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14. ABSTRACT
The study plan is to develop immortalized cultures of cells from the blood of veterans who are suffering from Gulf War Illness (GWI). A simple blood sample is taken from the soldier, and then transduced, using reliable established methods, to make the cells pluripotent. The pluripotent cells lines can then be treated with growth factors to differentiate them into a number of different cell types including neurons. The investigators will develop these cell lines from veterans of the Gulf War who got sick and also from veterans who did not get sick, so the two can be compared. Other scientists studying GWI will be able to use the cell lines for their own studies, which will maximize the effort of the biomedical community to rush medicines and treatment regimens to the veterans who are suffering. At the time of the final report, the pluripotent cell lines had been developed, stored in a repository, and their existence and availability made public through a publication in high visibility medical journal. That publication also provided a details on the rationale for creating the cell lines and how they can be used to address mechanistic questions about GWI, and test potential therapies. The second aim of the funded proposal was to use microtubule-based strategies to correct cellular defects observed in the cell lines when they are exposed to a toxicant regimen designed to mimic GWI. While no studies of this kind were performed on the cells lines created from the Gulf War veterans, because they were not yet ready when the microtubule work had to begin, those studies used pluripotent cell lines from non-veteran humans, as well as primary rodent cell lines. The results, published in a high-impact scientific journal, indicate that inhibitors of HDAC6, the chief microtubule de-acetylase in vertebrate cells, can correct a variety of cellular abnormalities resulting from the GWI toxicant regimen, including defects in dopamine release.

15. SUBJECT TERMS
microtubule, neuron, Gulf War Illness, hiPSC

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Table of contents

1 **INTRODUCTION:** Gulf War Illness (GWI) is thought to have its origins in exposure of soldiers serving in the 1991 war to various neurotoxicants, including pesticides, anti-nerve gas pills, and low level nerve gasses including sarin/cyclosarin. Studies to date have shown deficits in the movement of proteins and organelles within nerves by the process known as axonal transport, as well as aberrations in microtubules, which are the structural railways for axonal transport. Given the importance of microtubules, damage to them is suspected as a primary cause of neurodegeneration in GWI, as it is in many neurodegenerative diseases. Further investigation into this matter has a strong possibility of yielding effective treatment strategies. Animal models may not suffice to provide the needed insights, and therefore efforts to generate pluripotent cell lines from the veteran themselves will enable studies on microtubules to move forward more effectively, and will also provide a toolkit for other GWI researchers to use in the future, as the cell lines will be immortal once they are generated. The hypothesis is that exposure of neurons and/or neuroinflammatory cells to GW toxins caused long-lasting microtubule defects in neurons, and that these defects lead to a loss of microtubule mass, a change in the proportions of stable and labile microtubule mass, and/or flaws in the lattice of the microtubule that lead to abnormalities in how molecular motor proteins and other microtubule-related proteins interact with the microtubule. One objective is to develop new immortal lines of pluripotent cells derived from the blood of GW veterans themselves, so that the microtubule hypothesis (as well as other GWI hypotheses beyond the present proposal) can be tested. The other objective is to assess whether available microtubule-active drugs can correct these abnormalities and provide treatments for GWI.

2 **KEYWORDS:** *microtubule, neuron, Gulf War Illness, hiPSC*

3 **ACCOMPLISHMENTS:**

What were the major goals of the project?

- Aim 1. Develop human neurons or glial cells derived from human induced pluripotent stem cells (hiPSCs), originating from GW veterans with GWI and healthy GW veteran controls.
- Aim 2. Develop a microtubule-based strategy to treat impaired nervous system in GWI.

What was accomplished under these goals?

(I) In the Statement of Work, the first three of the six tasks were:

Task 1. Obtain necessary authorizations prior to initiation of human subjects' research

Task 2. Screening, recruitment and blood draw of Gulf War veterans

Task 3. Reprogram peripheral blood mononuclear cells (PBMCs) into induced pluripotent stem cells (iPSCs)

- 19 GW veterans were recruited (10 GWI cases, 9 controls) and blood samples were obtained. 5 hiPSC lines from GWI cases and 4 hiPSC lines from controls (veterans who were similarly exposed but who did not get GWI) were developed and frozen for experimental use. Blood cells were also frozen down so that they can be potentially used for direct conversion to neurons.
- A paper was published in *Neurology*, detailing the logic and reasoning of this approach, introducing the cell lines to the research community and the public, and describing in some detail how they can be used to study mechanistic hypothesis on the causes of GWI and how they can be used to test potential therapies. This

completes Aim 1, with the deliverables being the cell lines and the published article (see attached and below).

- Neurology Article: Reprogramming cells from Gulf War veterans into neurons to study Gulf War illness. Qiang L, Rao AN, Mostoslavsky G, James MF, Comfort N, Sullivan K, Baas PW. Neurology. 2017 May 16;88(20):1968-1975. doi: 10.1212/WNL.0000000000003938. PMID: 28507260
- Figure 1 of the article shows a schematic illustration of the how hiPSC lines are created from human somatic cells and differentiated into neurons, and also how somatic cells can be directly converted to neurons. The table on the following page shows the advantages and disadvantages of each of the two approaches. Figure 2 shows the pluripotent cell line from the lines that were created, first fluorescently labeled with pluripotency markers, and then after differentiation into neurons, with neuronal markers. Figure 3 is a schematic flow-chart guide on how to conduct GWI experiments using the cell lines. The journal requested that we contribute the cover illustration, which is similar to the neuronal parts of figure 2.

(II) In the Statement of Work, the last three of the six tasks were:

Task 4. Differentiate pluripotent stem cell lines into neurons and glia and conduct planned microtubule experiments

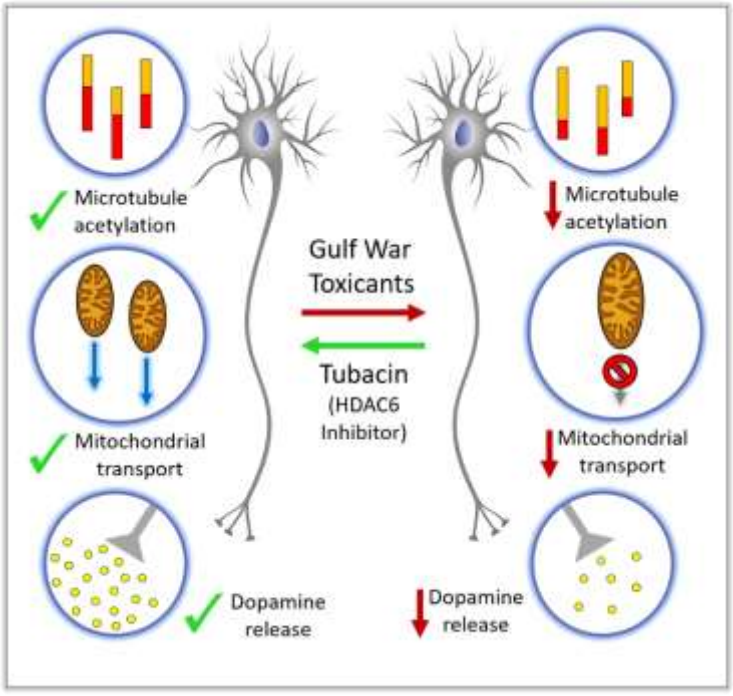
Task 5. Conduct drug treatment experiments with neurons and glia

Task 6. Data analysis and expected manuscripts to result from study

- As for Aim 2, due to limitations in time, no studies were conducted on glia, and the microtubule-based therapies tested were limited to one type of drug, namely HDAC6 inhibitors. Studies were completed on investigating the potential therapeutic benefit of HDAC6 inhibitors on improving cellular defects produced by exposure of neurons to a GWI toxicant regimen consisting of DFP (a sarin analog) and a hormone/chemical to mimic the physical stress of the battlefield. The results are highly encouraging; an HDAC6 inhibitor called tubacin reversed every microtubule abnormality we investigated as well as an abnormality in dopamine release caused by the toxicant regimen. These studies were partially funded by the present grant and partially funded by the GWI Consortium Grant to Dr. Kimberly Sullivan, through a subcontract to Dr. Baas. Some of the work was done on primary rat neurons (via funding of the other grant) and other work was done on human hiPSC lines (via funding of this grant). However, none of the published work was done on the hiPSC lines produced from the GW veterans. That part of the work is still ongoing. Thus, encouraging results related to Aim 2 have been obtained, with a published paper as a deliverable. In addition, we are now poised to use the GW cell lines for high-throughput analyses on a number of microtubule-based drugs.
- Traffic Article: Pharmacologically increasing microtubule acetylation corrects stress-exacerbated effects of organophosphates on neurons. Rao AN, Patil A, Brodnik ZD, Qiang L, España RA, Sullivan KA, Black MM, Baas PW. Traffic. 2017 Jul;18(7):433-441. doi: 10.1111/tra.12489. Epub 2017 May 25. PMID: 28471062.
- Figure 1 of the article shows that the GWI toxicant regimen results in a lower ratio of acetylated to total tubulin in both primary rat cortical neurons and hiPSCs differentiated into neurons. Figure 2 of the article shows that microtubule dynamics are perturbed in the neurons, using a live-cell imaging approach for directly

monitoring bouts of microtubule assembly. This figure also shows that the defects are mostly corrected by treatment with tubacin, an HDAC6 inhibitor. The idea of HDAC6 inhibition is to restore the microtubules to a more normal acetylation status. Figure 3 shows transport of mitochondria (with a live-cell imaging approach) is negatively impacted by the GWI toxicant regimen, with rescue of the defects by tubacin. Figure 4 shows that dopamine release (measured with HPLC) is negatively affected by the toxicant regimen, and corrected by tubacin. In all of the experiments, DFP alone produced the defects, but the defects were exacerbated by the inclusion of corticosterone or cortisol with the DFP.

- The following schematic summary of the results was published as a graphical abstract with the project:



The journal requested that we contribute the cover illustration, which is similar to the cover illustration that we contributed for the *Neurology* article.

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

- Over the course of the work, the Baas laboratory acquired an MD/PhD student (Philip Yates) who plans to pursue the next phases of the GWI work in the Baas laboratory for his doctoral dissertation.
- The completed published work in Traffic (related to Aim 2) was conducted by two doctoral students in the Baas laboratory, Anand Rao and Ankita Patil.
- Dr. Liang Qiang, a co-I on the grant, has now obtained his own CDMRP grant to study GWI, and as a result of acquiring the grant, he was promoted to Research Assistant Professor at Drexel University. Dr.

Qiang's grant utilizes the GW hiPSC cell lines to explore the hypothesis that GWI is a taopathy (i.e., it is a disease in which the microtubule-associated protein tau becomes abnormally phosphorylated, leading to cognitive degeneration).

How were the results disseminated to communities of interest?

- Dr. Baas has given interviews to the Drexel University media department on both of the published papers, after which news articles were published online through Drexel University, and released so that other media could pursue them. Google searches now can be done to read these news articles. URLs include:
  https://newsblog.drexel.edu/2017/06/06/reprogramming-veterans-blood-cells-to-study-gulf-war-illness/
  http://drexel.edu/now/archive/2017/June/Treating-Gulf-War-Illness/
  https://www.sciencedaily.com/releases/2017/06/170606135452.htm
  Dr. Sullivan gave a video interview of the study that can found at
  https://www.bu.edu/sph/2017/05/22/establishing-gulf-war-illness-human-stem-cell-biorepository/
- Ms. Patil will give a news conference at the 2017 Society for Neuroscience Meeting, where she will also deliver her poster on GWI. Dr. Sullivan will accompany Ms. Patil to the news conference.

4 **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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 to use for testing of mechanistic hypotheses and therapies.
- 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?

- Use of hiPSC lines is expanding to more applications directly related to diseases that afflict the human population.

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.

- **What was the impact on society beyond science and technology?**
  - Provides hope to veterans suffering from GWI, and addresses ongoing concerns about potential illnesses resulting from toxic exposures around the world.

5 **CHANGES/PROBLEMS:**

Changes in approach and reasons for change

- Nothing to report

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

- *Nothing to report*

Changes that had a significant impact on expenditures

- Nothing to report

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

- Nothing to report

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:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- Two manuscripts, as described above, were published in 2017.

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. http://sites.bu.edu/gwic/
- News articles and video, as indicated earlier, appear online.

Technologies or techniques

- The Neurology paper, indicated earlier, describes the logic of how to use the hiPSC lines for experiments on mechanism and therapy related to GWI.

Inventions, patent applications, and/or licenses

- Nothing to report

Other Products

- The bank of hiPSC cells has been produced

7 **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

- Peter Baas, Kimberly Sullivan, Liang Qiang, Anand Rao, Ankita Patil

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: Boston University School of Public Health**
- **Location of Organization:** 715 Albany Street, *Boston, MA*
- **Partner's contribution to the projec**t
  - **Financial support; -** none to report
  - **In-kind support** - None to report
  - **Facilities –** BUSPH stem cell center reprogrammed blood cells at expert facility, blood draws occurred at BU general clinical research unit, Subject recruitment occurred at BUSPH.
  - **Collaboration** – Subject recruitment, blood draws and reprogramming of stem cells occurred at BU.
  - **Personnel exchanges** - subject recruitment was done at BUSPH site with the BUSPH research assistant.

- **Other. –** none to report

8 **SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS

- n/a

QUAD CHARTS

- n/a

9 **APPENDICES**

- Attached are PDFs of the two published articles indicated above.

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WILEY Traffic

ORIGINAL ARTICLE

Pharmacologically increasing microtubule acetylation corrects stress-exacerbated effects of organophosphates on neurons

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Many veterans of the 1990-1991 Gulf War contracted Gulf War Illness (GWI), a multisymptom disease that primarily affects the nervous system. Here, we treated cultures of human or rat neurons with diisopropyl fluorophosphate (DFP), an analog of sarin, one of the organophosphate (OP) toxicants to which the military veterans were exposed. All observed cellular defects produced by DFP were exacerbated by pretreatment with corticosterone or cortisol, which, in rat and human neurons, respectively, serves in our experiments to mimic the physical stress endured by soldiers during the war. To best mimic the disease, DFP was used below the level needed to inhibit acetylcholinesterase. We observed a diminution in the ratio of acetylated to total tubulin that was correctable by treatment with tubacin, a drug that inhibits HDAC6, the tubulin deacetylase. The reduction in microtubule acetylation was coupled with deficits in microtubule dynamics, which were correctable by HDAC6 inhibition. Deficits in mitochondrial transport and dopamine release were also improved by tubacin. Thus, various negative effects of the toxicant/stress exposures were at least partially correctable by restoring microtubule acetylation to a more normal status. Such an approach may have therapeutic benefit for individuals suffering from GWI or other neurological disorders linked to OP exposure.

KEYWORDS

acetylation, axon, Gulf War Illness, microtubule, neuron, organophosphate, stress, tubacin

1 | INTRODUCTION

Gulf War Illness (GWI) is a chronic, multisymptom disorder that afflicts 25% to 32% of the nearly 700 000 US veterans who served in the 1990-1991 Gulf War.[1] Veterans with GWI struggle with unabated central nervous system (CNS) deficits including chronic headaches, memory and concentration problems, sleep difficulty, fatigue and mood alterations.[2-4] Epidemiological and intelligence data indicate that at least 100 000 GW veterans were probably exposed to the organophosphate (OP) nerve agents sarin and cyclosarin, which were released as fallout from demolitions of the ammunition depot at Khamisiyah, Iraq in March 1991.[5-7] While acute symptoms of OP toxicity have been characterized, only recently have the neurological consequences of exposure to low levels of OPs been studied.[8,9]

Understanding the impact on the CNS of low level OP exposure is important because OP pesticides represent the largest group of insecticides, and their use is widespread around the world, with >5 billion pounds of pesticides applied to crops, homes, schools, parks and forests.[10] In addition, the threat of OP nerve agent use in terrorism and war remains a concern. Growing evidence indicates a link between OP pesticide exposure and the development of Parkinson's and Alzheimer's diseases and amyotrophic lateral sclerosis,[11-15] necessitating a deeper investigation into the underlying mechanisms.

OP nerve agents are known to cause acute life-threatening acetylcholinesterase inhibition. Current thinking is that GWI is not due to acetylcholinesterase inhibition but rather due to subthreshold effects of OPs that produce a nonself-correcting state of neurodegeneration. Neuroinflammation, axonal transport deficits, microtubule impairments and dopaminergic changes have all been observed in

†These authors are co-first authors.

animal (rodent) and cell culture models of GWI at OP levels below those needed to inhibit acetylcholinesterase.[16-18] Additional animal studies have shown that the effects of OP exposures are exacerbated when coupled with physical restraint or chemically induced stressors designed to mimic the physical stress of the battlefield.[19]

We are interested in microtubule deficits that occur in the nervous system of veterans suffering from GWI, with the goal of developing microtubule-based therapies. OPs can affect a variety of proteins and pathways in cells, for example, by covalently binding to tyrosine and lysine residues. The impacts on microtubules and microtubule-related proteins are likely to be many, including effects on molecular motor proteins and microtubule stability and dynamics, as well as the binding to microtubules of microtubule-associated proteins such as tau.[20-24] In light of the complexity, the question becomes whether particular microtubule-related deficits can be identified that when corrected result in marked improvement of GWI symptoms.

Here, we pretreated cultured neurons with the stress hormone corticosterone (CORT) in the case of rat neurons, and cortisol in the case of human-derived neurons, and then exposed them to diisopropyl fluorophosphate (DFP), an OP compound used by researchers as a sarin-surrogate.[25] We then assessed various microtubule-related parameters such as microtubule dynamics and mitochondrial transport, using live-cell imaging, to ascertain potential deficits. We included in our studies analyses of dopamine release, which is a complex process that involves not only microtubules but many other factors as well.[26] Upon ascertaining that the toxin regimens resulted in diminished microtubule acetylation, we sought to determine whether a drug that increases microtubule acetylation could restore to normal observed deficits in microtubule dynamics and mitochondrial transport, as well as dopamine release. Improvement in dopamine release was taken as an indicator that this approach has the potential to effectively treat complex symptoms of GWI.

2 | RESULTS

2.1 | Pretreatment with CORT or cortisol intensifies DFP-induced aberrations in microtubule acetylation in human and rat neurons

Human neural stem cells were differentiated into neurons and allowed to mature and form networks in vitro for 1 month. These human neurons, or rat fetal cortical neurons grown for 8 to 14 days in culture, were exposed to DFP at concentrations of 20 or 200 nM with or without pretreatment with 2 μM cortisol (for the human neurons) or CORT (for the rat neurons), and changes in tubulin acetylation were assessed (Figure 1A). Human neuronal phenotype was confirmed by positive staining for βIII-tubulin, a neuron-specific tubulin isotype, and Tbr1, a transcription factor specific to glutamatergic forebrain neurons (Figure 1B). Immunostaining and western blot analyses both revealed a reduction in the ratio of acetylated to total tubulin after DFP exposure (Figures 1C,D and S1A,B, respectively). With the rat neurons, as described in Figure 1A, the ratio was reduced after 20 and 200 nM DFP treatment, fortifying the finding from human neurons. Pretreatment with CORT or cortisone exacerbated the effect (Figure 1E [top],F [left],G).

We sought to restore the acetylation status of the microtubules by inhibiting the tubulin deacetylating enzyme, HDAC6. Treatment with 1 μM tubacin significantly improved the ratio of acetylated to total tubulin after treatment with 20 nM DFP alone, but did not significantly restore the effects of 200 nM DFP alone or any CORT pretreatment groups. Treatment with 10 μM tubacin yielded significant improvements in the 20 and 200 nM DFP groups with and without CORT pretreatment (Figure 1E [bottom], F [right], G).

2.2 | Exposure to DFP alters microtubule dynamics in a manner exacerbated by CORT and correctable by tubacin

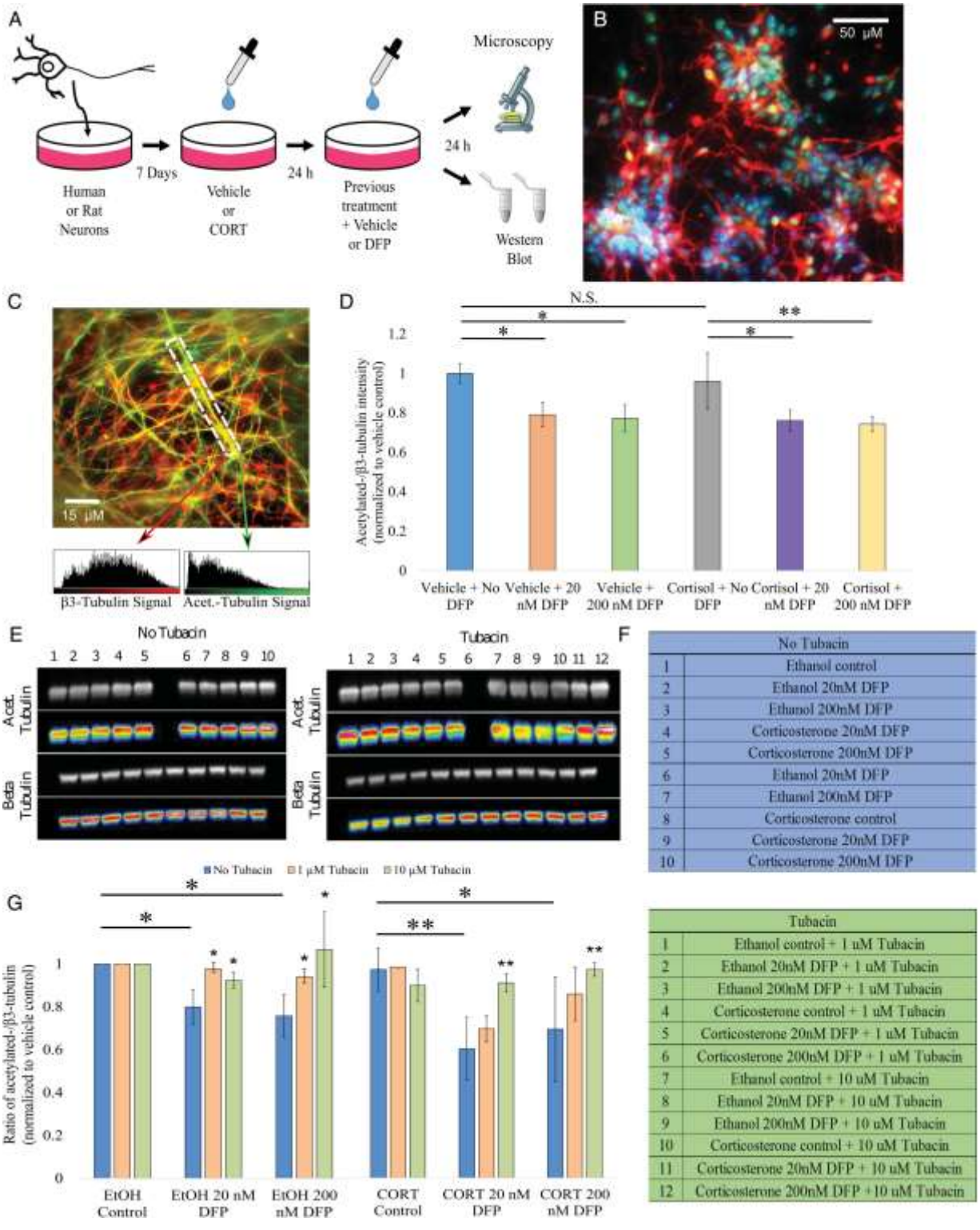
To explore microtubule dynamics, neurons were treated as before, but on DIV 9, green fluorescent protein (GFP)-EB3, a microtubule end-binding protein that affiliates with the growing tips of microtubules, was ectopically expressed and microtubule growth assessed 24 hours after DFP exposure. The excursion of GFP-EB3 at the plus end of the microtubule appears as a "comet" because of the gradual dissociation of EB3 molecules from the microtubules.[27] When neurons were treated with DFP alone, microtubule comet number was decreased at 20 nM (Figure 2B,C), but the rate of comet movement was not affected (Figure 2D; Movie S1). CORT pretreatment enhanced the effect of DFP, causing a reduction in comet number at 2 and 20 nM DFP, and yielding a reduction in comet rate at 20 nM DFP. Tubacin restored comet number to control levels but not rate (Figure 2D).

2.3 | Toxicant/stress exposure disrupts mitochondrial transport in tubacin-correctable manner

Mitochondria are the primary site of energy production and have been suggested to be the target of noncholinergic toxicity of OPs.[28] Past studies have indicated a link between exposure to higher concentrations of OP and oxidative stress and transport deficits after OP exposure.[29-31] Consistent with previous work, when neurons were treated with the DFP, transport of mitochondria was impaired and mitochondrial length increased, suggesting an increase in mitochondrial fusion. Mitochondria were stained with a live-cell mitochondrial dye called Mitotracker and live neurons were imaged (Figure 3A). An increase in stalled mitochondria was observed when neurons were treated with DFP, and the contribution to this effect was greater when cells were pretreated with CORT compared with DFP alone (Figure 3B, kymographs; Figure 3C, stacked bar graph; Movie S2). When treated with 10 μM tubacin, mitochondrial transport events were recovered (Figure 3D).

2.4 | Dopamine release is altered after DFP exposure in a manner exacerbated by CORT pretreatment

Dopamine and neurotransmission alterations have been reported after exposure to GW toxins,[1,4] which we hypothesize in part are due to changes in microtubules, which serve as tracks for synaptic vesicle



No Tubacin	
1	Ethanol control
2	Ethanol 20nM DFP
3	Ethanol 200nM DFP
4	Corticosterone 20nM DFP
5	Corticosterone 200nM DFP
6	Ethanol 20nM DFP
7	Ethanol 200nM DFP
8	Corticosterone control
9	Corticosterone 20nM DFP
10	Corticosterone 200nM DFP

Tubacin	
1	Ethanol control + 1 uM Tubacin
2	Ethanol 20nM DFP + 1 uM Tubacin
3	Ethanol 200nM DFP + 1 uM Tubacin
4	Corticosterone control + 1 uM Tubacin
5	Corticosterone 20nM DFP + 1 uM Tubacin
6	Corticosterone 200nM DFP + 1 uM Tubacin
7	Ethanol control + 10 uM Tubacin
8	Ethanol 20nM DFP + 10 uM Tubacin
9	Ethanol 200nM DFP + 10 uM Tubacin
10	Corticosterone control + 10 uM Tubacin
11	Corticosterone 20nM DFP + 10 uM Tubacin
12	Corticosterone 200nM DFP + 10 uM Tubacin

FIGURE 1 Pretreatment with corticosterone (CORT) or cortisol intensifies diisopropyl fluorophosphate (DFP)-induced aberrations in microtubule acetylation status in human and rat neurons. A, Schematic summary of experimental design. B, Validation of neuronal differentiation by immunostaining. βIII-tubulin (Biolegend Cat. #801202), which is specific to neurons and is significantly upregulated in mature neurons, is colored red. Tbr1, a transcription factor which serves as a marker for forebrain cortical glutamatergic neurons, is colored green, and 4', 6-Diamidino-2 Phenylindole, Dihydrochloride (DAPI), which stains nuclei, is blue. Scale bar represents 50 μm. C, Coimmunostaining of acetylated-tubulin (green; Sigma Cat. #T6793) and βIII-tubulin. The white checkered box represents the Region of Interest (ROI) to be analyzed with arrows pointing to the respective signal intensity values. Scale bar represents 15 μm. D, Bar graph depicting results from acetylated:total tubulin protein analysis of human neurons subjected to various conditions. E, Western blot acetylated-tubulin and βIII-tubulin bands from rat cortical neurons that after 24 hours with or without 10 μM tubacin treatment. F, Key identifying the conditions for each well (for easier identification, empty lanes seen were loaded with sample buffer alone). G, Bar graph depicting results from western blot analysis. Protein levels were normalized to cofilin (Abcam cat. # 42824) load control. Blots were cropped *$P < .05$; **$P < .01$; N.S., no significance found

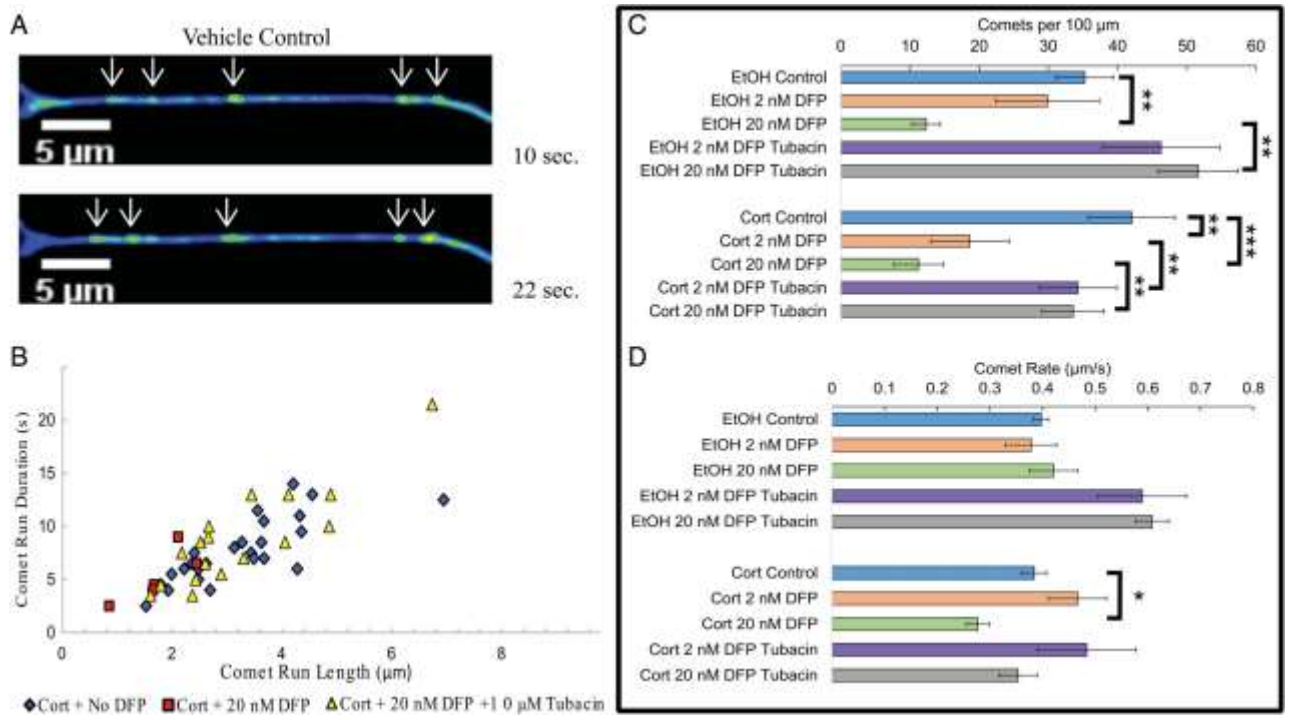


FIGURE 2 Exposure to diisopropyl fluorophosphate (DFP) alters microtubule dynamics in a manner exacerbated by corticosterone (CORT) and correctable by tubacin. A, Still frames from live-cell imaging movie of EB3 comets in rat cortical neurons that received vehicle with no DFP. Arrows indicate EB3 comets. B, Scatter plot of the observed polymerization events per axon (20 µm region of interest). C, Horizontal bar graph depicting the average number of comets per 100 µm across treatments with or without tubacin treatment. D, Horizontal bar graph depicting the average rate of observed comets across treatment groups with or without tubacin treatment. *$P < .05$; **$P < .01$; *** $P < .001$

transport. We cultured fetal ventral mesencephalic cells (dopamine precursor cells) and allowed them to grow for 7 days before submitting them to the treatment regimen (Figure 4A). At DIV 9, cultures were heterogeneous, with 27% ± 2% of control cells staining positive for tyrosine hydroxylase, a dopaminergic marker, and no significant difference across treatments (Figures 4B and S2A). Supernatant was collected from cultures that were exposed to DFP with or without CORT pretreatment, and subsequently treated with tubacin at 1 and 10 µM, or with vehicle. Supernatant was analyzed for dopamine content using high performance liquid chromatography (HPLC) (Figure 4C). Exposure to 2 and 20 nM DFP alone resulted in a significant reduction in extracellular dopamine (38% ± 5% and 40% ± 4%, respectively), and this reduction was even greater following CORT pretreatment (46% ± 5% and 55% ± 4%, Figure 4D). Treatment with 10 µM but not 1 µM tubacin was able to completely restore dopamine release after DFP alone, and partially restore release when cells were exposed to DFP after CORT pretreatment (Figure 4D). Tubacin did not significantly alter extracellular dopamine across control cells (Figure S2B).

3 | DISCUSSION

The present studies are the first to use cultured rodent and human-derived neurons to demonstrate that hormonal changes resulting from chemically induced stressors can exacerbate the negative consequences of OP exposure on microtubule and microtubule-related events in the axon. We used both human and rat neurons as a cross-validation because rat neurons are more amenable to established microtubule-related analyses, while human neurons are a better path toward therapy, given that some features of human neurodegeneration are not reflected in rodents.[32,33] In the future, an even more refined option would be to use induced pluripotent cell lines from the GW veterans themselves, given that genetic and possibly epigenetic factors potentially relevant to disease susceptibility would be preserved.[34] For now, the results on the human cells confirm that, as with the rat neurons, DFP causes a decrease in tubulin acetylation. The work on rat neurons indicates that pharmacologic restoration of tubulin acetylation, lowered by DFP ± CORT, rescues various cellular events impaired by DFP ± CORT.

While microtubule acetylation generally correlates with microtubule stability, the correlation depends upon the levels and activity of the enzyme(s) that acetylates tubulin after it becomes incorporated into the microtubule, as well as the enzyme(s) that deacetylates the tubulin after it is released from the microtubule during bouts of disassembly.[35-37] The live-imaging of microtubule dynamics shows DFP/CORT reduces the number of assembly excursions, which is not easily explained by diminished microtubule stability. Tubacin corrected for the deficit in microtubule dynamics, presumably because various proteins, such as microtubule-severing proteins, interact differently with the microtubule, depending on its acetylation status.[35] The finding that a higher tubacin concentration is needed to correct the acetylation deficit produced by higher DFP concentration suggests that the DFP/CORT may affect the levels or activity of the relevant enzymes. For example, if DFP/CORT increases HDAC6 levels or activity, a higher concentration of tubacin would be needed to inhibit the available enzyme.

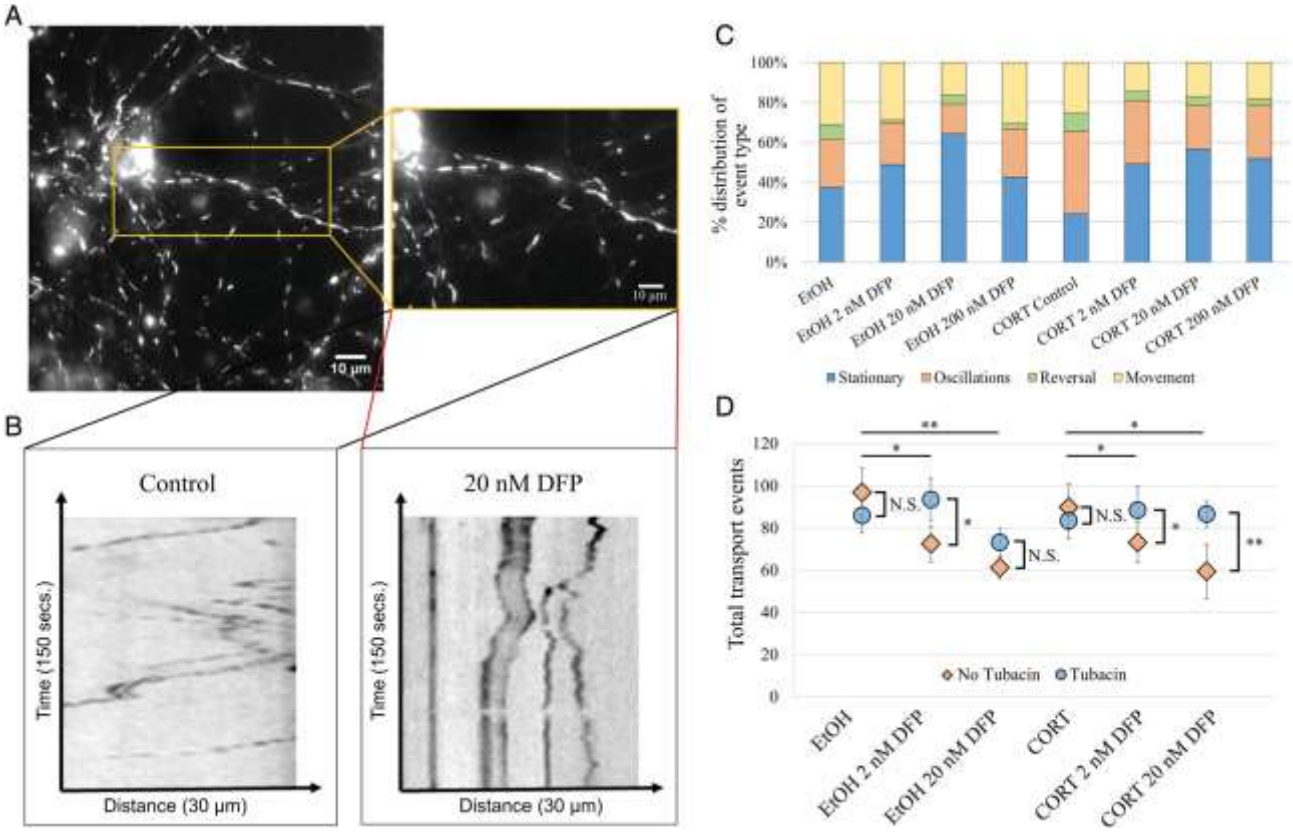


FIGURE 3 Toxicant/stress exposure disrupts mitochondrial transport in tubacin-correctable manner. A, Image of rat cortical neurons stained with Mitotracker Orange (Inset: magnified image of axon; ThermoFisher Scientific Cat. #M751). B, Representative kymographs depicting distance traveled over time for vehicle control (left) and ethanol + 20 nM diisopropyl fluorophosphate (DFP) (right). Cells were imaged for a total of 2.5 minutes at 1 frame per second. C, 100% stacked bar graph showing the distribution of transport event types across conditions. D, Dot plot depicting the average number of transport events across conditions with or without tubacin treatment. $^{*}P < .05$; $^{**}P < .01$

OPs used at subthreshold levels undoubtedly affect a variety of proteins and pathways, some of which are obviously microtubule-related and others of which are not as direct or obvious in their relationship to microtubules. In terms of therapy for sufferers of GWI, the question is whether restoring microtubule acetylation to a more normal status will translate into improvements in cognition and sleep, and help alleviate headaches and other symptoms of the disease. Deficits in mitochondria transport could explain some GWI symptoms, such as fatigue, and hence HDAC6 inhibitors might be helpful in treating those symptoms. We are especially encouraged by our dopamine release results, which suggest that a complex process directly related to neurotransmission, and one that could potentially go awry at many different levels, can be corrected by this treatment.

Reports exist in the literature of other neurodegenerative diseases that involve microtubule deacetylation, with symptoms improved in animal models by HDAC6 inhibitors.[38–41] In that regard, efforts are already underway to develop therapeutics that can be translated to human patients. Our next step is to test the effectiveness of such an approach on an animal model for GWI. From there, translation to human patients using new generations of HDAC6 inhibitors will be our goal.

In conclusion, the results presented here provide new mechanistic clues to the cellular basis of GWI, and point to a therapeutic strategy that may reverse neuronal decline in sufferers.

4 | MATERIALS AND METHODS

4.1 | Cell culture

4.1.1 | Rat cortical neurons

Primary cortical neurons were prepared as previously described.[42] Briefly, the pregnant Sprague-Dawley rat was euthanized on E18 in accordance with Public Health Services Policy on Humane Care and Use of Laboratory Animals, and fetuses were removed. The cortices from both hemispheres of each fetal rat brain were excised, dissociated and plated at a concentration of 500 000 cells per well in 6-well plates (for western blots) or 35 000 cells per well in glass-bottomed dishes (for live-cell imaging).

4.1.2 | Rat ventral mesencephalon neurons

The ventral mesencephalon was isolated from fetuses removed from a Sprague-Dawley rat on E14. Cells were isolated and dissociated according to a previously published protocol.[43]

4.1.3 | Human neural stem cells

Human induced-pluripotent stem cell (IPSC)-derived neural stem cells (ax0018), differentiation media, expansion media and maintenance media were purchased from Axol and cultures were established and differentiated into neurons according to the Axol Human IPSC-Derived Neural Stem Cell Protocol v5.0.

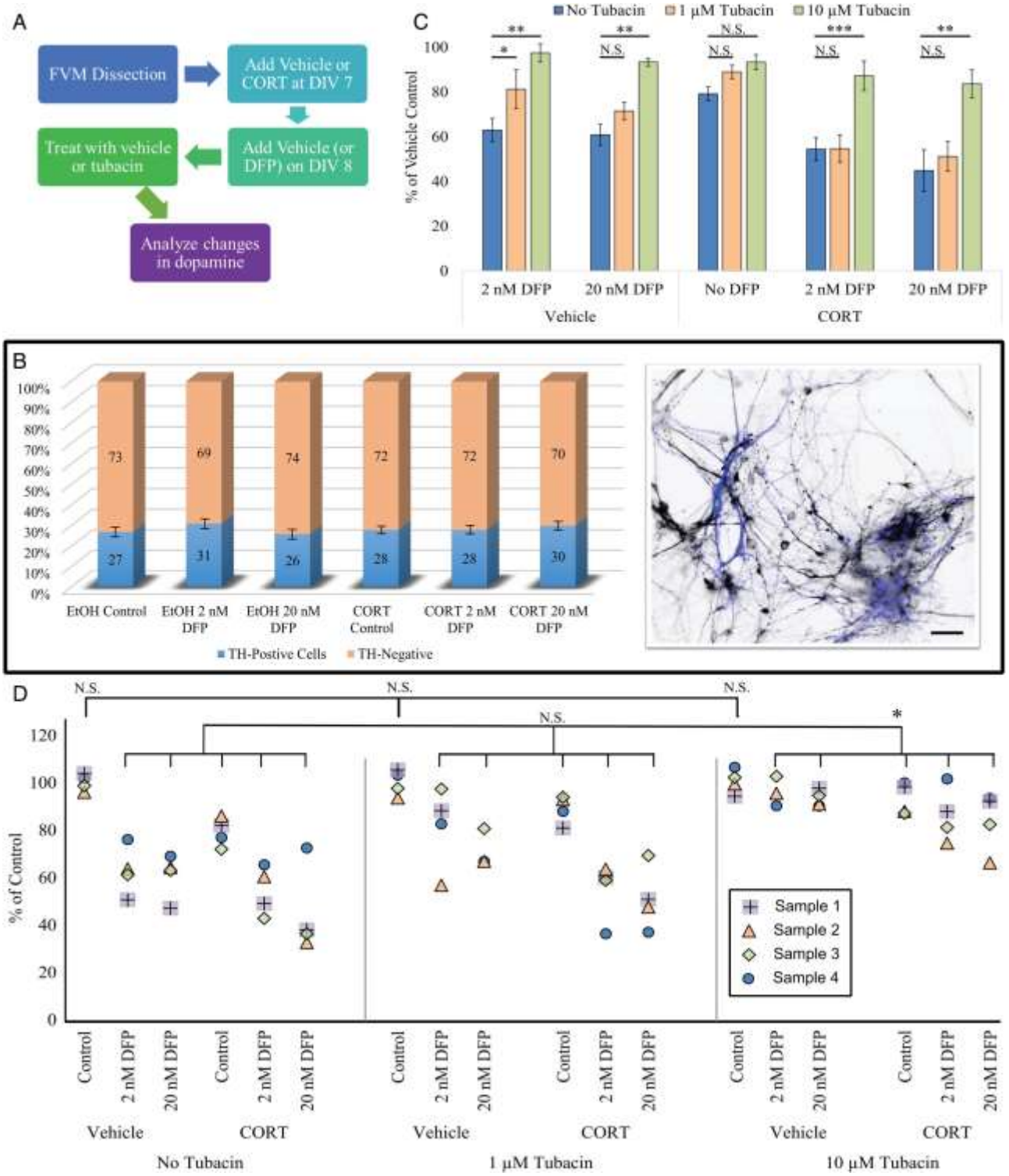


FIGURE 4 Dopamine release is altered after diisopropyl fluorophosphate (DFP) exposure in a manner exacerbated by corticosterone (CORT) pretreatment. A, Chart outlining design of dopamine release experiments. B, Left: Bar graph showing TH positive cells across DFP treatments. Right: Coimmunostain of fetal ventral mesencephalon neurons. Inverted dark cells are neurons stained for βIII-Tubulin, and blue cells represent neurons that are positive for tyrosine hydroxylase (EMD Millipore cat. #AB152). C, Bar graph showing dopamine release as a percent of vehicle control. Values were normalized to total TH positive neuron count. D, Dot plot of each sample (4 duplicates per condition, represented by different shape and color dots [see plot legend]) in the presence or absence of tubacin at 1 and 10 μM concentrations. Top brackets show no significant difference between EtOH controls. Bottom bracket shows no significant improvement in EtOH DFP or CORT DFP groups after treatment with 1 μM tubacin, and treatment with 10 μM tubacin yielded significant improvement in all groups when compared with control. * $P < .05$; ** $P < .01$; *** $P < .001$; N.S., no significance found. Scale bar represents 15 μm

4.2 | Treatment preparation and application

CORT or cortisol (Sigma), dissolved in ethanol to make a 2 mM stock solution, was diluted in culture media to make working concentrations. DFP (Sigma), dissolved in isopropanol to make a 100 μM stock solution, was diluted in culture media to make working concentrations. Experimental design is shown in Figure 1A. Cells were assayed at DIV 9 (no tubacin) or DIV 10 (24 h tubacin). The 2 lower concentrations of DFP used in this study are subthreshold (below the level need to inhibit acetylcholinesterase), while the 200 nM concentration may partially inhibit acetylcholinesterase.

4.3 | Live-cell imaging

4.3.1 | Assaying microtubule-based transport of mitochondria

Mitotracker CMH_2TMRos Orange was added directly into the media of primary cortical neuron cultures and incubated at 37°C for 25 minutes. After incubation, dishes were washed twice with serum-free media, and Fluorobrite DMEM imaging medium was added immediately before imaging. Dishes were imaged using a Zeiss AxioObserver Z1 inverted microscope equipped with a 1.4 NA 63X oil objective and an AxioCam mRm CCD camera. Images were captured every 1 second for 2.5 minutes at a fixed exposure time across conditions. Transport was analyzed using the MultiKymograph and DifferenceTracker plugins for (FIJI) ImageJ.

4.3.2 | Assessing changes in microtubule dynamics

Rat cortical neurons were plated at a density of 35 000 cells per well, allowed to grow for 8 days, and transfected to express GFP-tagged EB3 using Lipofectamine 2000 (ThermoFisher) according to the published product protocol. Cells were imaged using a Zeiss AxioObserver Z1 inverted scope equipped with a 1.46 NA ×100 oil objective and a Zeiss Axiocam 506 mono CCD camera. Images were captured every 1 second for a total of 2.5 minutes at a fixed exposure time across conditions. Movies were analyzed using the MultiKymograph plugin for FIJI (ImageJ).

4.4 | Determination of released dopamine after OP exposure

Extracellular dopamine was quantified by electrochemical detection-HPLC (HPLC-ECD). Reagents, chemicals, standard preparation and mobile phase were prepared as previously published.[44]

4.5 | Protein level analysis

4.5.1 | Whole cell lysate collection

Lysis buffer was prepared according to the Santa Cruz RIPA buffer lysis system (Santa Cruz; Cat. # sc-24948A) instructions with added phosphatase inhibitor. The media from culture wells were aspirated on DIV 10, and 200 μL of lysis buffer was added. Cells were detached using a cell scraper for 1 minute per well, and the lysis solution containing the cells was transferred to Eppendorf tubes. Lysate was incubated on ice for 45 minutes, sonicated and centrifuged at 10 000 revolutions per minute (RPM) for 10 minutes at 4°C. Supernatant was aliquoted and stored at −80°C until use.

4.5.2 | Protein quantification and western blotting

Protein content of each sample was quantified using the Thermo Scientific Pierce BCA Protein Assay Kit (ThermoFisher Scientific; Cat. # 23227). Western blotting was performed using standard methods, developed using the Bio-Rad Chemidoc MP Imaging System, and quantified using the Bio-Rad Image Lab software. Antibody information is provided in figure legends.

4.6 | Tubacin treatment

Tubacin (Cayman Chemical; Cat. # 537049-40-4) was used at a concentration of 1 or 10 μM, as established previously.[45] Tubacin was added into culture media immediately after the 24-hour DFP treatment and incubated with cells for 24 hours. Cells were then either lysed for western blotting or imaged to assess mitochondrial transport or microtubule dynamics. Supernatant was collected after 24 hours post tubacin.

4.7 | Experimental design and data analysis

Western blots and dopamine experiments were repeated 4 times to ensure reproducibility and power. Live-cell imaging experiments were performed using tissue from 3 different animals with at least 15 cells across 4 dishes per animal. Data were analyzed by blinded raters and statistical significance was assessed by student's *t* test, analysis of variance (ANOVA) followed by post hoc Bonferroni test, or the Kruskal-Wallis nonparametric ANOVA. All statistical analyses were performed using the IBM SPSS program.

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REFERENCES

1. Research Advisory Committee on Gulf War Veterans' Illnesses. *Gulf War Illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations*. Washington, D.C.: U.S. Government Printing Office; 2008.
2. Sullivan K, Krengel M, Proctor SP, Devine S, Heeren T, White RF. Cognitive functioning in treatment-seeking Gulf War veterans:

pyridostigmine bromide use and PTSD. *J Psychopathol Behav Assess*. 2003;25(2):95-103.
3. Proctor SP, Heaton KJ, Heeren T, White RF. Effects of sarin and cyclosarin exposure during the 1991 Gulf War on neurobehavioral functioning in US army veterans. *Neurotoxicology*. 2006;27(6):931-939.
4. White RF, Steele L, O'Callaghan JP, et al. Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: effects of toxicant exposures during deployment. *Cortex*. 2016;74:449-475.
5. Haley RW, Tuite JJ. Epidemiologic evidence of health effects from long-distance transit of chemical weapons fallout from bombing early in the 1991 Persian Gulf War. *Neuroepidemiology*. 2012;40(3):178-189.
6. Steele L, Sastre A, Gerkovich MM, Cook MR. Complex factors in the etiology of Gulf War illness: wartime exposures and risk factors in veteran subgroups. *Environ Health Perspect*. 2012;120(1):112.
7. Heaton KJ, Palumbo CL, Proctor SP, Killiany RJ, Yurgelun-Todd DA, White RF. Quantitative magnetic resonance brain imaging in US army veterans of the 1991 Gulf War potentially exposed to sarin and cyclosarin. *Neurotoxicology*. 2007;28(4):761-769.
8. Phillips KF, Deshpande LS. Repeated low-dose organophosphate DFP exposure leads to the development of depression and cognitive impairment in a rat model of Gulf War Illness. *Neurotoxicology*. 2016;52:127-133.
9. Ismail AA, Bodner T, Rohlman D. Neurobehavioral performance among agricultural workers and pesticide applicators: a meta-analytic study. *Occup Environ Med*. 2012;69(7):457-464.
10. EPA U. Pesticides Industry Sales and Usage 2006 and 2007 Market Estimates. Pesticides Industry Sales and Usage Reports 2011.
11. Yadav SS, Singh MK, Yadav RS. Organophosphates induced Alzheimer's disease: an epigenetic aspect. *J Clin Epigenet*. 2016; 2:1. doi: 10.21767/2472-1158.100010.
12. Wang A, Cockburn M, Ly TT, Bronstein JM, Ritz B. The association between ambient exposure to organophosphates and Parkinson's disease risk. *Occup Environ Med*. 2014;71(4):275-281. https://doi.org/10.1136/oemed-2013-101394.
13. Baltazar MT, Dinis-Oliveira RJ, de Lourdes Bastos M, Tsatsakis AM, Duarte JA, Carvalho F. Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases—a mechanistic approach. *Toxicol Lett*. 2014;230(2):85-103.
14. Hayden KM, Norton MC, Darcey D, et al. Occupational exposure to pesticides increases the risk of incident AD The Cache County Study. *Neurology*. 2010;74(19):1524-1530.
15. Merwin SJ, Obis T, Nunez Y, Re DB. Organophosphate neurotoxicity to the voluntary motor system on the trail of environment-caused amyotrophic lateral sclerosis: the known, the misknown, and the unknown. *Arch Toxicol*. 2017:1-14. doi: 10.1007/s00204-016-1926-1.
16. O'Callaghan JP, Kelly KA, Locker AR, Miller DB, Lasley SM. Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War Illness. *J Neurochem*. 2015;133(5):708-721.
17. Terry AV Jr, Gearhart DA, Beck WD Jr, et al. Chronic, intermittent exposure to chlorpyrifos in rats: protracted effects on axonal transport, neurotrophin receptors, cholinergic markers, and information processing. *J Pharmacol Exp Ther*. 2007;322(3):1117-1128.
18. Terry AV Jr, Callahan PM, Beck WD, et al. Repeated exposures to diisopropylfluorophosphate result in impairments of sustained attention and persistent alterations of inhibitory response control in rats. *Neurotoxicol Teratol*. 2014;44:18-29.
19. Parihar VK, Hattiangady B, Shuai B, Shetty AK. Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. *Neuropsychopharmacology*. 2013;38(12):2348-2362.
20. Jiang W, Duysen EG, Hansen H, Shlyakhtenko L, Schopfer LM, Lockridge O. Mice treated with chlorpyrifos or chlorpyrifos oxon have organophosphorylated tubulin in the brain and disrupted microtubule structures, suggesting a role for tubulin in neurotoxicity associated with exposure to organophosphorus agents. *Toxicol Sci*. 2010;115(1):183-193.
21. Seifert J, Casida JE. Possible role of microtubules and associated proteases in organophosphorus ester-induced delayed neurotoxicity. *Biochem Pharmacol*. 1982;31(11):2065-2070.
22. Lockridge O, Schopfer LM. Review of tyrosine and lysine as new motifs for organophosphate binding to proteins that have no active site serine. *Chem Biol Interact*. 2010;187(1):344-348.
23. Gearhart DA, Sickles DW, Buccafusco JJ, Prendergast MA, Terry AV. Chlorpyrifos, chlorpyrifos-oxon, and diisopropylfluorophosphate inhibit kinesin-dependent microtubule motility. *Toxicol Appl Pharmacol*. 2007;218(1):20-29.
24. Grigoryan H, Schopfer LM, Peeples ES, et al. Mass spectrometry identifies multiple organophosphorylated sites on tubulin. *Toxicol Appl Pharmacol*. 2009;240(2):149-158.
25. Li Y, Lein PJ, Liu C, et al. Spatiotemporal pattern of neuronal injury induced by DFP in rats: a model for delayed neuronal cell death following acute OP intoxication. *Toxicol Appl Pharmacol*. 2011;253(3):261-269.
26. Gardiner J, Overall R, Marc J. The microtubule cytoskeleton acts as a key downstream effector of neurotransmitter signaling. *Synapse*. 2011;65(3):249-256.
27. Stepanova T, Slemmer J, Hoogenraad CC, et al. Visualization of microtubule growth in cultured neurons via the use of EB3-GFP (end-binding protein 3-green fluorescent protein). *J Neurosci*. 2003;23(7):2655-2664.
28. Masoud A, Kiran R, Sandhir R. Impaired mitochondrial functions in organophosphate induced delayed neuropathy in rats. *Cell Mol Neurobiol*. 2009;29(8):1245-1255.
29. Soltaninejad K, Abdollahi M. Current opinion on the science of organophosphate pesticides and toxic stress: a systematic review. *Med Sci Monit*. 2009;15(3):RA75-RA90.
30. Morfini GA, Burns M, Binder LI, et al. Axonal transport defects in neurodegenerative diseases. *J Neurosci*. 2009;29(41):12776-12786.
31. Gao J, Naughton SX, Wulff H, et al. Diisopropylfluorophosphate impairs the transport of membrane-bound organelles in rat cortical axons. *J Pharmacol Exp Ther*. 2016;356(3):645-655.
32. Mestas J, Hughes CCW. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172(5):2731-2738.
33. Götz J, Deters N, Doldissen A, et al. A decade of tau transgenic animal models and beyond. *Brain Pathol*. 2007;17(1):91-103.
34. Qiang L, Rao AN, Mostoslavsky G, et al. Reprogramming cells from Gulf War veterans into neurons to study Gulf War illness. *Neurology*. 2017;88:1968-1975.
35. Howes SC, Alushin GM, Shida T, Nachury MV, Nogales E. Effects of tubulin acetylation and tubulin acetyltransferase binding on microtubule structure. *Mol Biol Cell*. 2014;25(2):257-266.
36. Kull FJ, Sloboda Roger D. A slow dance for microtubule acetylation. *Cell*. 2014;157(6):1255-1256.
37. Zilberman Y, Ballestrem C, Carramusa L, Mazitschek R, Khochbin S, Bershadsky A. Regulation of microtubule dynamics by inhibition of the tubulin deacetylase HDAC6. *J Cell Sci*. 2009;122(19):3531-3541.
38. Wang Z, Leng Y, Wang J, et al. Tubastatin A, an HDAC6 inhibitor, alleviates stroke-induced brain infarction and functional deficits: potential roles of α-tubulin acetylation and FGF-21 up-regulation. *Sci Rep*. 2016;6:19626.
39. d'Ydewalle C, Krishnan J, Chiheb DM, et al. HDAC6 inhibitors reverse axonal loss in a mouse model of mutant HSPB1-induced Charcot-Marie-Tooth disease. *Nat Med*. 2011;17(8):968-974.
40. Iwata A, Riley BE, Johnston JA, Kopito RR. HDAC6 and microtubules are required for autophagic degradation of aggregated huntingtin. *J Biol Chem*. 2005;280(48):40282-40292.
41. Rivieccio MA, Brochier C, Willis DE, et al. HDAC6 is a target for protection and regeneration following injury in the nervous system. *Proc Natl Acad Sci USA*. 2009;106(46):19599-19604.
42. Pacifici M, Peruzzi F. Isolation and culture of rat embryonic neural cells: a quick protocol. *J Vis Exp*. 2012;63:e3965.
43. Pruszak J, Just L, Isacson O, Nikkhah G. Isolation and culture of ventral mesencephalic precursor cells and dopaminergic neurons from rodent brains. *Curr Protoc Stem Cell Biol*. 2009;Chapter 2:Unit 2D 5. doi: 10.1002/9780470151808.sc02d05s11.
44. Brodnik ZD, Jaskiw GE. Effect of mobile phase pH on the function of other optimization parameters in an HPLC-ECD assay of biogenic

amines and their metabolites. *J Liq Chromatogr Relat Technol*. 2015;38(4):467-471.
45. Sudo H, Baas PW. Acetylation of microtubules influences their sensitivity to severing by katanin in neurons and fibroblasts. *J Neurosci*. 2010;30(21):7215-7226.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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MEDICAL HYPOTHESIS

Reprogramming cells from Gulf War veterans into neurons to study Gulf War illness

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ABSTRACT

Gulf War illness (GWI), which afflicts at least 25% of veterans who served in the 1990–1991 war in the Persian Gulf, is thought to be caused by deployment exposures to various neurotoxicants, including pesticides, anti–nerve gas pills, and low-level nerve agents including sarin/cyclosarin. GWI is a multisymptom disorder characterized by fatigue, joint pain, cognitive problems, and gastrointestinal complaints. The most prominent symptoms of GWI (memory problems, poor attention/concentration, chronic headaches, mood alterations, and impaired sleep) suggest that the disease primarily affects the CNS. Development of urgently needed treatments depends on experimental models appropriate for testing mechanistic hypotheses and for screening therapeutic compounds. Rodent models have been useful thus far, but are limited by their inability to assess the contribution of genetic or epigenetic background to the disease, and because disease-vulnerable proteins and pathways may be different in humans relative to rodents. As of yet, no postmortem tissue from the veterans has become available for research. We are moving forward with a paradigm shift in the study of GWI, which utilizes contemporary stem cell technology to convert somatic cells from Gulf War veterans into pluripotent cell lines that can be differentiated into various cell types, including neurons, glia, muscle, or other relevant cell types. Such cell lines are immortal and will be a resource for GWI researchers to pursue mechanistic hypotheses and therapeutics. *Neurology*® 2017;88:1968–1975

GLOSSARY

DFP 5 di-isopropyl fluorophosphates; GW 5 Gulf War; GWI 5 Gulf War illness; GWIC 5 Gulf War Illness Consortium; hiN 5 human induced neurons; hiPSC 5 human induced pluripotent stem cells; OP 5 organophosphate; PB 5 pyridostigmine bromide.

Gulf War illness (GWI) encompasses a constellation of debilitating symptoms experienced by over 25% of the nearly 700,000 US soldiers who served in the 1990–1991 war.[1] The symptoms are primarily deficits in CNS functioning, but also include gastrointestinal and musculoskeletal complaints. The CNS symptoms, which include diminished short-term memory, poor attention/concentration, chronic headaches, fatigue, and impaired sleep, are consistent with chronic exposure to neurotoxicants including organophosphate (OP) pesticides and nerve agents.[2–4] Gulf War (GW) veterans were exposed to toxicants including OP pesticides, pyridostigmine bromide (PB) anti–nerve gas pills, and low-level sarin nerve agents.[1,5] However, a mechanistic explanation for the association of these toxicants to GWI remains undetermined, and there are no current treatments that have substantially improved cognitive functioning or other chronic health problems of veterans with GWI. Urgency is heightened by the fact that environmental pollutants, biological warfare, and terrorism could lead to far greater numbers of patients with diseases with similar etiology to GWI.

As with any human disease, mechanistic studies in the preclinical laboratory have started with animal models, with rodents favored over invertebrate models such as flies or worms. In one early rodent model, rats were exposed to PB, the sarin-surrogate di-isopropyl fluorophosphate (DFP), and the insect repellent N,N-diethyl-meta-toluamide (DEET), and then assessed for behavioral and histologic deficits.[6,7] Deficits were noted with or without combining the

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neurotoxicant exposures with a physical stressor, namely temporary restraint of the animal,[8,9] but were heightened with the addition of the physical stressor.[9,10] This observation led to the view that chronic GWI results from the combined effects of physical stressors with the toxicants.[9] Subsequent studies have used corticosterone to simulate the effects of physical stressors in combination with DFP to create an animal model of chronic GWI.[11] Also relevant is the fact that veterans exposed to the same deployment-related conditions may or may not have contracted GWI, suggesting that genetic or epigenetic factors may have contributed to any individual soldier's susceptibility to the illness.[12] In fact, recent animal models have shown miRNA and other epigenetic changes in GWI-exposed animal models.[13]

Despite progress, there has been concern about the continued use of animal models in GWI research by the National Academy of Sciences Institute of Medicine.[14] This is because human diseases often involve proteins and pathways that are not well-reflected in rodents, and because animal work cannot appropriately take into account myriad genetic and epigenetic factors relevant to human disease. In light of these issues, there is value in studying human cells, especially if they come directly from GW veterans. Toward this end, the US Department of Defense has funded us to generate a bank of human induced pluripotent stem cells (hiPSC) from the blood of veterans with GWI that can be used for mechanistic studies and to test therapeutic compounds. hiPSC are self-renewing cell lines generated by reprogramming terminally differentiated cells so that they become pluripotent, similar to embryonic stem cells. These cells can then be treated with various growth and patterning factors that induce them to differentiate into different lineages, including various types of neurons and non-neuronal cells of the nervous system, as well as other cell types such as muscle. hiPSC are gaining popularity as models for a number of different human diseases, including neurodegenerative disorders.[15]

The advantages of hiPSC are that they (1) are human cells, and therefore have the human proteins and pathways that may not be appropriately reflected in animal models; (2) are from the patients themselves, which means that they harbor the array of genetic factors that may contribute to the disease or susceptibility of the disease; (3) may retain some of the epigenetic factors that might also contribute[16]; (4) can be differentiated into a vast array of cell types and can also be built into complex organoid and tissue ensembles reflective of parts of the body such as the brain[17,18]; (5) enable rapid and high-throughput experiments and screens of therapeutic compounds; and (6) can be passaged an unlimited number of times so that each stock can be expanded indefinitely, without concern of stocks running out or changing due to too many passages. In addition, results obtained with these cells can be compared against results of parallel studies on an array of neurodegenerative diseases that might share elements of their etiology with GWI.

WHAT DO WE KNOW ABOUT GWI FROM RODENT MODELS AND CLINICAL STUDIES? Rodent studies of GW-relevant OP neurotoxicants have shown deficits in the axonal transport of proteins and organelles, as well as alterations of microtubules, which form the structural tracks for axonal transport.[19–22] It has been reported that rodents exposed to GW-relevant (OP) pesticides and nerve agents display brain microtubules with fewer associated proteins than in a normal situation, leading to reduced microtubule width.[19–23] This reduced association of such proteins to the microtubules may be due to modifications in those proteins or to changes in the tubulin backbone of the microtubule. Molecular motor proteins move less efficiently on the microtubules, and the microtubules may be less stable without their normal complement of associated proteins.[20,24] Mitochondria are carried down the axon by axonal transport and supply the energy needed for axonal functioning. Several studies have now shown that GW-relevant OP exposures affect multiple facets of axonal transport and mitochondrial dynamics that could lead to GW symptoms of cognitive complaints and fatigue.[21,24,25] Alterations in axonal transport mechanisms can result in deficits ranging from delayed information processing and cognitive complaints to the development of neurodegenerative conditions such as those observed in patients with Alzheimer disease and amyotrophic lateral sclerosis.[26,27]

Chronic low-level OP exposures similar to what GW veterans experienced have been associated with mitochondrial damage as a result of oxidative stress and neuroinflammation.[28] One hypothesis of GWI

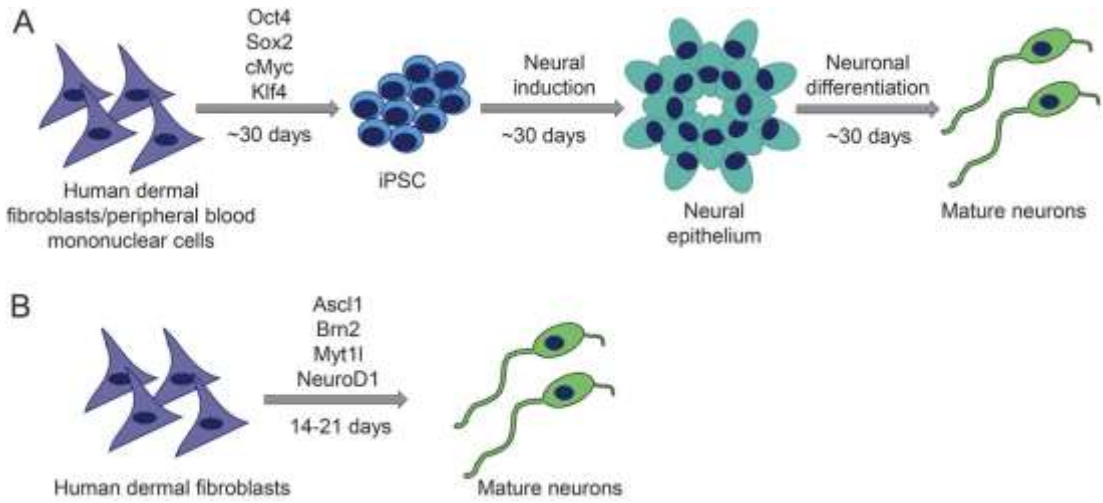
suggests that toxicant exposures caused the chronic symptoms of GWI by directly damaging microtubules or mitochondria in a manner that the neurons and other affected cells are not equipped to self-repair.[29,30] A second GWI hypothesis suggests that GW toxicants affected the brain in an additive manner to cause chronic, ongoing neuroinflammation. This chronic neuroinflammation is then thought to negatively affect microtubules and other cellular structures (including mitochondria), pathways, and mechanisms relevant to axonal transport.[11,29,30] These 2 hypotheses of GWI, which are not mutually exclusive, remain difficult to assess in current clinical models.

Recent clinical studies comparing brain imaging and cognitive functioning in GW veterans have shown significant evidence of altered CNS functioning in veterans with GWI.[5,31–33] However, overlap between individual GWI cases and controls on the outcome measures has precluded their use as objective diagnostic markers. Mechanistic studies have been impeded also by a lack of available postmortem brain tissue.

HUMAN INDUCED NEURONS—BRIDGING THE GAP WITH A PARADIGM SHIFT Seminal work showed that a cocktail of 4 pluripotency factors in viral expression vectors can effectively reprogram human somatic cells to a pluripotent cell fate within weeks.[34] Toward modeling various neurologic disorders, neurons of various types have been generated from hiPSC, such as dopaminergic, spinal motor, cortical glutamatergic, and GABAergic neurons.[35] Phenotypes corresponding to human neurodegenerative disorders that have not been reproduced in laboratory animals have, in some instances, been reproduced in hiPSC.[36] A major advantage of patient-derived cells is that many diseases cannot be traced to just one gene being mutated, but rather are due to complex interactions of genes, or genes that remain unknown. Other diseases or susceptibility to the disease are not entirely genetically based but may be due at least in part to epigenetic factors. Patient-derived cells preserve the complete genetic composition of the patient and may also preserve some epigenetic modifications.[16]

More recently, direct conversion of somatic cells into neurons has been achieved by altering the complement of transcription and growth factors. These directly converted neurons, termed human induced neurons (hiN), have advantages and disadvantages compared to neurons from the hiPSC approach (figure 1 and table). The extensive cloning process used in the hiPSC approach may overwhelm subtle disease phenotypes present in patient cells, and if so, this problem can be avoided with hiN. Also, because hiN bypass the pluripotent state, they preserve more epigenetic factors,[37] and also obviate the need for the analysis of multiple colonies from individuals. Recently, age-related transcriptome analyses in hiPSC-derived neurons and hiN from individuals of different ages suggested that certain epigenetic states/signatures, such as those being induced by aging, were erased or reset to the embryonic stage in the case of hiPSC but were largely retained in directly reprogrammed hiN.[37] However, current hiN approaches also hold some critical liabilities: for instance, the cells seem to be relatively immature and more vulnerable; only a few neuronal subtypes have been achieved through direct conversion; and they are not expandable, which limits their utilization for any genetic manipulation.

Figure 1 Schematic of neuronal induction using 2 different reprogramming strategies



(A) Neuronal induction from human induced pluripotent stem cells (iPSC). (B) Direct conversion from somatic cells (e.g., fibroblasts) to mature neurons.

Table Comparison of human induced neurons (hiN) and human induced pluripotent stem cells (hiPSC)

	hiN	hiPSC
Transcription factors	Ascl1, Brn2, Myt1l, NeuroD1	Oct4, Sox2, Klf4, c-Myc
Destination cell type	Neurons	Pluripotent stem cells
Modeling system	Neuronal	Various tissue-specific cells
Efficiency	Relatively high	Low
Duration of the process, wk	2–4	.12
Procedure complexity	Low	High
Colony formation and selection	No	Yes
Aging signature	Retained	Erased
Tumorigenesis	None	High

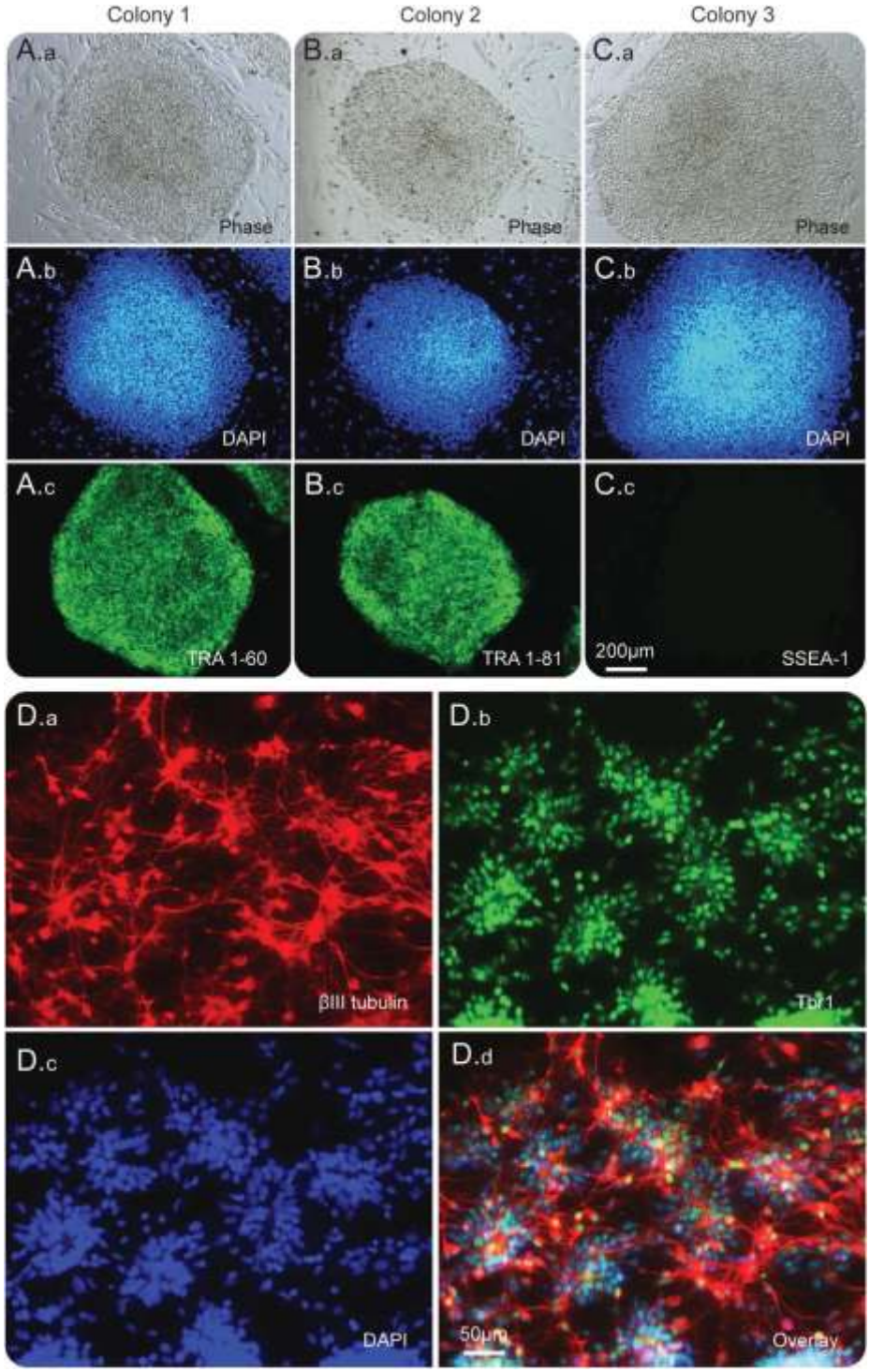
We are obtaining peripheral blood from GW veterans with GWI and also from GW veterans without GWI as controls. Mononuclear cells are being isolated from the peripheral blood and are being reprogrammed into hiPSC. These immortalized cell lines are being frozen and stored in a repository to be used for our own studies related to microtubules and axonal transport, and will also be made available for collaborative projects on other potential mechanistic hypotheses and therapeutic strategies across the GWI research community. Although we are prepared to carry out skin biopsy procedures to collect the skin fibroblasts from the recruited veterans for hiN conversion, extra mononuclear cells are also being isolated and frozen from blood for testing of direct conversion methods. With permission from each veteran donor, demographic information is being tallied as well as the particular symptoms experienced by those with GWI.

RECRUITMENT PROCESS AND INFORMED CONSENT We are currently recruiting blood donors from the 300 study participants from the Gulf War Illness Consortium (GWIC), funded by the US Department of Defense (PI: Kimberly Sullivan). At the Boston GWIC site, 175 study participants are being recruited. The human neuron studies are considered a follow-up to the GWIC study and include one clinic visit for the study participant. A participant is considered eligible if he or she is a 1990–1991 GW veteran who does not have any relevant medical exclusions. GWI cases are determined if the veteran meets the Kansas GWI criteria by endorsing moderately severe or multiple symptoms in 3 of 6 health symptom domains (pain, fatigue, neurologic/cognitive/mood, skin, gastrointestinal, respiratory) on the Kansas questionnaire.[38] Veterans are required to have served in the Gulf War for any period between August 1990 and July 1991 and to have participated in the GWI consortium main study. Exclusionary criteria include a history of prior CNS or major psychiatric disorders that may affect cognitive function (e.g., epilepsy, stroke, brain tumor, multiple sclerosis, Parkinson disease, Alzheimer disease, schizophrenia). GW veterans who meet criteria for GWI based on the Kansas GWI case criteria (i.e., cases) or GW veterans who do not meet criteria for GWI based on Kansas criteria (controls) but also do not meet any Kansas exclusion criteria and have participated in the GWIC main study are eligible to participate. GW veterans who have met any of the exclusion criteria for the Kansas GWI case criteria are excluded from this study. Veterans meeting the Kansas criteria but without any symptoms of GWI are taken into the study as controls. The veterans are informed and they agree in writing that their stem cells will become immortalized and used for future treatment and mechanistic studies of GWI.

The number of cell lines that we are funded to generate (4–6 cell lines from veterans with GWI and 4–6 from veterans without GWI) are not sufficient to evaluate the contribution of such factors as race, sex, or genetic background to the disease. For this reason, recruited veterans for the cell lines are primarily male, Caucasian, and age-matched, although future potential funding may allow for an expansion of the number of cell lines from veterans of a broader range of demographic backgrounds. The present number of cell lines is also insufficient to draw strong conclusions on the potential relevance of genetic or epigenetic differences among them. However, clinical data, such as neural imaging, obtained from the same veterans may yield some initial insights into the severity and nature of the disease corresponding to each of the cell lines. Especially notable differences among the cell lines may provide clues that can be pursued in the future with greater numbers of cell lines.

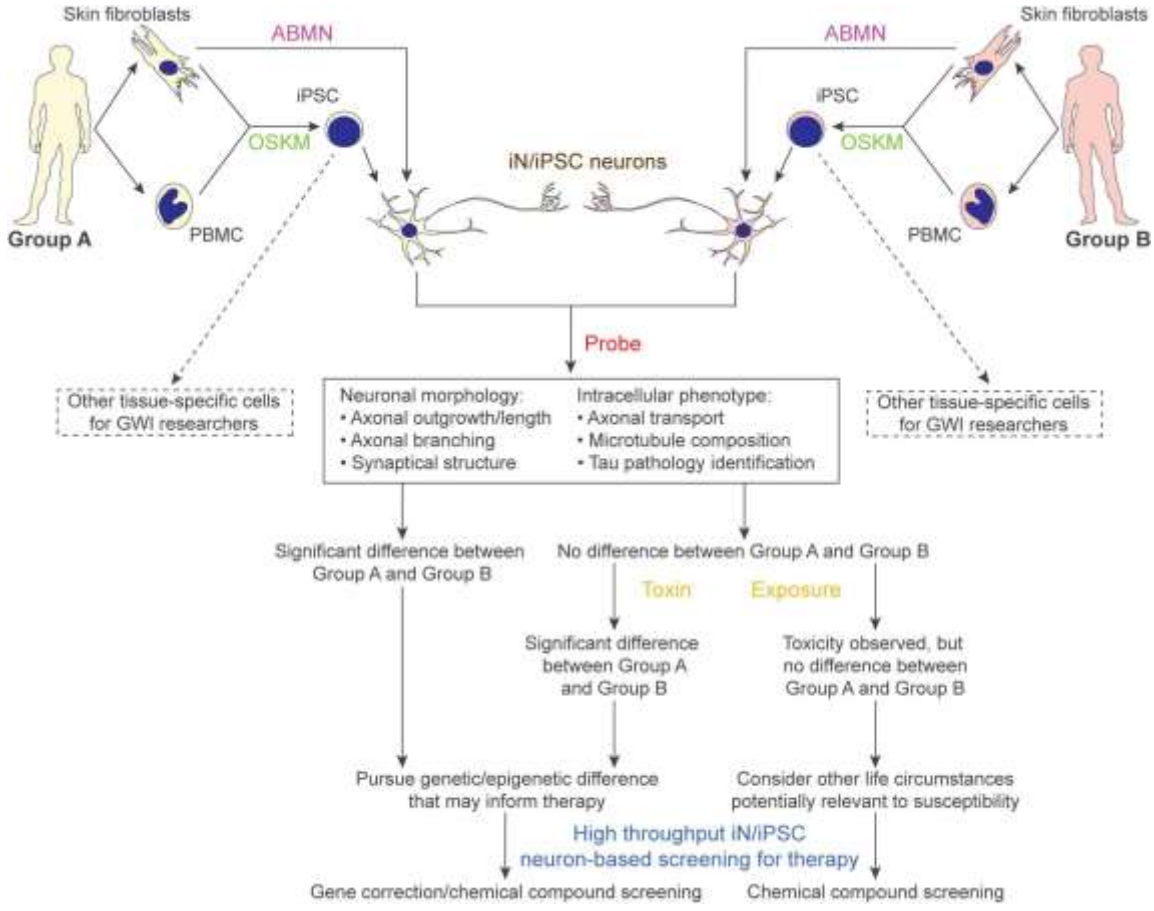
CONTROL SAMPLES A challenging element to our plan was the decision regarding what to use for control sample hiPSC cells. We considered using cells from veterans of similar age who had not been deployed to the GW. However, epidemiologic studies indicate that comparing deployed ill vs deployed non-ill samples have proven more effective in identifying epidemiologic, cognitive, and biomarker differences between groups.[1,5] We decided, for 2 reasons, on cells from veterans who were also deployed to the GW but who did not develop GWI. First, we simply did the best that we could to choose cells as close as possible to the cells from GWI cases. Second, we considered the possibility that the individuals who contracted GWI might be genetically or epigenetically predisposed to contracting the illness from the neurotoxicant exposures, and hence that

Figure 2 Human induced pluripotent stem cells (hiPSC) validation and neuronal differentiation



A.a–A.c, B.a–B.c, and C.a–C.c are 3 individual iPSC colonies from 1 of the recruited veterans (1039-2). A.a, B.a, and C.a are phase-contrast images for the 3 individual colonies; A.b, B.b, and C.b are DAPI (nuclear stain) counterstains; colony 1 and 2 are immunostained with pluripotency marker TRA 1-60 (A.c) and TRA 1-80 (B.c). C.c from colony 3 shows that SSEA-1 (only expressed in murine iPSC) is not expressed in the hiPSC; also this indicates that the hiPSC are undifferentiated, since the expression of SSEA-1 is upregulated during hiPSC differentiation. D.a–D.d are the immunostaining validation of the neuronal differentiation of the hiPSC. D.a is the immunostaining for βIII tubulin and D.b is the immunostaining for Tbr1. βIII tubulin is a neuronal specific tubulin whose expression is significantly upregulated in mature neurons; and Tbr1 is a transcription factor which serves as a marker for forebrain cortical glutamatergic neurons. D.c and D.d are DAPI counterstain image and the overlay image.

Figure 3 Schematic flow chart for planned experimental regimen



Group A, Gulf War (GW) veterans without GW illness (GWI); group B, GW veterans with GWI. The types of assessments that will be done on the cells depend on the hypothesis being pursued. Our initial plan, to probe phenotypes mentioned in the chart, will be to measure lengths of axons after toxicant exposures, as well as to count branches along the axons as normalized per 100 mm, quantify synapses by performing patch-clamp recording to quantify spontaneous activities and by immunostaining for synaptophysin or synapsin, examine axonal transport by performing live-cell imaging using fluorescent markers for various organelles, and examine microtubule structure by immunocytochemistry and Western blotting using antibodies to various tubulin variants and microtubule-associated proteins. We are especially interested in studying tau, a microtubule-associated protein that dissociates from microtubules to form abnormal filaments in a number of human neurodegenerative diseases, a pathologic phenomenon not well reflected in rodent neurons.[36] One-way analyses of variance will be used for all the statistic quantitations with n ˛ 50. ABMN 5 transcription factors Ascl1, Brn2, Myt1l, and NeuroD1; fibs 5 fibroblasts; iN 5 induced neurons; iPSC 5 induced pluripotent stem cells; OSKM 5 transcription factors Oct4, Sox2, Klf4, and c-Myc; PBMC 5 peripheral blood mononuclear cells.

there may be something to be learned from comparing cells from equally exposed veterans who either did or did not get sick.[13] By no means did we think that cells from any human being would be unresponsive to the relevant toxicants, but perhaps the sensitivity would be greater in one group compared to the other, or perhaps the reaction would be qualitatively different. For example, neurons derived from the veterans who contracted the illness might react to the toxicants by hyperphosphorylating tau, a microtubule-related protein that becomes aberrantly phosphorylated in a number of neurodegenerative conditions,[36] but the same would not be true of the neurons from the veterans who did not contract the illness. This might be due to a predisposition of the former group to factors that contribute to tauopathies such as Alzheimer disease. Along these lines, there may be a certain threshold of the toxicants that kills any neuron, but below that threshold, the cells of some people can recover while the cells of other people cannot and hence acquire GWI.[39] As noted earlier, a greater number of cell lines will most likely be needed to draw strong conclusions, but the current number may provide an initial set of clues.

In the case of GWI, it is not known whether newly differentiated neurons (or other cell types) from blood cells will "have the disease" or essentially start fresh, without the disease. If the former is the case, then we can simply compare cell lines from veterans with GWI against veterans without GWI, with no need to re-expose them to GW toxicants. Such a scenario might be due, for example, to chromosomal or epigenetic damage that affects cells from multiple systems (i.e., hepatic cells, muscle cells, neurons).[13] More likely, the newly differentiated neurons will represent the status of the veteran prior to exposure

to the toxicants, and the neurons will have to be exposed to GW toxicants (as well as cortisol to model the physical stressors of the war) to mimic the disease phenotype.

EXPERIMENTAL PARADIGM Mononuclear cells from the veterans are being isolated from the peripheral blood and reprogrammed into hiPSC at the Center for Regenerative Medicine at Boston University. Additional peripheral blood mononuclear cells have been stored for potential direct conversion procedures. If necessary, these individuals can be recruited for potential skin biopsies in order to obtain skin fibroblasts for the direct conversion procedures. Our first round of studies will be on cells differentiated into cortical forebrain neurons. Figure 2 shows hiPSC validation and neuronal differentiation from the hiPSC (see figure legend for details). The outline of our experimental strategy is displayed as a flow chart in figure 3. Briefly, neurons from the 2 groups will be compared without the toxicants for differences in such readouts as morphology and axonal transport. Should differences be observed, we will further probe for the possibility of genetic pre-deposition or epigenetic variations in the 2 groups.[13] However, should no differences be obtained, the toxicants, such as DFP or PB, will be added into the neuronal cultures from the 2 groups followed by the experimental analyses.

A challenge worth mentioning is the difficulty of reproducing the levels or complexity of toxicant exposures in the culture dish to match those experienced by soldiers in the battlefield. For this reason, experiments need to be performed with a range of toxicant concentrations, with informed knowledge of the biochemistry of the particular toxicant. For example, OPs are known to inhibit acetylcholinesterase above a certain threshold concentration, but subthreshold levels are likely more relevant to GWI.[21,25,40] Especially when more cell lines become available, attention will be given to variability not only between subject groups but also among individual lines, as line-to-line differences could provide insight into contributing factors to GWI that may be correlated to symptom severity.

A NOTE TO PHYSICIANS Research on the mechanisms of GWI will allow for the development of novel therapies that will benefit future patients with toxicant exposures similar to GWI. At present, our best chances of helping current patients with GWI is rapid-throughput studies on already available and approved therapeutics. Our cell lines are optimal for this purpose, and we look forward to coordinating with physicians who can rapidly translate our most promising results into urgently needed therapy for the veterans. The best progress can be made through an interactive relationship between scientists working in the laboratory on the human cell lines and physicians working directly with veterans in the clinic.

AUTHOR CONTRIBUTIONS

Liang Qiang: Stem cell expert who designed the studies and wrote the manuscript. Anand N. Rao: Student conducting primary research on stem cells for Gulf War illness. G. Mostoslavsky: Director of the stem cell facility commissioned to produce the cell lines. Marianne F. James: Scientist in the stem cell facility responsible for the cell lines. Nicole Comfort: Assistant to Dr. Sullivan in working with the veterans to obtain their blood for the production of the cell lines. Kimberly Sullivan: Gulf War illness expert and coordinator of the effort to obtain blood from veterans for the production of the cell lines; contributed to writing manuscript. Peter W. Baas: Leader of the project and wrote the manuscript with Liang Qiang.

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DISCLOSURE

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

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REFERENCES

1. RAC. Gulf War Illness and the Health of Gulf War Veterans: Scientific Findings and Recommendations. Washington, DC: US Government Printing Office; 2008.
2. Ismail AA, Bodner T, Rohlman D. Neurobehavioral performance among agricultural workers and pesticide applicators: a meta-analytic study. Occup Environ Med 2012;69:457–464.
3. Ross SM, McManus I, Harrison V, Mason O. Neurobehavioral problems following low-level exposure to organophosphate pesticides: a systematic and meta-analytic review. Crit Rev Toxicol 2013;43:21–44.
4. Abou-Donia MB, Abou-Donia MM, ElMasry EM, Monro JA, Mulder MF. Autoantibodies to nervous system-specific proteins are elevated in sera of flight crew members: biomarkers for nervous system injury. J Toxicol Environ Health A 2013;76:363–380.
5. White RF, Steele L, O'Callaghan JP, et al. Recent research on Gulf War illness and other health problems in veterans of the 1991 Gulf War: effects of toxicant exposures during deployment. Cortex 2016;74:449–475.
6. Abou-Donia MB. Neurotoxicity resulting from coexposure to pyridostigmine bromide, DEET, and permethrin: implications of Gulf War chemical exposures. J Toxicol Environ Health A 1996;48:35–56.
7. Abou-Donia MB, Wilmarth KR, Abdel-Rahman AA, Jensen KF, Oehme FW, Kurt TL. Increased neurotoxicity following concurrent exposure to pyridostigmine bromide, DEET, and chlorpyrifos. Toxicol Sci 1996;34:201–222.

8. Abdel-Rahman A, Abou-Donia S, El-Masry E, Shetty A, Abou-Donia M. Stress and combined exposure to low doses of pyridostigmine bromide, DEET, and permethrin produce neurochemical and neuropathological alterations in cerebral cortex, hippocampus, and cerebellum. J Toxicol Environ Health A 2004;67:163–192.
9. Parihar VK, Hattiangady B, Shuai B, Shetty AK. Mood and memory deficits in a model of Gulf War illness are linked with reduced neurogenesis, partial neuron loss, and mild inflammation in the hippocampus. Neuropsychopharmacology 2013;38:2348–2362.
10. Abdullah L, Evans JE, Bishop A, et al. Lipidomic profiling of phosphocholine containing brain lipids in mice with sensorimotor deficits and anxiety-like features after exposure to Gulf War agents. Neuromolecular Med 2012;14:349–361.
11. O'Callaghan JP, Kelly KA, Locker AR, Miller DB, Lasley SM. Corticosterone primes the neuroinflammatory response to DFP in mice: potential animal model of Gulf War illness. J Neurochem 2015;133:708–721.
12. Steele L, Lockridge O, Gerkovich MM, Cook MR, Sastre A. Butyrylcholinesterase genotype and enzyme activity in relation to Gulf War illness: preliminary evidence of gene-exposure interaction from a case–control study of 1991 Gulf War veterans. Environ Health 2015;14:1.
13. Pierce LM, Kurata WE, Matsumoto KW, Clark ME, Farmer DM. Long-term epigenetic alterations in a rat model of Gulf War illness. Neurotoxicology 2016;55:20–32.
14. Medicine Io, National Academies of Sciences E, Medicine. Gulf War and Health: Volume 10: Update of Health Effects of Serving in the Gulf War, 2016. Washington, DC: The National Academies Press; 2016.
15. Soldner F, Jaenisch R. Medicine: iPSC disease modeling. Science 2012;338:1155–1156.
16. Kim K, Doi A, Wen B, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285–290.
17. Choi SH, Kim YH, Hebisch M, et al. A three-dimensional human neural cell culture model of Alzheimer's disease. Nature 2014;515:274–278.
18. Kim YH, Choi SH, D'Avanzo C, et al. A 3D human neural cell culture system for modeling Alzheimer's disease. Nat Protoc 2015;10:985–1006.
19. Prendergast MA, Self RL, Smith KJ, et al. Microtubule-associated targets in chlorpyrifos oxon hippocampal neurotoxicity. Neuroscience 2007;146:330–339.
20. Jiang W, Duysen EG, Hansen H, Shlyakhtenko L, Schopfer LM, Lockridge O. Mice treated with chlorpyrifos or chlorpyrifos oxon have organophosphorylated tubulin in the brain and disrupted microtubule structures, suggesting a role for tubulin in neurotoxicity associated with exposure to organophosphorus agents. Toxicol Sci 2010;115: 183–193.
21. Terry AV Jr, Stone JD, Buccafusco JJ, Sickles DW, Sood A, Prendergast MA. Repeated exposures to subthreshold doses of chlorpyrifos in rats: hippocampal damage, impaired axonal transport, and deficits in spatial learning. J Pharmacol Exp Ther 2003;305:375–384.
22. Grigoryan H, Lockridge O. Nanoimages show disruption of tubulin polymerization by chlorpyrifos oxon: implications for neurotoxicity. Toxicol Appl Pharmacol 2009; 240:143–148.
23. Gearhart DA, Sickles DW, Buccafusco JJ, Prendergast MA, Terry AV Jr. Chlorpyrifos, chlorpyrifos-oxon, and diisopropylfluorophosphate inhibit kinesin-dependent microtubule motility. Toxicol Appl Pharmacol 2007;218:20–29.
24. Terry AV Jr, Gearhart DA, Beck WD Jr, et al. Chronic, intermittent exposure to chlorpyrifos in rats: protracted effects on axonal transport, neurotrophin receptors, cholinergic markers, and information processing. J Pharmacol Exp Ther 2007;322:1117–1128.
25. Terry AV Jr, Beck WD, Warner S, Vandenhuerk L, Callahan PM. Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrfos or diisopropylfluorophosphate. Neurotoxicol Teratol 2012; 34:1–8.
26. Falnikar A, Baas PW. Critical roles for microtubules in axonal development and disease. Results Probl Cell Differ 2009;48:47–64.
27. Morfini GA, Burns M, Binder LI, et al. Axonal transport defects in neurodegenerative diseases. J Neurosci 2009;29: 12776–12786.
28. Binukumar B, Bal A, Sunkaria A, Gill KD. Mitochondrial energy metabolism impairment and liver dysfunction following chronic exposure to dichlorvos. Toxicology 2010;270:77–84.
29. Golomb BA. Oxidative stress and mitochondrial injury in chronic multisymptom conditions: from Gulf war illness to autism spectrum disorder. Nature Proc. Epub 2012 Jan 31.
30. Koslik HJ, Hamilton G, Golomb BA. Mitochondrial dysfunction in Gulf war illness revealed by 31Phosphorus magnetic resonance spectroscopy: a case-control study. PLoS One 2014;9:e92887.
31. Chao LL, Abadjian L, Hlavin J, Meyerhoff DJ, Weiner MW. Effects of low-level sarin and cyclosarin exposure and Gulf War illness on brain structure and function: a study at 4T. Neurotoxicology 2011;32:814–822.
32. Heaton KJ, Palumbo CL, Proctor SP, Killiany RJ, Yurgelun-Todd DA, White RF. Quantitative magnetic resonance brain imaging in US army veterans of the 1991 Gulf War potentially exposed to sarin and cyclosarin. Neurotoxicology 2007;28:761–769.
33. Sullivan K, Krengel M, Proctor SP, Devine S, Heeren T, White RF. Cognitive functioning in treatment-seeking Gulf War veterans: pyridostigmine bromide use and PTSD. J Psychopathol Behav Assess 2003;25:95–103.
34. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861–872.
35. Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. Cell 2008;134:877–886.
36. Israel MA, Yuan SH, Bardy C, et al. Probing sporadic and familial Alzheimer's disease using induced pluripotent stem cells. Nature 2012;482:216–220.
37. Mertens J, Paquola AC, Ku M, et al. Directly reprogrammed human neurons retain aging-associated transcriptomic signatures and reveal age-related nucleocytoplasmic defects. Cell Stem Cell 2015;17:705–718.
38. Steele L. Prevalence and patterns of Gulf War illness in Kansas veterans: association of symptoms with characteristics of person, place, and time of military service. Am J Epidemiol 2000;152:992–1002.
39. Furlong CE. Genetic variability in the cytochrome P450–paraoxonase 1 (PON1) pathway for detoxication of organophosphorus compounds. J Biochem Mol Toxicol 2007;21:197–205.
40. Gao J, Naughton SX, Wulff H, et al. Diisopropylfluorophosphate impairs the transport of membrane-bound organelles in rat cortical axons. J Pharmacol Exp Ther 2016; 356:645–655.

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2⌘D⌘ ,I ⇧VDPH⇧ DV ,WHP ▪ ⇕⌘▪ VR VWDWH⌘

2⌘E⌘ 6HOI↓H[SODQDWRU\⌘

2⌘F⌘ 3URFXUHPHQW ,QVWUXPHQW ,GHQWLILFDWLRQ ↲3,,) QXPEHU RI FRQWUDFW ↲')$56 2♦⌧⌘→♦♦3)⌘

2⌘G⌘ WKURXJK ←⌘H⌘ 6HOI↓H[SODQDWRU\⌘

←⌘I⌘ 7KH QDPH DQG DGGUHVV RI WKH HPSOR\HU RI HDFK LQYHQWRU QRW HPSOR\HG E\ WKH FRQWUDFWRU RU VXEFRQWUDFWRU LV QHHGHG EHFDXVH WKH *RYHUQPHQW▫V ULJKWV LQ D UHSRUWHG LQYHQWLRQ PD\ QRW EH GHWHUPLQHG VROHO\ E\ WKH WHUPV RI WKH ⇧3DWHQW 5LJKWV⇧ FODXVH LQ WKH FRQWUDFW⌘

([DPSOH ▪: ,I DQ LQYHQWLRQ LV PDGH E\ D *RYHUQPHQW HPSOR\HH DVVLJQHG WR ZRUN ZLWK D FRQWUDFWRU▪ WKH *RYHUQPHQW ULJKWV LQ VXFK DQ LQYHQWLRQ ZLOO EH GHWHUPLQHG XQGHU ([HFXWLYH 2UGHU ▪♦♦☒⇡⌘

([DPSOH 2: ,I DQ LQYHQWLRQ LV PDGH XQGHU D FRQWUDFW E\ MRLQW LQYHQWRUV DQG RQH RI WKH LQYHQWRUV LV D *RYHUQPHQW HPSOR\HH▪ WKH *RYHUQPHQW▫V ULJKWV LQ VXFK DQ LQYHQWRU▫V LQWHUHVW LQ WKH LQYHQWLRQ ZLOO DOVR EH GHWHUPLQHG XQGHU ([HFXWLYH 2UGHU ▪♦♦☒⇡▪ H[FHSW ZKHUH WKH FRQWUDFWRU LV D VPDOO EXVLQHVV RU QRQSURILW RUJDQL]DWLRQ▪ LQ ZKLFK FDVH WKH SURYLVLRQV RI 3← 8⌘6⌘&⌘ 2♦2↲H) ZLOO DSSO\⌘

←⌘J⇕⌘▸) 6HOI↓H[SODQDWRU\⌘

←⌘J⌘↲2) 6HOI↓H[SODQDWRU\ ZLWK WKH H[FHSWLRQ WKDW WKH FRQWUDFWRU RU VXEFRQWUDFWRU VKDOO LQGLFDWH▪ LI NQRZQ DW WKH WLPH RI WKLV UHSRUW▪ ZKHWKHU DSSOLFDWLRQV ZLOO EH ILOHG XQGHU HLWKHU WKH 3DWHQW &RRSHUDWLRQ 7UHDW\ ↲3&7) RU WKH (XURSHDQ 3DWHQW &RQYHQWLRQ ↲(3&)⌘ ,I VXFK LV NQRZQ▪ WKH OHWWHUV 3&7 RU (3& VKDOO EH HQWHUHG DIWHU HDFK OLVWHG FRXQWU\⌘

⇡⌘D⌘ 6HOI↓H[SODQDWRU\⌘

⇡⌘E⌘ 6HOI↓H[SODQDWRU\⌘

⇡⌘F⌘ 6HOI↓H[SODQDWRU\⌘

⇡⌘G⌘ 3DWHQW 5LJKWV &ODXVHV DUH ORFDWHG LQ)$5 ←2⌘22→⌘

⇡⌘H⌘ 6HOI↓H[SODQDWRU\⌘

⇡⌘I⌘ 6HOI↓H[SODQDWRU\⌘

→⌘ &HUWLILFDWLRQ QRW UHTXLUHG E\ VPDOO EXVLQHVV ILUPV DQG GRPHVWLF QRQSURILW RUJDQL]DWLRQV⌘

→⌘D⌘ WKURXJK →⌘G⌘ 6HOI↓H[SODQDWRU\⌘