

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 = di-isopropyl fluorophosphates; **GW** = Gulf War; **GWI** = Gulf War illness; **GWIC** = Gulf War Illness Consortium; **hiN** = human induced neurons; **hiPSC** = human induced pluripotent stem cells; **OP** = organophosphate; **PB** = 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 contribute¹⁶; (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.

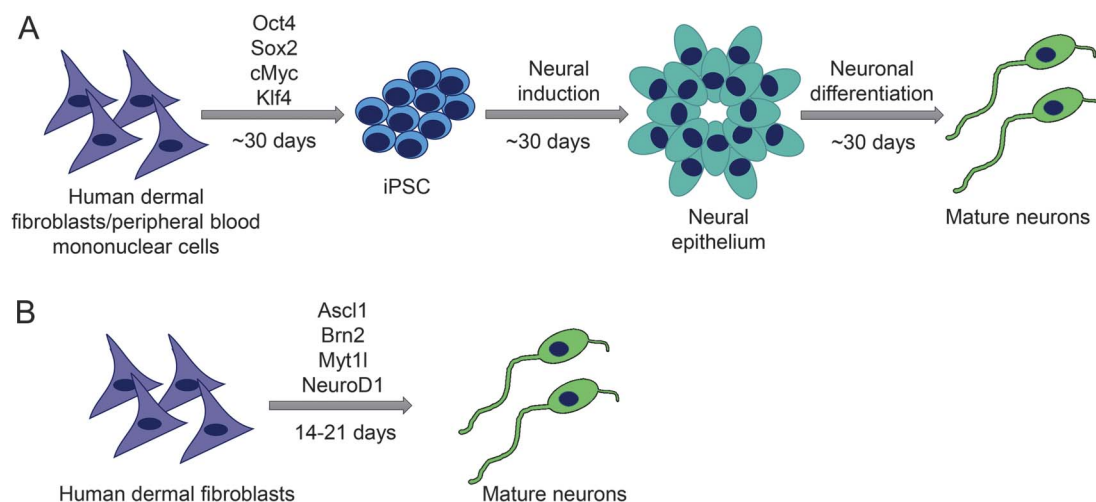
Recent clinical studies comparing brain imaging and cognitive functioning in GW veterans have shown significant evidence of altered CNS functioning in veterans with GWI.^{5,31–33} However, overlap between individual GWI cases and controls on the outcome measures has precluded their use as objective diagnostic 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

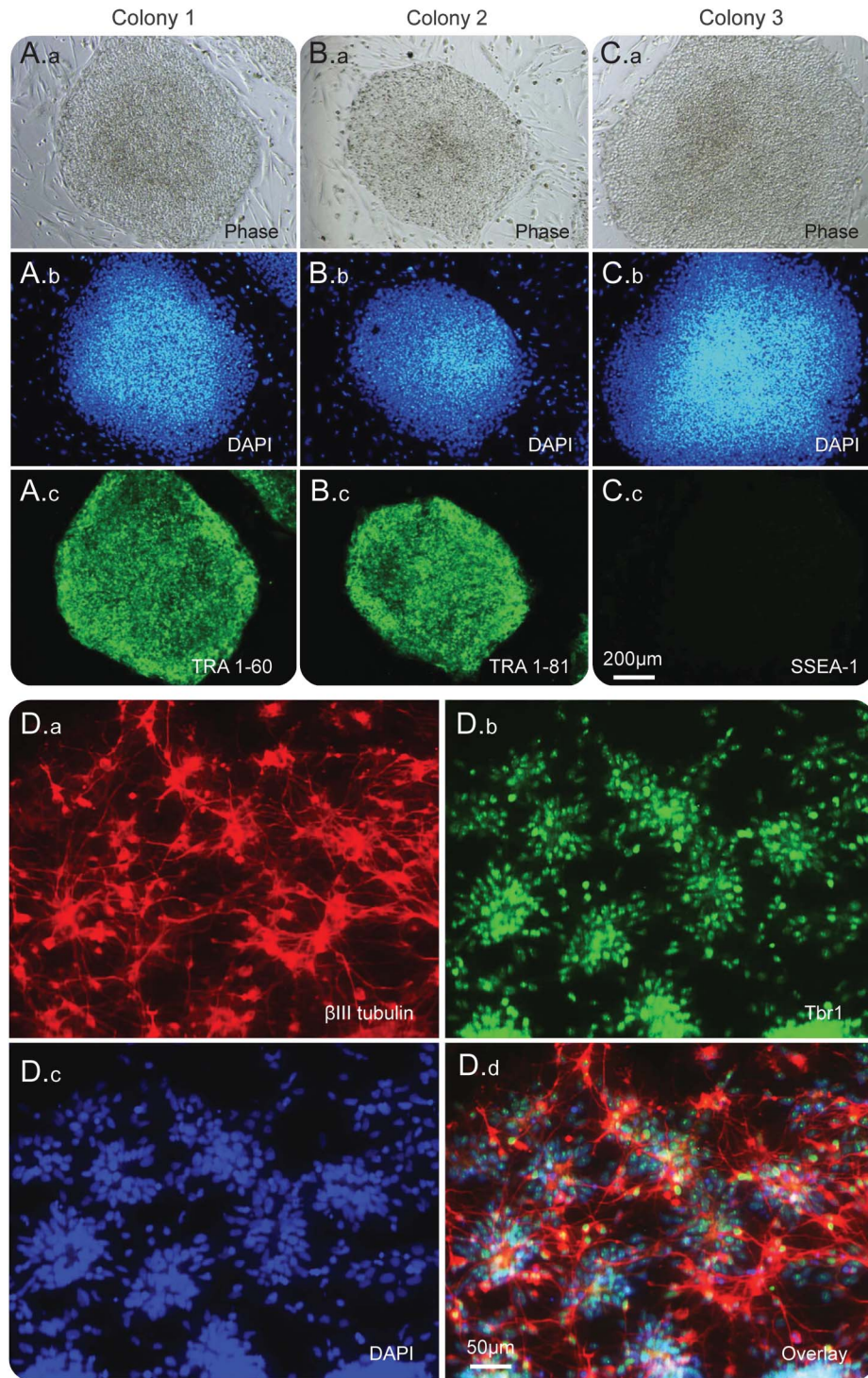
We are currently recruiting blood donors from the 300 study participants from the Gulf War Illness Consortium (GWIC), funded by the US Department of Defense (PI: Kimberly Sullivan). At the Boston GWIC site, 175 study participants are being recruited. The human neuron studies are considered a follow-up 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.

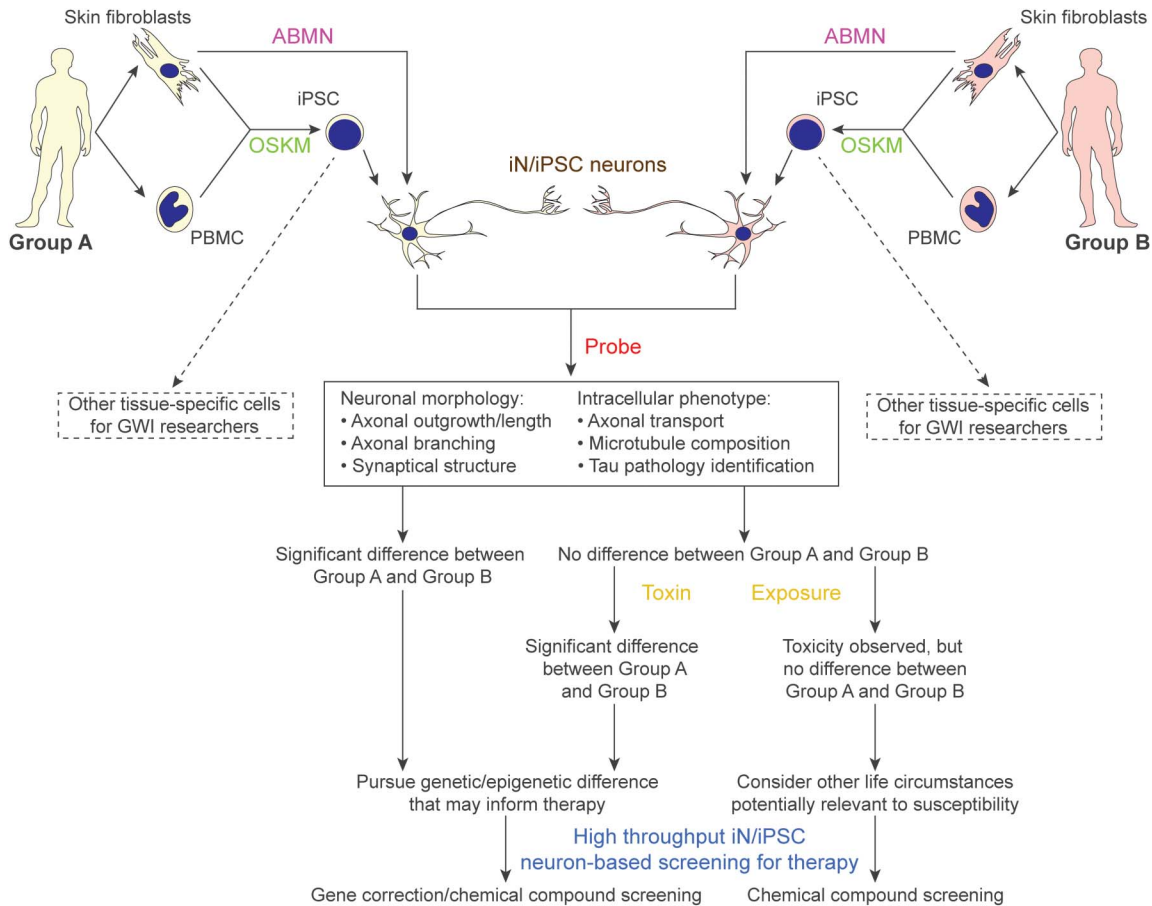
CONTROL SAMPLES A challenging element to our plan was the decision regarding what to use for control sample hiPSC cells. We considered using cells from veterans of similar age who had not been deployed to the GW. However, epidemiologic studies indicate that comparing deployed ill vs deployed non-ill 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 β III tubulin and D.b is the immunostaining for Tbr1. β III 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 μm , 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 = transcription factors Ascl1, Brn2, Myt1l, and NeuroD1; fibs = fibroblasts; iN = induced neurons; iPSC = induced pluripotent stem cells; OSKM = transcription factors Oct4, Sox2, Klf4, and c-Myc; PBMC = 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

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