**Supplemental Material**

**Cellular and Molecular Life Sciences**

# A cellular approach to understanding and treating Gulf War Illness

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**Materials and Methods**

hiPSC generation and culture. All cells were cultured in 5% CO2 at 37°C. The hiPSCs were initially cultured on mouse embryonic fibroblast (MEF) feeder cells for 2-3 passages before transitioning to a feeder-free culture. One day before thawing hiPSCs, MEFs (Gibco, A34180) were thawed and plated onto 6-well plates (500,000 per well) pre-coated with attachment factor (Gibco, S-006-100) for 45 minutes at 37°C, and then cultured overnight in MEF medium [for 100ml: 89ml DMEM (Gibco, 10566-016), 10ml embryonic stem cell qualified fetal bovine serum (ES-FBS) (Gibco, 16141061), 1ml minimum non-essential amino acids (MNEAA) (Gibco, 11140-050), and 100µl beta-mercaptoethanol (Gibco, 21985-023)]. The next day one vial of hiPSCs was thawed and cells were plated onto one well of a 6-well plate of MEFs with 10µM ROCK inhibitor Y-27632 (Tocris, 1254) and cultured in hiPSC medium [for 500ml: 390ml DMEM/F12 (Gibco, 11330-032), 100ml Knockout serum replacement (Gibco, 10828028), 5ml Glutamax (Gibco, 25030-081), 5ml MNEAA, 500µl beta-mercaptoethanol, and 20ng/ml bFGF (Peprotech, 100-18B) added fresh daily]. hiPSCs at 80% confluency were passaged weekly with Collagenase Type IV (Stem Cell Technologies, 07909). For passaging, hiPSC medium was removed, cells were washed with D-PBS without $Ca^{2+}$ or $Mg^{2+}$, and then incubated with collagenase for 5 minutes at 37°C. Collagenase was removed, cells were washed again with D-PBS, and a cell scraper was used to collect the hiPSC colonies, which were then passaged onto a new plate in hiPSC medium with 10µM Y-27632. The hiPSCs were transitioned from a feeder-dependent culture to a feeder-free culture after 2-3 passages of the initial cultures. Feeder-free hiPSCs were cultured in mTeSR1 (Stem Cell Technologies, 85857) on ES-qualified Matrigel (Corning, 354277) coated plates. Matrigel was diluted 1:75 in cold DMEM/F12 and coated on a 6-well plate for 2 hours at 37°C before thawing or passaging hiPSCs. Before passaging or directed differentiation, hiPSC colonies were selected to remove areas of spontaneous differentiation. Passaging the feeder-free hiPSCs with collagenase was the same method as described above for the feeder-dependent hiPSCs.

Neuronal differentiation and GW toxicant treatment. hiPSC lines from 3 healthy GW veterans (controls) and 4 veterans with GWI (cases) were differentiated into forebrain glutamatergic neurons by methods

adapted with modifications from previously established protocols [32]. hiPSC colonies were dissociated in Accutase (Stem Cell Technologies, 07920) for 5 minutes at 37°C and cells were counted using a hemocytometer. Cells were centrifuged at 0.4 x g for 5 minutes at room temperature and resuspended using the STEMdiff SMADi Neural Induction Kit (Stem Cell Technologies, 08581). Before plating cells, one well of an AggreWell 800 plate (Stem Cell Technologies, 34811) was washed with anti-adherence rinsing solution (Stem Cell Technologies, 07010) and centrifuged at 2000 x g for 5 minutes at room temperature. 3 million hiPSCs were plated into one well of the AggreWell plate (10,000 cells per microwell) in 2ml from the STEMdiff SMADi Neural Induction Kit supplemented with 10µM Y-27632 and low-bFGF (4ng/ml). The AggreWell plate was centrifuged at 100 x g for 3 minutes at room temperature to collect the cells at the bottom of the microwells and then placed in the 37°C incubator. On days 1-4, one half of the medium was changed using the STEMdiff SMADi Neural Induction Kit supplemented with low-bFGF. On day 5, embryoid bodies (EBs) were resuspended in the AggreWell plate using a cut P-200 pipette tip, replated into 3-wells of a 6-well plate coated with ES-Matrigel, and cultured using the STEMdiff SMADi Neural Induction Kit supplemented with 10µM Y-27632. On days 6-9, the proportion of medium from the STEMdiff SMADi Neural Induction Kit was reduced by 1/4 each day and replaced with neural progenitor cell (NPC) medium [for 500ml: 237.5ml DMEM/F12, 237.5ml Neurobasal Medium (Gibco, 21103-049), 5ml N2 supplement (Gibco, 17502-048), 10ml B27 supplement without Vitamin A (Gibco, 12587-010), 5ml MNEAA, 5ml Glutamax, 500µl 2-Mercaptoethanol)] supplemented with 200µM ascorbic acid (Sigma-Aldrich, A5960) and the dual-SMAD inhibitors 10µM SB431542 (Tocris, 1614) and 250nM LDN193189 (Tocris, 6053). On days 10-11 the full NPC medium with supplements was changed. On day 12, neural rosettes were selected by incubating with STEMdiff Neural Rosette Selection Reagent (Stem Cell Technologies, 05832) for 2 hours at 37°C. The neural rosettes were dislodged from the plate by expelling medium directly at the neural rosettes using a P-1000 pipette. The neural rosettes were collected and centrifuged at 0.4 x g for 5 minutes at room temperature, and then resuspended in NPC medium supplemented with 200µM ascorbic acid, 500µM dibutyryl cAMP (Sigma-Aldrich, D0627), and 10µM Y-27632. NPCs were plated on Matrigel (Corning, 354234) coated plates. On days 13-15, NPC medium

supplemented with 200µM ascorbic acid, 500µM dibutyryl cAMP, and 10µM DAPT (Tocris, 2634) was changed. On day 16, neural progenitor cells were replated onto the final culture platform that was coated overnight in 0.01% poly-l-ornithine (Sigma-Aldrich, P4957) at room temperature, washed four times with $ddH_2O$ then incubated overnight with $ddH_2O$ at 37°C, and then coated with 10µg/ml laminin (Invitrogen, 23017015) diluted in D-PBS without $Ca^{2+}$ or $Mg^{2+}$ for 4 hours at 37°C. NPCs were detached via Accutase for 5 minutes at 37°C, counted using a hemocytometer, and then plated in neural differentiation medium (the same N2/B27 NPC medium but with B27 containing Vitamin A (Gibco, 17504-044)) supplemented with ascorbic acid, dibutyryl cAMP, and DAPT, and Y-27632 only on the day of plating. NPCs were differentiated for 6 days in this medium before long term culture in N2 neuronal maturation medium [for 500ml: 485ml DMEM/F12, 5ml N2 supplement, 5ml MNEAA, 5ml Glutamax, 500µl beta-mercaptoethanol) supplemented with ascorbic acid, dibutyryl cAMP, 20ng/ml BDNF (Peprotech, 450-02), and 20ng/ml GDNF (Peprotech, 450-10)] for 3 weeks before mature neurons were treated with our GW toxicant regimen. The regimen consisted of three days of 2µM hydrocortisone (Cortisol) (Sigma-Aldrich, H0888), dissolved in 10% ethanol in ddH2O, followed by two days of 2µM Cortisol plus 200nM diisopropyl fluorophosphate (DFP) (Sigma-Aldrich, D0879), dissolved in isopropanol. After two days in Cortisol+DFP, one half of the medium was changed, and then cells were studied three days later (8 days after first cortisol exposure).

Evaluation of cell viability and plasma membrane integrity in cells exposed to GW toxicants.

*1. MTT assay*: Cell culture medium was discarded and replaced with 50µl of MTT Reagent mixed with 50µl of culture media and incubated at 37°C for 3 hours. After incubation, the MTT Reagent-supplemented media was removed and replaced with 150µl of MTT Solvent. The plate was wrapped in aluminum foil and placed on an orbital shaker at room temperature for 15 minutes or until all of the crystals dissolved. The absorbance was read on a Tecan Infinite M200Pro spectral plate reader at a wavelength of 590nm for optical density. Analysis was conducted following the manufacturer's instructions to calculate percent cytotoxicity.

*2. LDH assay*: 10µl of ddH2O or 10X Lysis Buffer was added to the appropriate wells containing 100µl of N2 neuronal maintenance medium and incubated at 37°C for 45 minutes. 50µl of sample medium was added to a new clear 96-well flat-bottom plate and mixed with 50µl of LDH Reaction Mixture and incubated for 30 minutes at room temperature protected from light. 50µl of Stop Solution was added to each well and then the absorbance was read on a Tecan spectral plate reader at wavelengths of 490nm and 680nm for optical density. Analysis was conducted following the manufacturer's instructions to calculate percent cytotoxicity.

Extracellular tau ELISA analysis. Cell culture supernatant was collected with protease inhibitors (Thermo Scientific, 78430) and phosphatase inhibitors (Thermo Scientific, 78428) and analyzed fresh for extracellular human total tau (Invitrogen, KHB0041) and [pT231] human phosphorylated tau (Invitrogen, KHB8051) according to the manufacturer's instructions. 50µl of cell culture supernatant was mixed with 50µl of Standard Diluent Buffer and incubated at room temperature for 2 hours. After thorough washing, samples were incubated with 100µl of human total tau biotin or human [pT231] tau biotin for 1 hour at room temperature. After thorough washing, samples were incubated with 100µl of anti-Rabbit IgG Streptdavidin-HRP for 30 minutes at room temperature. After thorough washing, samples were incubated with 100µl of Stabilized Chromogen and incubated for 30 minutes at room temperature in the dark. 100µl of Stop solution was added to each well and then the absorbance was read on a Tecan spectral plate reader at a wavelength of 450nm for optical density. Analysis was conducted according to the manufacturer's instructions to calculate the concentration of extracellular human total tau or [pT231] human phosphorylated tau in each sample.

Extracellular glutamate analysis. Cell culture supernatant was collected with protease inhibitors and analyzed fresh for extracellular glutamate using the glutamate assay kit (Sigma-Aldrich, MAK004) according to the manufacturer's instructions. All reagents were equilibrated to room temperature before

use. 50µl of cell culture supernatant was mixed with 100µl of glutamate reaction mix and incubated for 30 minutes at 37°C protected from light. After incubation, the absorbance was read on a Tecan spectral plate reader at a wavelength of 450nm for optical density. Analysis was conducted following the manufacturer's instructions to calculate the concentration of glutamate in each sample.