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TITLE: Sigma-1 Receptor Agonists as a Novel Therapeutic for Brain Mitochondrial Dysfunction in Gulf War Syndrome

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14. ABSTRACT
Gulf War Syndrome or Illness (GWI) is a unique chronic health disorder with multiple symptoms including cognitive difficulties, fatigue, muscular pain, and gastrointestinal problems. GWI is believed to be associated with prolonged or excessive exposure to various pesticides and pyridostigmine bromide (PB). This toxicant exposure causes disrupted mitochondrial function in neurons and thus cognitive difficulties in GWI. Treatments that target at restoring mitochondrial function in neurons and improving cognitive abilities in GWI veterans are needed. Sigma-1 receptor (S1R) agonists have the potential to restore mitochondrial function. This study will test therapeutic efficacy of selected S1R agonists for cognitive difficulties in an established GWI mouse model.

15. SUBJECT TERMS
Gulf War Syndrome or Illness (GWI), mitochondrial dysfunction, , sigma-1 receptor

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1. **INTRODUCTION:**
Gulf War Syndrome or Illness (GWI) is a unique chronic health disorder with multiple symptoms including cognitive difficulties, fatigue, muscular pain, and gastrointestinal problems. GWI is believed to be associated with prolonged or excessive exposure to various pesticides and pyridostigmine bromide (PB). This toxicant exposure causes disrupted mitochondrial function in neurons and thus cognitive difficulties in GWI. Treatments that target at restoring mitochondrial function in neurons and improving cognitive abilities in GWI veterans are needed. Sigma-1 receptor (S1R) agonists have the potential to restore mitochondrial function. Thus, this study will test therapeutic efficacy of selected S1R agonists for cognitive difficulties in an established GWI mouse model. There are four specific aims for this study: 1) to evaluate the efficacy of selected S1R agonists in improving cognitive degradation in a GWI mouse model, 2) to characterize the impact of S1R agonist treatments on brain energy metabolism of GWI mice, 3) to characterize the effect of S1R agonists on mitochondrial function that impacts metabolic homeostasis and neuroplasticity, and 4) to investigate the interaction between S1R and the selected agonists.

2. **KEYWORDS:**
Gulf War Syndrome or Illness (GWI)
mitochondrial dysfunction
sigma-1 receptor

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Specific Aim 1: Evaluate the efficacy of selected S1R agonists in improving GWI induced cognitive degradation	**Timeline**	**Status**
Major Task 1	Months	
Subtask 1: Agent exposure and treatments Total # of mice: 80 [Male mice (C57BL/6) from Charles River, Inc.; 64 for agent exposure and treatments; 16 for control]	1-16	Pending. As of September 5, 2018, institution-supported lab renovation for animal experiments was funded. A contractor was selected to conduct the lab renovation. Animal experiments were planned to begin in November 2018 to avoid possible Hurricane related evacuation, which would disrupt the animal study (26

		weeks).
Subtask 2: Evaluate the efficacy of two S1R agonists and co-enzyme Q10 (CoQ10) on restoration of learning and memory in GWI mice Total # of mice: 80	16-18	Pending.
Subtask 3: Assess the effect of two S1R agonists and CoQ10 on restoring motor coordination in GWI mice Total # of mice: 80	16-18	Pending.
Milestone(s) Achieved: Measurement of the efficacy of two S1R agonists and CoQ10 in restoring cognitive capabilities (learning, memory, and motor coordination) impaired in GWI mouse model.	*18*	
Local IACUC Approval	1-3	Completed. Received initial and amendment IACUC (add 10 mice for pilot run) approvals dated September 1, 2017 and May 3, 2018, respectively.
Milestone Achieved: ACURO Approval	3-6	Completed. Received ACURO approval dated November 13, 2017.
Specific Aim 2: Characterize the impact of S1R agonist treatments on brain energy metabolism of GWI mice		
Major Task 2: Characterize the impact of S1R agonist treatments as well as CoQ10 on brain energy metabolism of GWI mice		
Subtask 1: Characterize the effect of S1R agonists on brain energy metabolite profiling Total # of mice: 40 [32 treated GWI mice, 8 control mice]	19-25	Pending
Milestone(s) Achieved: 1) Verification of S1R agonists' impact on energy metabolism in different brain regions; 2) Measurement of differential impact of S1R agonists and CoQ10 on brain energy metabolism	*25*	

Specific Aim 3: Characterize the effect of S1R agonists on mitochondrial functions		
Major Task 3: Characterize the effect of S1R agonists on mitochondrial functions that impact metabolic homeostasis and neuroplasticity		
Subtask 1: Prepare and culture motor cortex neurons	19-21	Pending.
Subtask 2: Characterize the impact of S1R agonists on mitochondrial function	21-23	In preparation. Conducted cell viability assays using a human neuroblastoma cell line SH-SY5Y to evaluate the capabilities of selected S1R agonists in restoring mitochondrial functions. Optimized experimental conditions such as different dye type and concentration for characterizing mitochondrial function using confocal microscopy.
Subtask 3: Quantify the effect of S1R agonists on Bcl-2 and BDNF signaling pathways	23-25	In preparation. Examined S1R protein expression levels for the selected S1R agonists with different concentrations in the SH-SY5Y cell lines.
Milestone(s) Achieved: 1) Verification of S1R agonists' impact on mitochondrial function; 2) Quantification of differential protein expression level of BDNF and Bcl-2 in neuronal mitochondria	*25*	
Specific Aim 4: Investigate the interaction between S1R and its agonists		
Major Task 4: Investigate the interaction between S1R and its agonists using NMR spectroscopy		
Subtask 1: Determine the backbone structure of S1R using NMR spectroscopy	1-18	In progress. As of September 28, 2018, we were optimizing human protein

		over-expression in other expression systems to reduce the amount of oligomers in the overexpressed S1R protein.
Subtask 2: Locate S1R agonist binding sites and characterize its conformational changes upon activation	18-36	In progress. Performed a series of point mutagenesis on human S1R, which was based on published S1R crystal structure, to disrupt possible agonist binding.
Milestone(s) Achieved: Determination of high resolution solution structure of S1R and S1R-agonist interfaces	*36*	

What was accomplished under these goals?

The overall goal of this study is to investigate the therapeutic efficacy of selected S1R agonists in relieving cognitive degradation in an established GWI mouse model. During this first reporting period (September 1, 2017-September 30, 2018), the PI, co-PI, and their team followed the approved SOW to prepare and conduct the proposed experiments. For the Specific Aims 1 and 2, we successfully received approvals from both SSU's IACUC and DoD's ACURO. The animal protocol and its amendment, which added 10 mice for pilot run, were submitted to SSU's IACUC and ACURO. Because the animal facility at SSU has not been used since 2015, the PI and his team worked diligently with SSU's Office of Sponsored Research Administration to prepare the University's animal facility for implementing Aims 1 and 2. Despite this year's tight budget, the University's administrators, including SSU's College Dean Dr. Mustafa, Associate Vice President for Research Dr. Chetty, and Provost Dr. Laney, secured funds for renovation of a dedicated new lab to accommodate the proposed behavioral and tissue culture studies. The renovation is expected to finish by the end of November 2018.

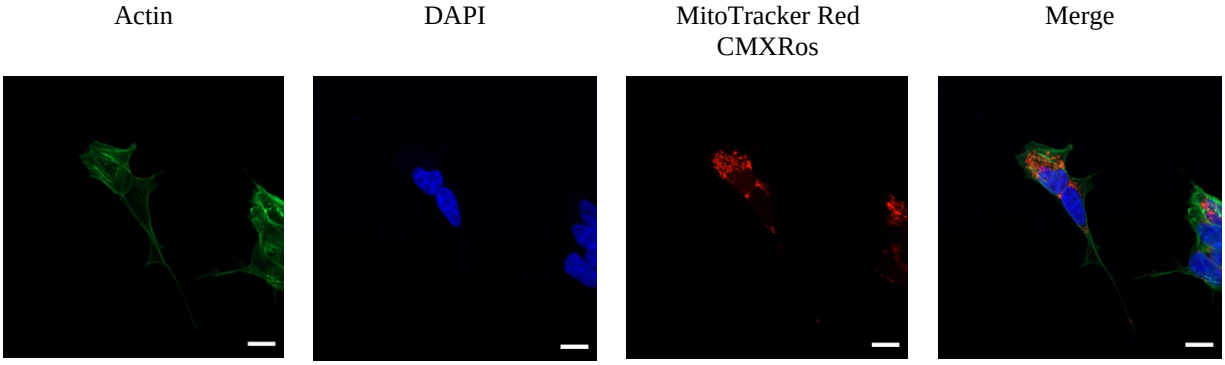
For Aim 3, we have conducted a series of experiments using a model cell line SH-SY5Y to compare and select appropriate dyes and their concentrations for quantifying mitochondrial function, including membrane potentials and calcium capacities, in neuronal cells. Figure 1 shows representative confocal micrographs of SH-SY5Y cells. We selected the appropriate range of MitoTracker Red CMXRos for staining neurons in Aim 3. Additionally, we tested the concentration effect of selected S1R agonists on SH-SY5Y's mitochondria function and selected a suitable range for in vitro studies.

For Aim 4, we are experimenting different protein overexpression systems to reduce oligomerization of S1R, which will reduce line width in NMR spectra and subsequently lead to more accurate assignments of resonances in NMR spectra. Our western blot showed that the S1R dimer and oligomers were formed in overexpressed S1R protein prepared from E. coli

system. We are testing a number of other overexpression systems, including insect cell and yeast systems, to reduce S1R oligomer formation. Additionally, we conducted site-directed mutagenesis at S1R oligomerization sites to further increase monomeric S1R for NMR spectroscopy.

All these efforts during the first reporting period laid a solid foundation for carrying out the planned experiments in all four aims.

Figure 1. Optimization of MitoTracker Red CMXRos concentration (20-500 nM) for mitochondrial function studies. The confocal images were acquired on a Zeiss LSM 800 equipped with a 63 X objective. Concentration of dye used: 400 nM; Scale bar: 10 μm.



What opportunities for training and professional development has the project provided?
During this reporting period, a ROTC cadet with a major in Forensic Science was trained in this research project. Besides being supported by this grant, this senior student was trained in biochemistry/cell biology lab skills and lab management skills. The PI also recommended the student for a summer internship (May-July 2018) in Georgia Bureau of Investigation (GBI).

The PI, co-PI, research associate, and the undergraduate trainee have completed the required CITI training on Responsible Conduct of Research (RCR).

How were the results disseminated to communities of interest?
Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period, we expect to have lab space ready for conducting planned experimental work, especially for Aims 1 and 2. For these two Aims, we will first conduct a pilot run involving 10 mice to ensure all chemical agent exposure, treatments, behavioral tests, and tissue handling are conducted properly before implementing the full study that involves 80 mice.

Additionally, we will adjust Agilent LC-MS experimental conditions for metabolic profiling (Aim 2) using cultured primary mouse cortical neurons.

For Aim 3, we will first use primary mouse cortical neurons to verify whether the selected dye conditions for characterizing mitochondrial function work for primary cells. We will also optimize dye conditions for live cell imaging, western blot for semi-quantification of Bcl-2 and BDNF expression levels, and RT-qPCR for quantifying their gene expression levels. The outcomes of these experiments will ensure optimized experimental conditions for characterizing cortical neurons from our GWI mouse models.

For Aim 4, we expect to obtain the mutated S1R recombinant protein that lacks oligomers for determining its NMR solution structure in the next reporting period. We will also collect and compare ^{1}H, ^{15}N hsqc spectrum of this mutated S1R protein with that of wild type S1R to ensure the mutant is properly folded for structure determination.

4. **IMPACT:**

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. **CHANGES/PROBLEMS:**

Changes in approach and reasons for change:
Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:
Nothing to Report.

6. **PRODUCTS:**
Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
What individuals have worked on the project?

September 1, 2017 – December 31, 2017

Name	Role	Nearest person month worked	Contribution
Dr. Kai Shen	PI	0.5	Prepared and submitted animal protocols to SSU's IACUC and DoD's ACURO; planned behavioral experiments (Aims 1 & 2); recruited a research associate; conducted S1R receptor recombinant protein overexpression in E coli system (Aim 4); recruited and trained an undergraduate student in literature research and basic lab skills.
Dr. Meharvan Singh	co-PI	0.2	Helped the PI prepare animal protocols, process grant related paperwork, and plan animal experiments.
Kenneth Nealy	Undergraduate student	2	Conducted protein overexpression under the supervision of the PI. Helped manage the lab inventories.

January 1, 2018 – April 30, 2018

Name	Role	Person month worked	Contribution
Dr. Kai Shen	PI	0.5	Supervised a research associate to optimize cell culture conditions for SH-SY5Y cells, prepare cell differentiation, and conduct cell viability assays; worked with the research associate and the department to set up a temporary tissue culture lab (Aims 2 & 3); optimized S1R receptor overexpression to reduce S1R oligomer for NMR experiments; trained the undergraduate student in hands-on lab skills (Aim 4).
Dr. Meharvan Singh	co-PI	0.3	Helped the PI and his SSU team on optimizing cell culture conditions and cell differentiation protocols.
Mr. Harshavardhan Kenche	Research Associate	2	Optimized cell culture for neuroblastoma cells and evaluated cell viability; worked with the PI to start a temporary tissue culture lab; assisted the PI in student lab training and managing day-to-day lab operations.
Kenneth Nealy	Undergraduate student	2	Under the supervision of the PI and research associate, the student learned cell culture techniques and assisted with cell passage and lab management.

May 1, 2018 – August 1, 2018

Name	Role	Nearest person month worked	Contribution

Dr. Kai Shen	PI	1	Supervised and worked with the research associate to select a set of dyes and optimize their concentrations for characterizing mitochondrial function (Aim 3); overexpressed S1R protein in different protein expression systems (insect cells/E. coli); designed mutants to decrease S1R oligomer content for NMR experiments (Aim 4).
Dr. Meharvan Singh	co-PI	0.4	Advised the PI's team in dye selection and mitochondria insults.
Mr. Harshavardhan Kenche	Research Associate	1.5	Conducted characterization of mitochondrial function using different dyes on SH-SY5Y cells; evaluated the effect of one mitochondrial insult.

August 1, 2018 – September 30, 2018

Name	**Role**	**Nearest person month worked**	**Contribution**
Dr. Kai Shen	PI	0.2	Supervised the Research Associate and the undergraduate student; worked with the team members on two mitochondrial insults for evaluating S1R agonist on rescuing cell viability.
Dr. Meharvan Singh	co-PI	0.1	Advised the PI on mitochondrial insult selection and experimental conditions.
Mr. Harshavardhan Kenche	Research Associate	1	Evaluated two mitochondrial insults on cell viability and mitochondrial function.
Kenneth Nealy	Undergraduate student	1	Continued cell culture work and assisted with lab management by organizing group meetings and benchwork schedules for student researchers.

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?

During the next reporting period, we plan to include Dr. Serdikoff, associate professor of behavioral science, from Social Science department to help us assess data of behavioral tests.

8. **SPECIAL REPORTING REQUIREMENTS COLLABORATIVE AWARDS:**

None.

9. **APPENDICES:** None.