AWARD NUMBER: W81XWH-18-1-0750

TITLE: Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans

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REPORT DATE: Oct 2019

TYPE OF REPORT: Annual Report

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Oct-2019	Annual	09/30/2018 - 09/29/2019

4. TITLE AND SUBTITLE

Microtubule-Based Therapy for Neurodegeneration in Gulf War Illness: Studies with hiPSC-Derived Neurons from Gulf War Veterans

5a. CONTRACT NUMBER
W81XWH-18-1-0750

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Drexel University
Philadelphia, PA

Augusta University
Atlanta, GA

Boston University
Boston, MA

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have generated a bank of human induced pluripotent stem cells (hiPSCs) from the blood of Gulf War veterans (some with and some without Gulf War Illness), and we have conducted research on rat and human neurons on the microtubule-based mechanisms underlying neuronal deficits in GWI, thus enabling us to move forward on microtubule-based therapies. Toward the goal of rushing effective drug therapies to the sufferers of GWI, we propose to utilize the hiPSCs in rapid-throughput analyses of microtubule-active drugs that are either already FDA-approved or in advanced clinical trials.

15. SUBJECT TERMS

microtubule, HDAC6, Kinesin-5, neuron, Gulf War Illness, hiPSC

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Gulf War Illness (GWI) is a chronic debilitating disorder suffered by veterans of the first Gulf War. Accumulating evidence suggests that GWI is caused by exposure to low doses of organophosphate pesticides and nerve agents like sarin, in combination with the stress of the battlefield. Animal and cell studies have revealed that exposure to GW-relevant toxicants induces abnormalities in neuronal microtubules (MTs) and axonal transport. The MTs have fewer associated proteins than normal, leading to reduced MT width. These alterations result in MTs that are less stable and cause molecular motor proteins to move less efficiently on the MTs, thus impairing axonal transport of secretory vesicles carrying neurotransmitters to be released at the synapse, as well as impairing the transport of cellular organelles such as mitochondria that provide the energy required to power the biochemical reactions necessary for the functioning of the axon. Several studies have shown how GW-relevant organophosphate exposure affects multiple facets of axonal transport and mitochondrial dynamics and can lead to GWI symptoms of fatigue and cognitive complaints such as slowed information processing speeds. The purpose of this study is to screen two different categories of MT-based therapies that might be able to improve organophosphate-induced alterations in mitochondrial health and dynamics and neurotransmitter release, thus improving the cognitive defects suffered by veterans with GWI. We are using human-induced pluripotent stem cells (hiPSCs) that we previously generated from the veterans themselves, both with and without GWI, as well as an animal model of GWI. The plan was to differentiate the hiPSCs into multiple different types of neurons that are relevant to the symptoms of GWI, and then study how the two different kinds of MT-based therapies can alleviate the organophosphate-induced defects in mitochondrial health and dynamics and neurotransmitter release. The most effective treatments would then be taken to the animals to ascertain whether defects in behavioral tasks relevant to the symptoms of GWI are ameliorated. The ultimate goal is to rush treatments to the veterans as soon as possible.

2. KEYWORDS: microtubule, HDAC6, Kinesin-5, neuron, Gulf War Illness, hiPSC

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Aim 1. Conduct analyses on GWI hiPSC lines, differentiated into 4 different kinds of neurons relevant to GWI symptoms, and ascertain the degree to which each of the 4 HDAC6 inhibitors and/or the 4 kinesin-5 inhibitors rectify deficits in neurotransmitter release and/or prevent mitochondria defects caused by DFP/cortisol.

Aim 2. Test the benefits of CoQ10 as per Aim 1, and then ascertain whether the most effective microtubule-based drugs from Aim 1 synergize with coQ10 to improve the outcome even more.

Aim 3. Take the very best treatment results from the first two aims into an animal model for GWI to ascertain the potential benefits of the treatments on GWI symptoms at the histological and behavioral levels.

Major Task 1: Columbia University Stem Cell Facility will differentiate the GW hiPSC lines in our repository into neuroblasts partially committed to the four neuronal fates. **33% completed**

Major Task 2: Initial screen of HDAC6 and kinesin-5 inhibitors on neuronal cultures differentiated from GW hiPSC lines to ascertain optimal low and high dosages. **25% completed**

Major Task 3: Ascertain effects on neurotransmitter release produced by DFP/cortisol of two concentrations of 4 HDAC6 drugs and two concentrations of 4 kinesin-5 drugs on four subtypes of neurons differentiated from sick and non-sick GW veterans. **20% completed**

Major Task 4: Ascertain effects on mitochondria produced by DFP/cortisol of two concentrations of 4 HDAC6 drugs and two concentrations of 4 kinesin-5 drugs on four subtypes of neurons differentiated from sick and non-sick GW veterans. **25% completed**

Major Task 5: Ascertain whether coQ10 augments the benefits of drugs shown to be most beneficial (in terms of correcting mitochondrial and neurotransmitter defects caused by DFP/cortisol) in Tasks 3 and 4. **0% completed**

Major Task 6. Conduct behavioral studies on rat (Sprague Dawley) animal model for GWI to ascertain potential benefits of drug and coQ10 treatments. **25% completed**

Major Task 7: Data analysis and expected manuscripts. **25% completed**

What was accomplished under these goals?

Human-induced pluripotent stem cells (hiPSCs) are powerful tools for the study of mechanisms of human disease and for screening therapies in human cells in the laboratory. One of the outstanding questions in the GWI field is why some soldiers developed GWI while their similarly exposed mates did not, and whether the soldiers who developed GWI had a vulnerability or predisposition that was exacerbated by exposure in the field to GWI toxicants, thus increasing the likelihood of developing GWI. In this study, we are using hiPSCs generated from veterans of the first Gulf War, both with (case) and without (control) GWI, to test microtubule-based therapies that can be rushed to suffering veterans.

The hiPSCs previously generated from GW veterans with (case) and without (control) GWI were cultured in mTeSR1 media (Stem Cell Technologies) on Matrigel-coated plates (Corning), and then validated for their pluripotency capabilities via immunostaining for pluripotency markers (Figure 1) and ability to differentiate into the three germ layers (Figure 2). The three-germ layer differentiation was carried out per the manufacturer's instructions using the Human Pluripotent Stem Cell Functional Identification Kit (R&D Systems).

All 9 hiPSC lines (4 control and 5 case) were sent to the Columbia Stem Cell Core Facility to differentiate into neural progenitor cells (NPCs) for multiple types of neurons relevant to the symptoms of GWI. These neuronal subtypes are glutamatergic, cholinergic, dopaminergic, and serotonergic neurons. In our lab at Drexel University, we are further differentiating the NPCs into mature neurons in order to conduct the experiments outlined in this proposal. Columbia has

successfully differentiated all 9 hiPSC lines into glutamatergic NPCs and we have successfully differentiated the NPCs into mature glutamatergic neurons that were immunostained for glutamatergic markers (Figure 3). The Columbia facility is preparing to move on to the next neuronal subtypes. This phase of the study should proceed rapidly in the next year as the facility continues to work with the hiPSCs.

We first differentiated the hiPSCs into glutamatergic neurons because glutamate is the primary excitatory neurotransmitter in the brain and is intricately involved in synaptic plasticity, learning, and memory, which links back to some of the cognitive symptoms of GWI. Moreover, GW-relevant organophosphates have been shown to induce abnormalities in the glutamatergic system. We cultured the glutamatergic neurons for four weeks before exposing them to our previously characterized model of neurotoxicant exposure in Gulf War Illness by using a regimen combining the stress hormone cortisol with an analog of sarin, Diisopropyl fluorophosphate (DFP). We exposed neurons to 2 μM Cortisol for three days, followed by two days of 2 μM Cortisol plus 200nM DFP, and then studied the cells three days later. We started with a DFP concentration of 200nM that we used in our previous studies and which has been documented as just below the level that inhibits acetylcholinesterase (GWI is thought to be caused by low-level organophosphate exposures that do not inhibit acetylcholinesterase). This regimen of Cortisol+DFP was what we previously used to document alterations in mitochondrial health and dynamics and neurotransmitter release. However, as described below, we may have to backtrack and alter our GWI toxicant regimen (longer toxicant exposures, for example) in order to obtain a sufficiently strong phenotype about which we can assess the effects of the MT-based therapies.

The reviewers on our proposal cautioned us against attempting too much work in the three-year timeframe of the grant and recommended that we consider eliminating the kinesin-5 drugs in favor of a concentrated focus on the HDAC6 drugs, as well as eliminating the coQ10 component of the studies. We have come to agree with the wisdom of the reviewers in downscaling our ambitious plan, and so we have completely dropped the coQ10 component of the studies and have reduced the number of drugs being tested in each category to the prototypical inhibitors of HDAC6 (tubastatin) and kinesin-5 (monastrol) used in the laboratory. We settled on tubastatin as the best HDAC6 drug because of solid precedent in recent literature and because of the high costs of purchasing the other drugs. At present, we are not requesting changes to our Statement of Work (SOW) other than the deletion of the coQ10 component as we believe we can make substantial progress on everything else we proposed in the remaining funding period.

We began our experiments investigating impairments in mitochondrial health and dynamics due to our GWI toxicant regimen because we have had success with this assay in the past. For this experiment, the cell permeable dye tetramethylrhodamine, ethyl ester (TMRE) was added to the cell culture media for 30 minutes prior to live cell imaging. TMRE is actively taken up by mitochondria with an intact membrane potential, and so the dye can be used to assess mitochondrial health and to track mitochondrial transport.

We acquired several hours of live-imaging movies of mitochondrial behaviors in glutamatergic neurons from multiple repeats of multiple hiPSC lines with and without the GWI toxicant

regimen, and with and without tubacin or monastrol. The movies were gathered blind and are being quantified blind. While theoretically we have all the mitochondrial movies needed to pull together an appropriate and publishable statistical analysis, we have thus far not seen as dramatic effects as we did on rat neurons or during our preliminary studies on human neurons. We are attempting to quantify parameters in different ways to draw out meaningful effects of the GWI toxicant regimen so that we can then assess the effectiveness of the drugs.

For the mitochondrial health and dynamics assay, we are investigating GWI toxicant-induced changes in:

1. Mitochondria fluorescence intensity
2. Mitochondria length
3. Number of mitochondria per length of axon
4. Percent of axon occupied by mitochondria
5. Percent of mitochondria that are moving compared to stationary
6. Distance traveled by mitochondria
7. Velocity of mitochondria
8. Mitochondria processivity: run length, number of stops

Alternatively, as stated above, we may have to backtrack and alter our GWI toxicant regimen (longer toxicant exposures, for example) in order to obtain a sufficiently strong phenotype about which we can assess the effects of the drugs.

As outlined in the proposal, the neurotransmitter experiments are being led by Dr. Espana from Drexel. We have purchased glutamate detection kits that enable rapid and reliable detection both in the cells themselves and in the cell culture media into which they release neurotransmitters. We anticipate that this should go smoothly over the next several months.

The animal work, being performed on sub-contract to Dr. Terry at Augusta University, is underway, but there has been difficulty in obtaining reproducible memory defects as previously observed by Dr. Maier. Dr. Terry has tried several different GWI toxicant regimens using several different behavioral tasks, but the memory deficits are proving to be much harder to document than we had anticipated. In discussions with Dr. O'Callaghan, we have been cautioned that memory defects in GWI rodent models are very subtle. Dr. Terry has administered corticosterone in the drinking water to rats for 7 days (based on their weight and 24-hour water consumption) and then administered a single subcutaneous injection of DFP on Day 8, followed by behavioral testing on Day 11 in the novel object recognition task or the Morris water maze task. After observing no differences between groups, Dr. Terry is now trying a multiple dosing procedure on the animals by giving 5-10 injections of DFP over 1-2 weeks to strengthen the phenotype. Once Dr. Terry is able to settle on a GWI toxicant regimen that produces a robust memory deficit phenotype, Dr. Terry can also test the animals on the 5-choice serial reaction time task that can generate a lot of meaningful data. The reason for the delay in starting this behavioral test is that the task is very labor intensive and takes two months of daily training before administering the GWI toxicants, so Dr. Terry has been cautious about starting such a long study while still settling on the appropriate GWI toxicant regimen.

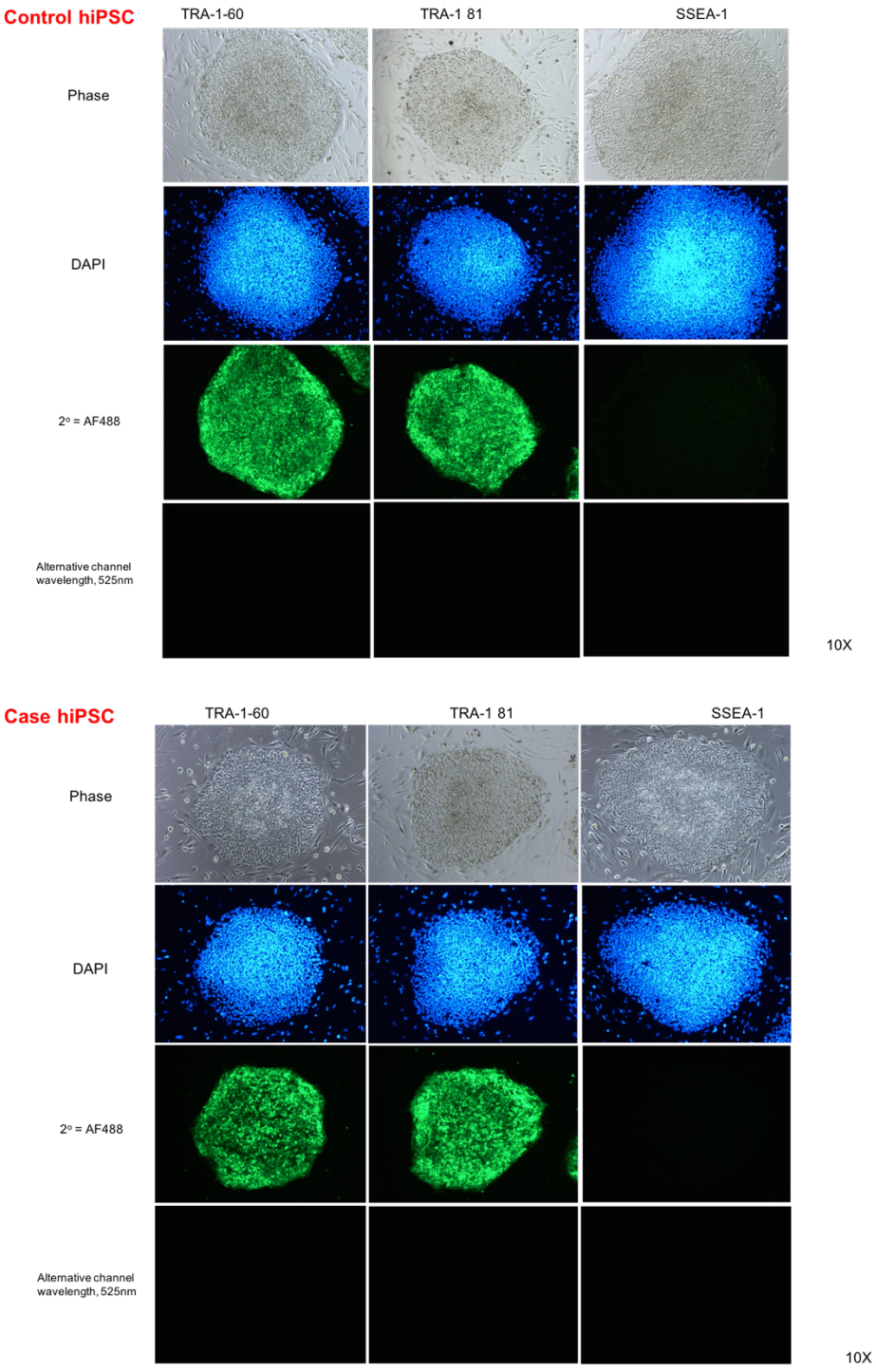

Figure 1. Pluripotency validation of hiPSCs generated from Gulf War veterans without (control) or with (case) GWI. The top row shows phase-contrast images of the hiPSC colonies. The second row in blue shows DAPI staining for all nuclei. The third row in green shows the specific pluripotency marker being tested. The bottom row shows another channel with no staining. The first two columns show the pluripotency markers TRA-1-60 and TRA-1-81 that are positive in hiPSCs, while the third column shows the marker SSEA-1 that is downregulated in hiPSCs.

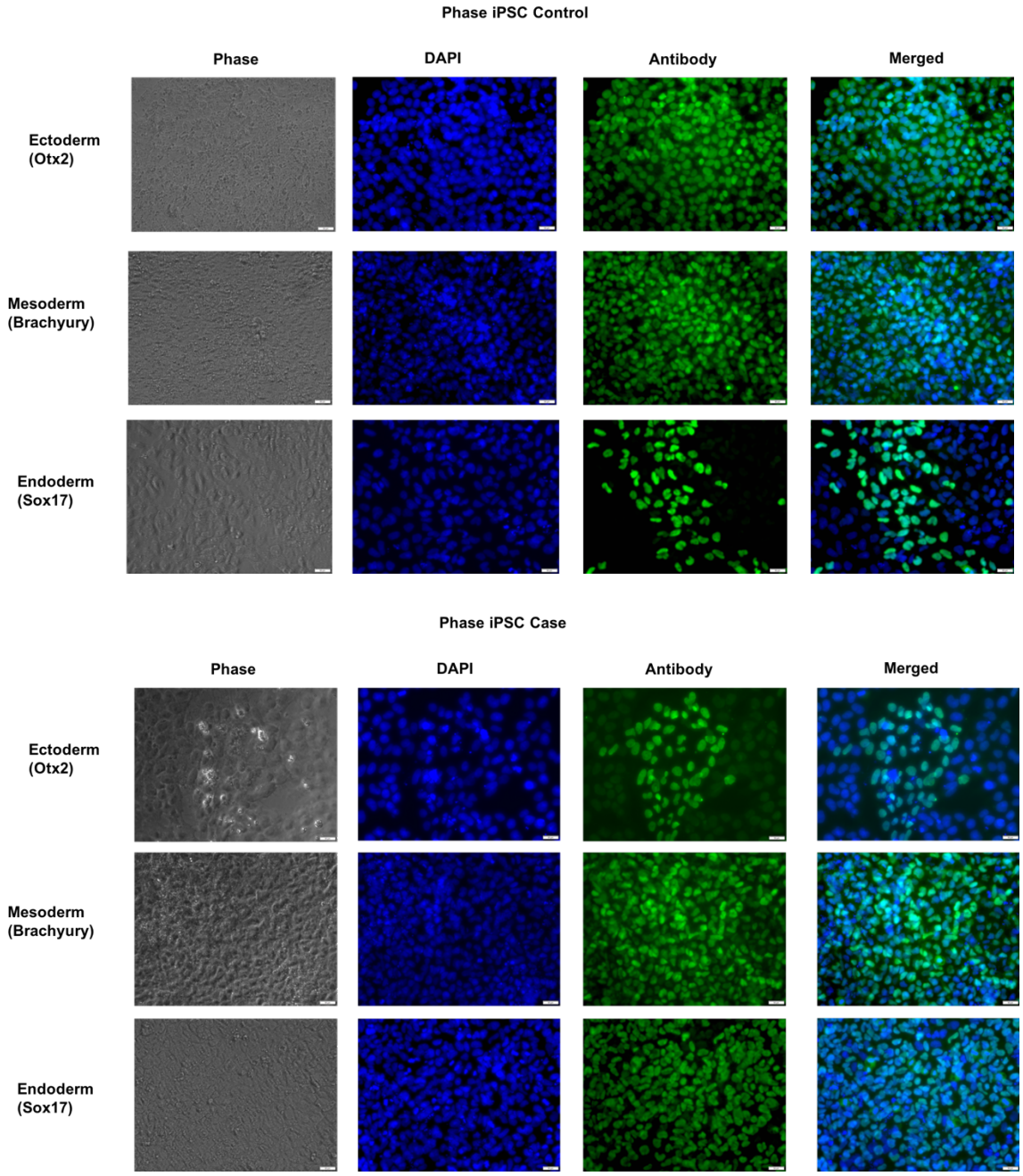

Figure 2. Three germ layer differentiation of hiPSCs generated from Gulf War veterans without (control) or with (case) GWI. The hiPSCs were differentiated into the three germ layers and stained with the appropriate markers Otx2, Brachyury, and Sox17 for ectoderm, mesoderm, and endoderm, respectively. The first column shows phase-contract images of the differentiated cells. The second column in blue shows DAPI staining for nuclei. The third column in green shows the specific germ layer marker being tested. The last column shows the merged blue and green channels.

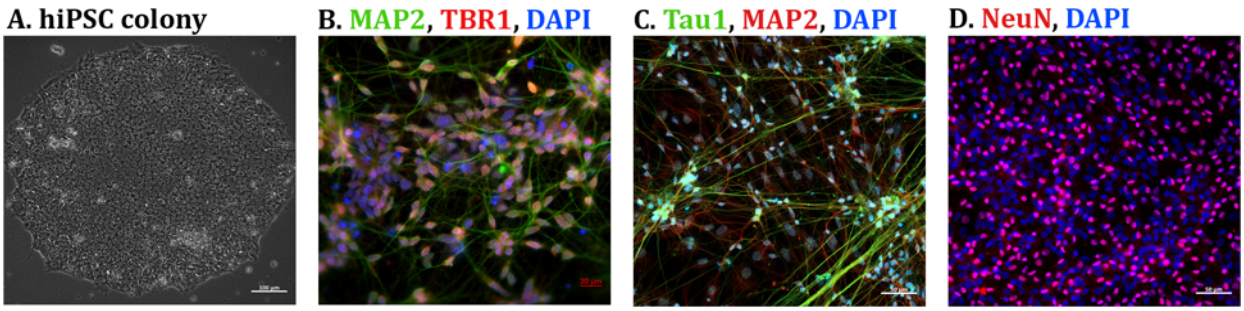

Figure 3. Validation of hiPSC-derived neurons. A. hiPSC colony. **B.** Microtubule-associated protein 2 (MAP2) labels neuronal processes. TBR1 is a transcription factor for mature forebrain glutamatergic neurons. DAPI labels the nuclei of all cells. **C.** Tau1 stains for total tau in mature neurons. **D.** NeuN is a mature neuronal nuclear marker.

What opportunities for training and professional development has the project provided?

- Philip Yates, an MD/PhD student in our lab, is working on the project as part of his dissertation, which he will defend in roughly one year.

How were the results disseminated to communities of interest?

- We presented a poster at the 2019 Society for Neuroscience Meeting. The abstract follows below:

  Microtubule-based mechanisms and therapies for Gulf War Illness using hiPSC-derived neurons and organoids. Gulf War Illness (GWI) is a chronic multisystem disorder suffered by at least 25% of the nearly 700,000 U.S. veterans who fought in the 1990-1991 Gulf War. Central nervous system (CNS) symptoms include chronic fatigue, reduced information processing speeds, memory deficits, chronic headaches, and impaired mood and sleep. Evidence suggests that GWI is caused by the combination of the stress of the battlefield and exposure to organophosphate pesticides and nerve agents at concentrations below the threshold that inhibit acetylcholinesterase, thus implicating novel biological targets. We posit there is a constellation of cellular changes in the central nervous system contributing to the long-lasting symptoms of GWI. We are interested in elucidating these aberrant cellular phenomena, screening potential therapies that can be rushed to suffering veterans, and investigating any cellular differences that might help explain why some veterans suffer from GWI while their similarly exposed mates do not. To tackle these questions, we are using human induced pluripotent stem cells (hiPSCs) derived from veterans of the first Gulf War, both from healthy veterans and from veterans with GWI. We are differentiating the hiPSCs into glutamatergic neurons and then exposing them to the GWI regimen of Cortisol plus Diisopropyl fluorophosphate (DFP), an analog of sarin. In one line of investigation, we are screening microtubule-based therapies that might correct previously documented changes in microtubule (MT) related

processes, including alterations in MT stability, axonal transport, and neuronal activity. We are testing inhibitors of the tubulin-specific histone deacetylase 6 (HDAC6), which we have already shown can correct many of these MT-based alterations in primary rodent neurons, and inhibitors of kinesin-5, which we have shown can correct some of the axonal transport deficits. However, a mechanistic explanation for these MT-based alterations is still unclear. We hypothesize that pathology of the microtubule-associated protein tau might contribute to some of the MT-related deficits. Exposure to Cortisol plus DFP dramatically increases total tau and hyperphosphorylated tau, and now we are testing whether tau knockdown can correct the alterations in MT stability, axonal transport of mitochondria, and neuronal activity. This line of investigation opens the door to therapies from the tauopathy field. Lastly, we are growing forebrain cerebral organoids to model GWI in a more complex system and to examine alterations in neurogenesis, cortical laminations, synapses, and glial activation.

What do you plan to do during the next reporting period to accomplish the goals?

- We are determined that the next reporting period will be one of rapid progress on data-gathering, processing and publication. We know that progress has been slow during the past cycle, but we do think the next cycle should be one of rapid progress, during which we can write and submit for publication the HDAC6 component of the grant – both on cells and the animal model.
- Time permitting, we will work on the kinesin-5 component of the grant, and also see if we can make some progress on testing different types of neurons, as per our original plan.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

- There is now a bank of hiPSCs available to the GWI community (via BBRAIN) to use for testing of mechanistic hypotheses and therapies. We are expanding its capabilities continuously through more knowledge, resources and collaborative opportunities.
- Pre-clinical studies on mechanism and therapy for GWI have been limited until now to animals that are imperfect models for human disease. These new human cell lines will provide a major resource for GWI researchers.

What was the impact on other disciplines?

- Nothing to report

What was the impact on technology transfer?

- The resource of GWI hiPSC cells will not be used for commercial profit but will instead be made available for collaborative projects with GWI investigators via BBRAIN. The partial differentiation into neuroblasts as well as our ongoing development of organoid technology add to the technical expertise of the BBRAIN resource, as well as the cells themselves.

What was the impact on society beyond science and technology?

- Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

- Nothing to report

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

- As noted in some detail in earlier sections, we have had to scale back our ambitions for the project. There was simply too much work proposed which involved two different drug targets, multiple drugs for each target, and four different types of neurons. We are not requesting a change in the SOW, but we are handling this more realistically and will probably wind up with significant progress on tubastatin in glutamatergic neurons and modest progress on the rest. As per the advice of the reviewers, there is less focus on kinesin-5 as a target and we have dropped the CoQ10 component.
- The animal work is going slowly because the memory defect phenotype has been hard to reproduce. We are working on it, and by the end of our work, this should be a huge asset to the GWI community if we can figure out and document the best way to do this.

Changes that had a significant impact on expenditures

- Nothing to report

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

- Nothing to report

Significant changes in use or care of human subjects

- Nothing to report

Significant changes in use or care of vertebrate animals.

- Nothing to report

Significant changes in use of biohazards and/or select agents

- Nothing to report

6. PRODUCTS:

- As indicated above, a poster was presented at the SFN meeting in 2019.
- We anticipate a major manuscript to be submitted around April 2020 with the hiPSC-derived glutamatergic neurons with analyses on mitochondrial behaviors and glutamate, and also with tau data relating to our earlier grant with Dr. Liang Qiang as PI.

Website(s) or other Internet site(s)

- The GWI Consortium that gave rise to the project has a website that will be used in the future to disseminate the progress and availability of the hiPSC lines. This is now BBRAIN.

Technologies or techniques

- We are working on organoid technology to add to our BBRAIN repository.

Inventions, patent applications, and/or licenses

- Nothing to report

Other Products

- The bank of hiPSC cells is now stored at BBRAIN, and we are adding technologies to it, such as organoids.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Peter Baas
Project Role	PI
Researcher Identifier	ORCID iD is 0000-0002-1272-4538
Nearest person month worked	1
Contribution to Project	Dr. Baas oversees the entire project and coordinates the various elements of the project. He is in charge of planning experiments, interpreting data, and providing regular updates to the Department of Defense (DoD) on the progress.
Funding Support	This grant.

Name	Liang Qiang
Project Role	Co-I
Researcher Identifier	Orcid iD is 0000-0001-9896-602X
Nearest person month worked	1
Contribution to Project	Dr. Qiang is specifically in charge of the hiPSC differentiation experiments. He also participates in data analysis and interpretation.
Funding Support	This grant.

Name	Alvin Terry
Project Role	Co-I
Researcher Identifier	ORCID iD is 0000-0003-2071-4767
Nearest person month worked	1

Contribution to Project	Dr. Terry is in charge of the animal work.
Funding Support	This grant.

Name	Kimberly Sullivan
Project Role	Co-I
Researcher Identifier	Orcid iD is: 0000-0001-7940-6123
Nearest person month worked	1
Contribution to Project	Dr. Sullivan serves as a GWI expert for the project.
Funding Support	This grant.

Name	Rodrigo Espana
Project Role	Co-I
Researcher Identifier	Orcid iD is orcid.org/0000-0003-2403-6342
Nearest person month worked	1
Contribution to Project	Dr. Espana is in charge of the neurotransmitter release experiments.
Funding Support	This grant.

Name	Philip Yates
Project Role	Graduate Student
Researcher Identifier	ORCID ID is: 0000-0001-7025-2092
Nearest person month worked	6
Contribution to Project	Philip performs all of the hiPSC cell experiments, analysis, and interpretation.
Funding Support	NIH T32 to Drexel University

Name	Ankita Patil
Project Role	Graduate Student
Researcher Identifier	none
Nearest person month worked	2
Contribution to Project	Ankita performs the mitochondria experiments and analysis.
Funding Support	Dean's Fellowship from Drexel University

Name	Ramnik Gill
Project Role	Undergraduate Student
Researcher Identifier	none
Nearest person month worked	2
Contribution to Project	Ramnik assists with mitochondria analysis.
Funding Support	Work-Study funds from Drexel University

Name	Daniel Beck
Project Role	technician
Researcher Identifier	none
Nearest person month worked	4
Contribution to Project	Mr. Beck is working on the animal studies in Dr. Terry's lab.
Funding Support	This grant.

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

- Nothing to report

What other organizations were involved as partners?

- **Organization Name:** Augusta University
- **Location of Organization:** Atlanta, GA
- **Partner's contribution to the projec**t
  - **Collaboration**: Dr. Alvin Terry has a sub-contract on the grant to conduct the animal work in his laboratory.

- **Organization Name:** Boston University
- **Location of Organization:** Boston, MA
- **Partner's contribution to the projec**t
  - **Other:** Dr. Kimberly Sullivan has a small salary component on the grant to act as an advisor, as well as Director of BBRAIN.

Dr. Peter Baas (Principal Investigator, Drexel University), is an expert on microtubules in the nervous system and a member of the GWIC. Dr. Liang Qiang (co-Investigator, Drexel University) serves as the team's hiPSC expert, as he did for the development of our hiPSC repository. Dr. Rodrigo España (co-Investigator, Drexel University) acts as the neurotransmitter expert for the proposed project, as he did on a recently published paper with Dr. Baas. Dr. Kimberly Sullivan (Co-Investigator, Boston University), the leader of the GWIC, will serve as the GWI expert on the project. She is a behavioral neuroscientist who has studied the cognitive, neuroimaging and neurotoxicological correlates of GWI and is a co-investigator on several currently funded GWI treatment trials. Dr. Sullivan is in close contact with clinicians, basic researchers and the veterans themselves who are relevant to GWI. Dr. Alvin Terry (co-Investigator, Medical College of Georgia at Augusta University) has conducted multiple studies both *in vitro* and *in vivo* that are relevant to GWI OPs, and acts as the pharmacology expert for the proposed project. Dr. Terry is conducting the animal experiments in Aim 3.

In addition (but with no form subcontract arrangements), Dr. Nancy Klimas (Consultant, Nova Southeastern University), Principal Investigator of a multi-site study of CoQ10 in GWI serves as a clinical consultant. Dr. Steven Maier (Consultant, University of Colorado) developed a memory test for the rat GWI animal model developed by Dr. James O'Callaghan (CDC/NIOSH/HELD). Drs. Maier and O'Callaghan are advising us in using this model and memory test in the Terry Laboratory. Members of the team meet often in web meetings.

This is as outlined in the proposal. Nothing has changed.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

- n/a

QUAD CHARTS

- n/a

9. APPENDICES

- n/a