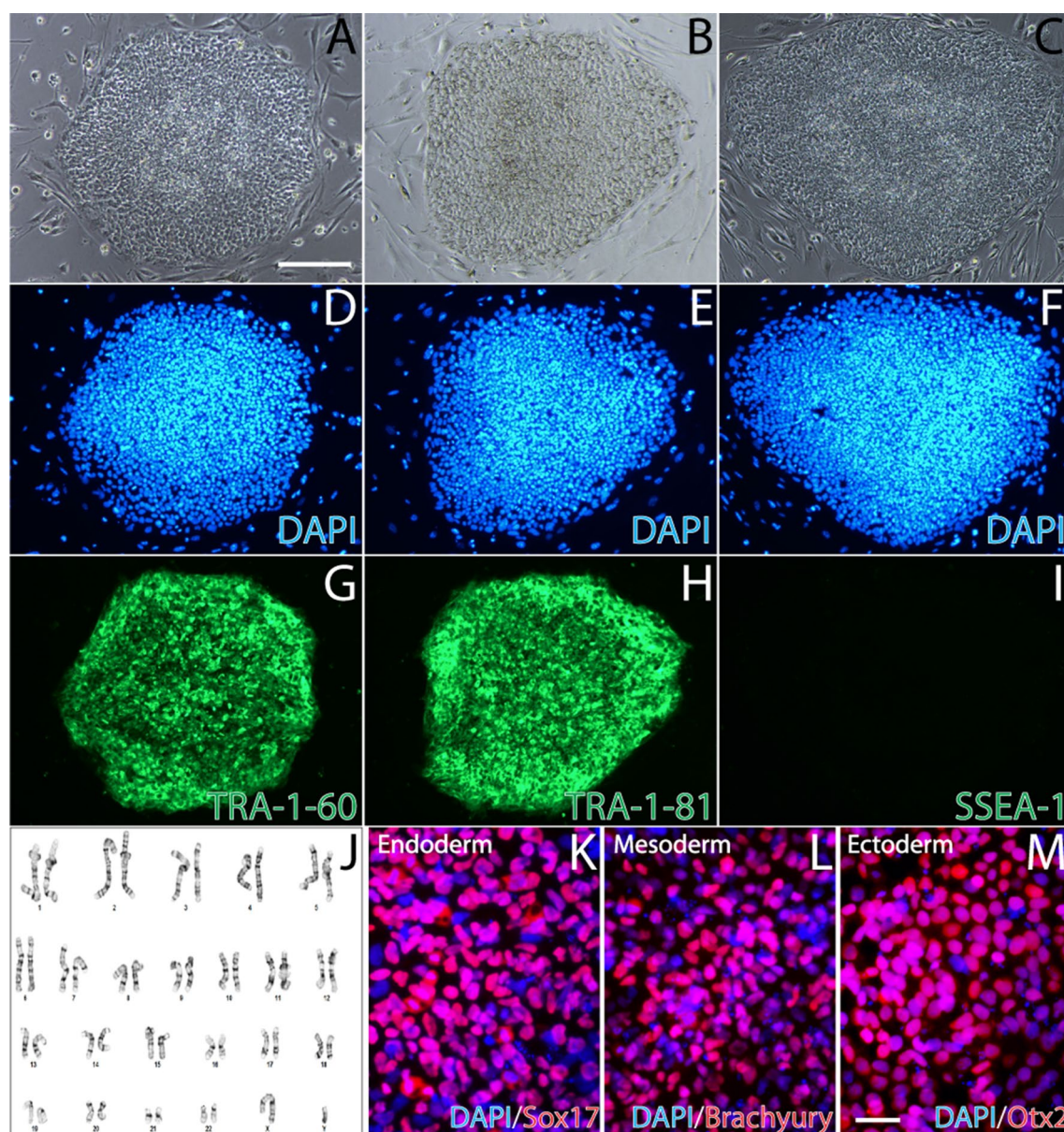


Fig. 1 Pluripotency validation of GW veteran-derived hiPSCs. **a–c** Phase-contrast images of the hiPSC colonies. Scale bar, 100 μ m. **d–f** DAPI staining. **g–i** Immunostaining for the pluripotent stem cell surface markers TRA-1-60 and TRA-1-81, and negative staining for the

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

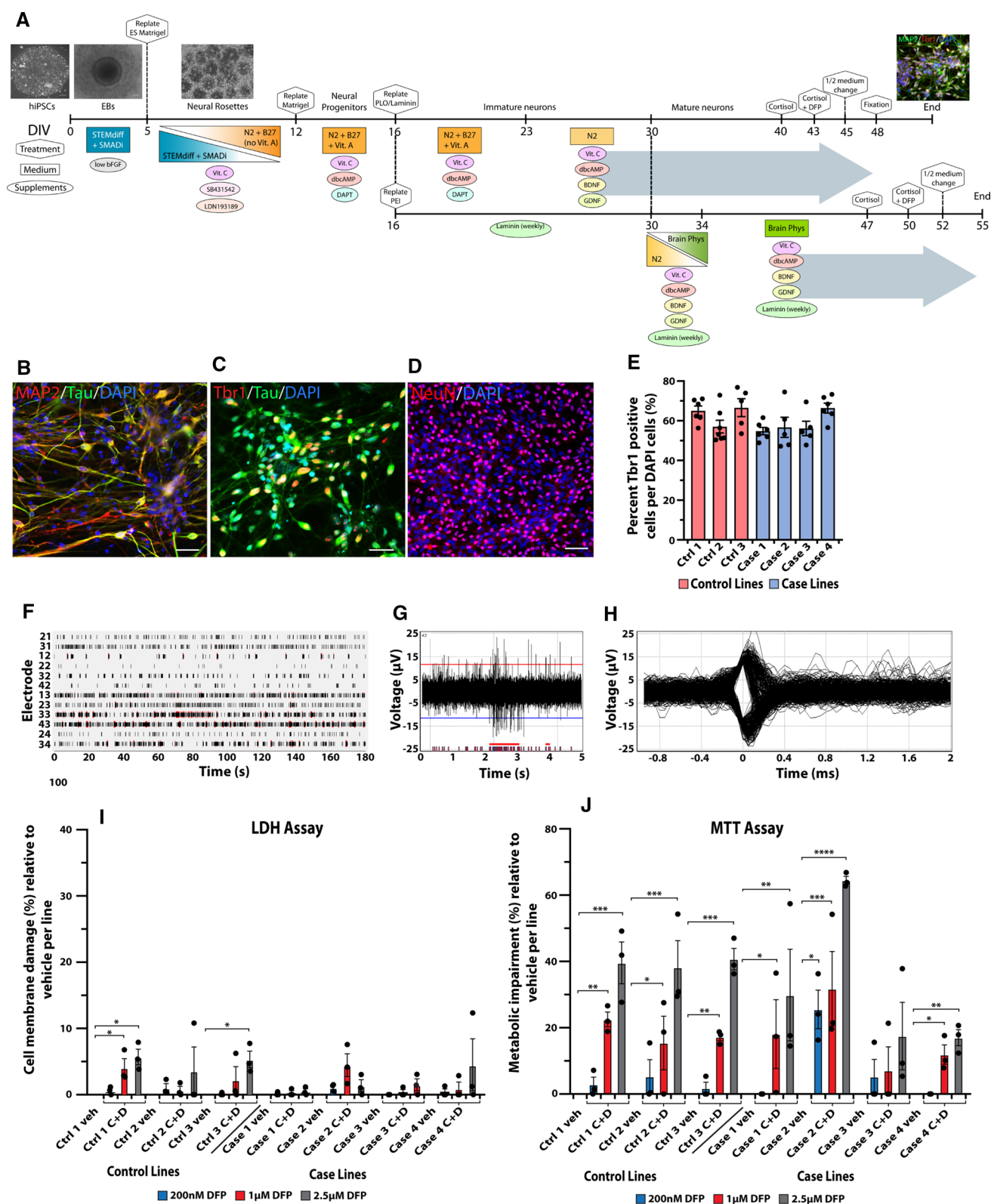


Fig. 2 Validation of forebrain glutamatergic neuronal differentiation from veteran-derived hiPSCs and GW toxicant regimen. **a** Schematic of the neuronal differentiation protocol with phase contrast and immunocytochemical images relevant to each stage. The large gray arrows represent the culture conditions 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

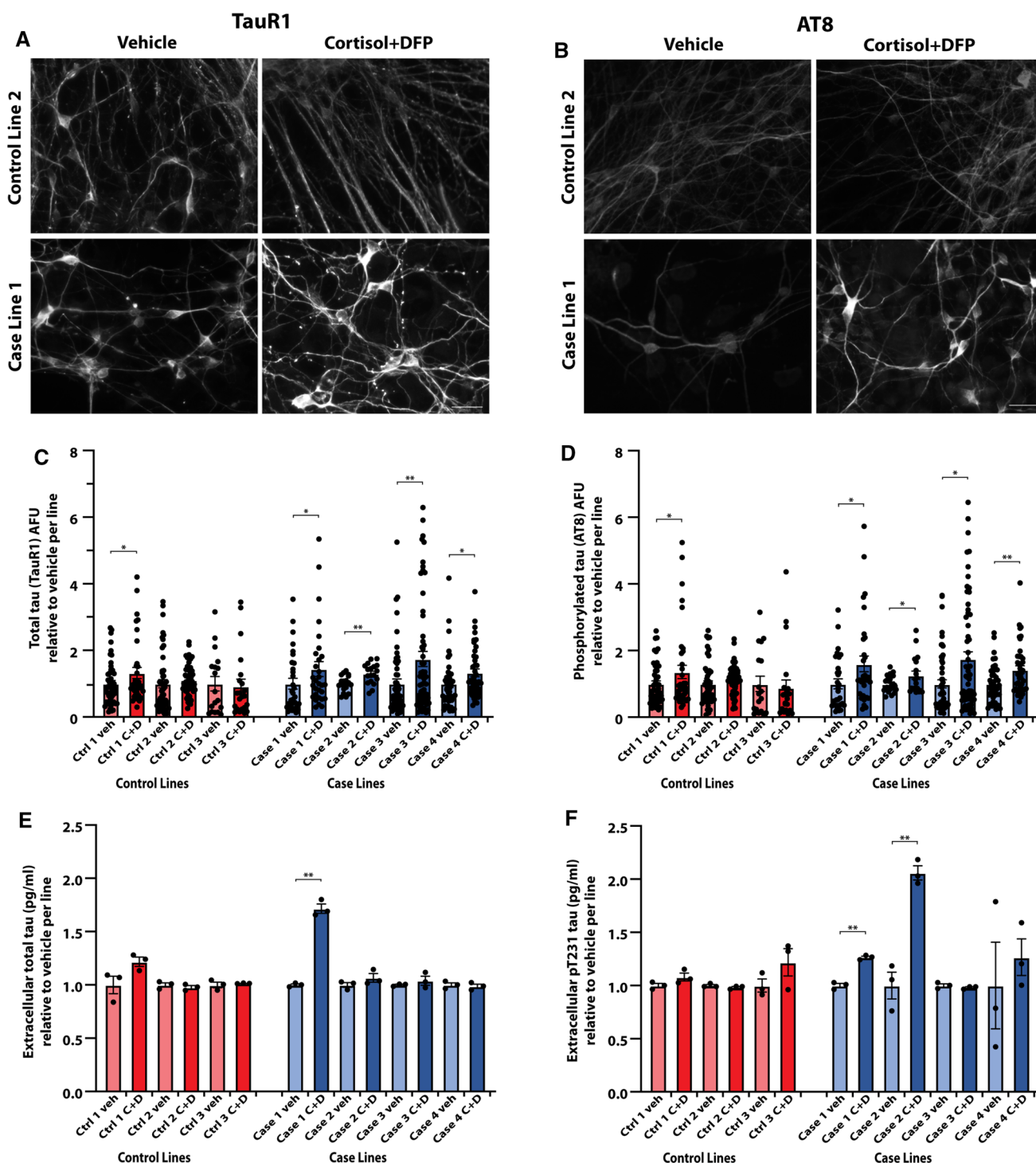
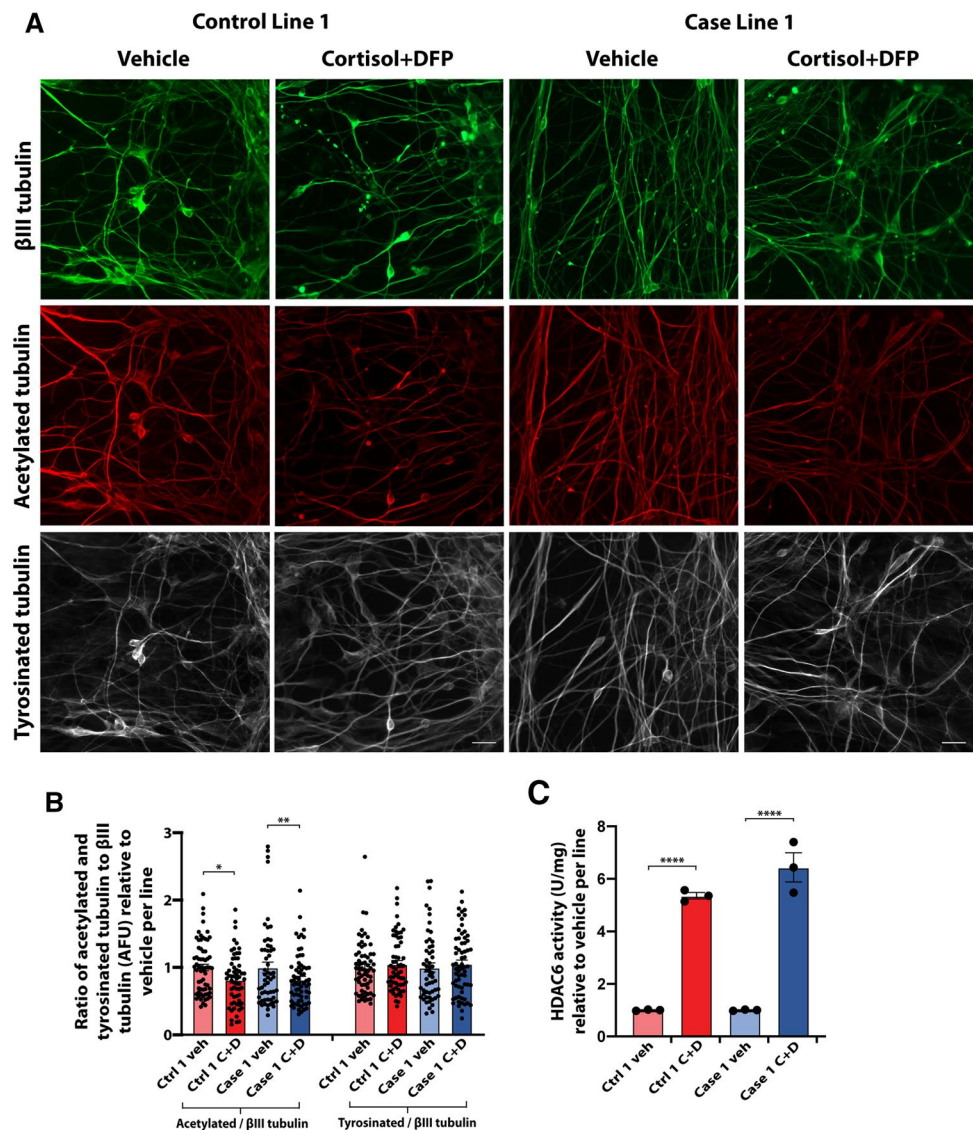


Fig. 3 hiPSC-derived glutamatergic neurons from veterans with GWI are more susceptible to develop tau pathology after toxicant exposure. **a, b** Representative images of hiPSC-derived glutamatergic neurons from one control line and one case line exposed to 2 μ M cortisol plus 200 nM DFP, and immunostained 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

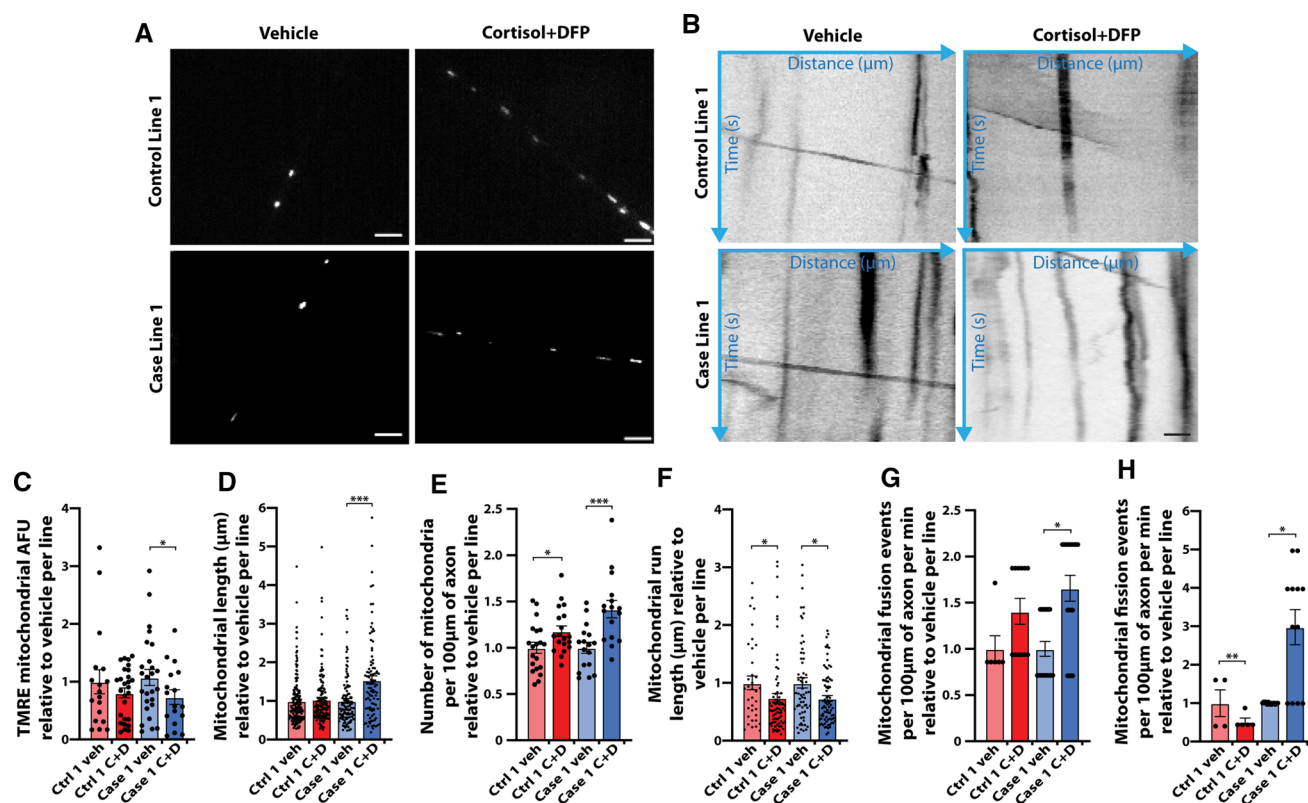


Fig. 5 Altered mitochondrial health, dynamics, and transport in hiPSC-derived glutamatergic neurons exposed to GW toxicants. hiPSC-derived glutamatergic neurons from one control line and one case line exposed to vehicle (veh) or 2 μ M cortisol plus 200 nM DFP (C+D) were incubated with the cell permeable 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].

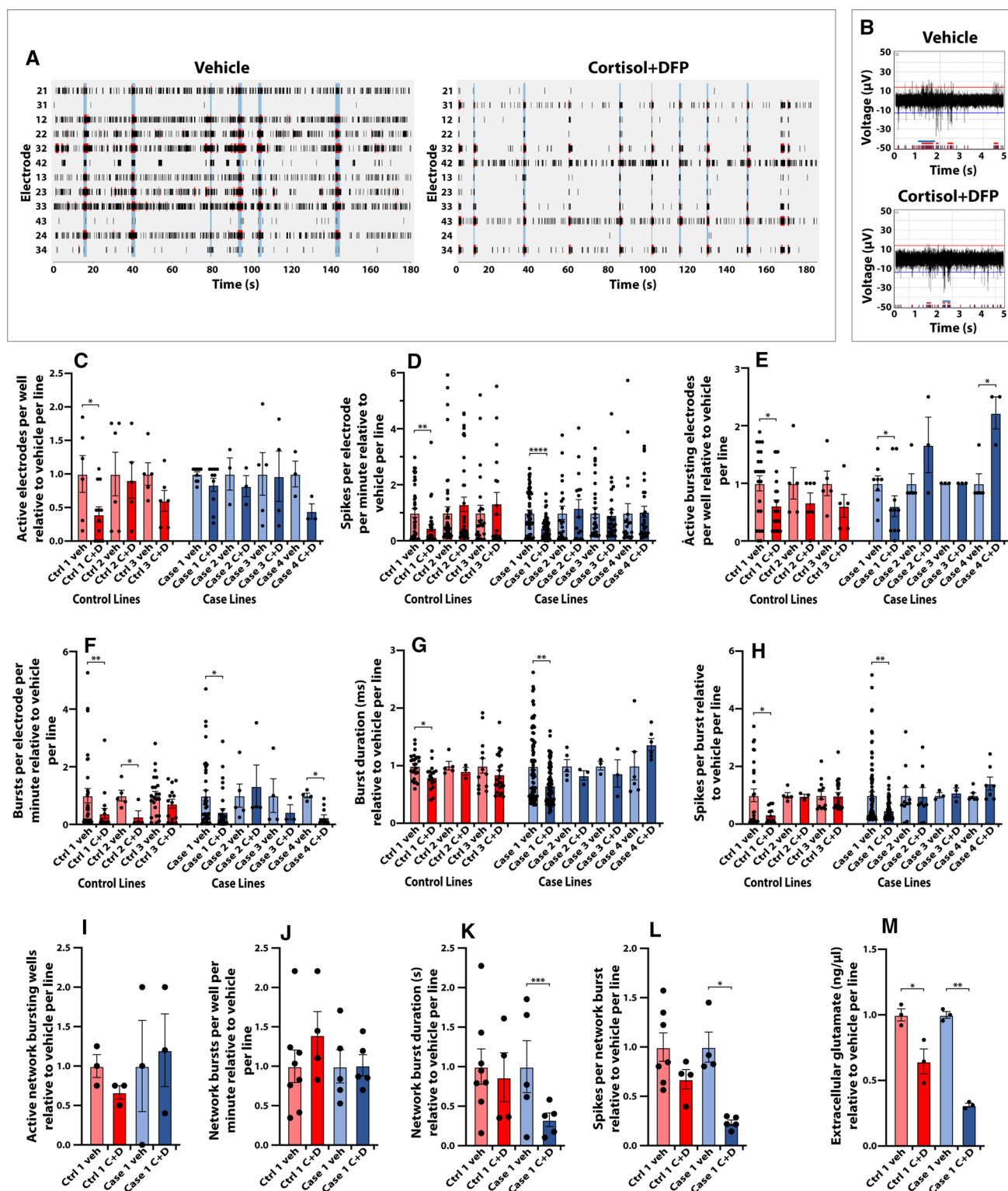
Discussion

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

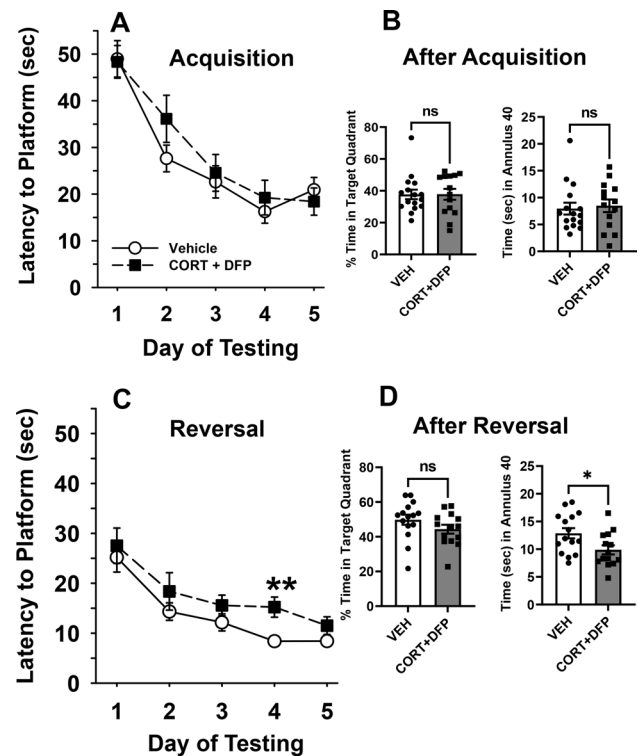


Fig. 7 GW toxicants impair reversal learning and memory in adult rats. Effects of 7 days of exposure to CORT (corticosterone) plus a single injection of DFP 1.5 mg/kg on day seven on a water maze repeated acquisition procedure beginning 48 h after the injection. **a** The graph illustrates the results 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

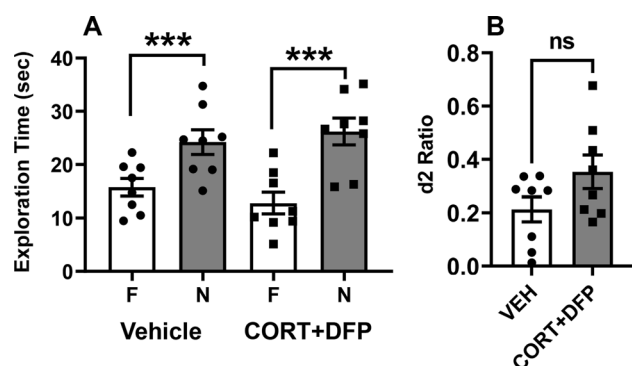
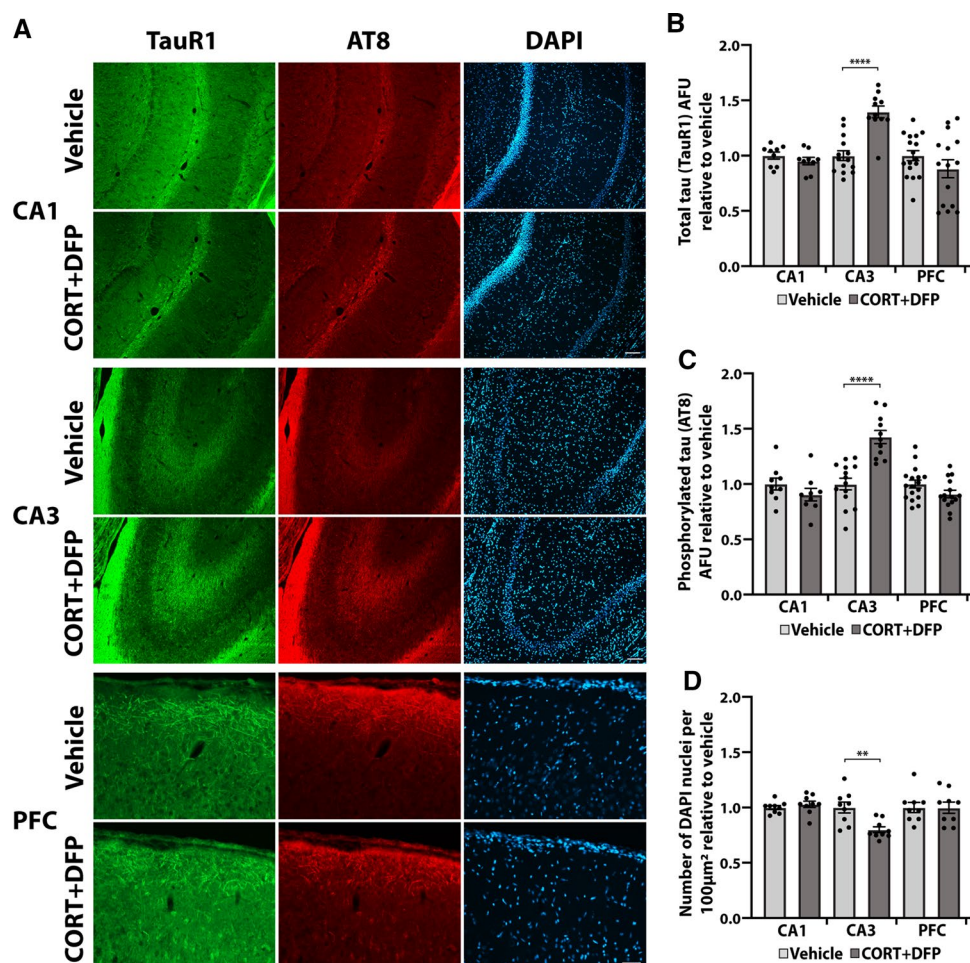


Fig. 8 GW toxicants do not alter object recognition memory in adult rats. Effects of 7 days of exposure to CORT (corticosterone) plus a single injection of DFP 1.5 mg/kg on day 7 on performance of a spontaneous novel object recognition (NOR) procedure 48 h after the injection. **a** Bars on the graph 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

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

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. ** $p < 0.01$, **** $p < 0.0001$



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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