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TITLE: Pyridostigmine Bromide, the Enteric Nervous System, and Functional Gastrointestinal Disorders in Gulf War Illness

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CONTRACTING ORGANIZATION: Michigan State University

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14. ABSTRACT: Gulf War illness (GWI) is a chronic, multi-symptom disorder with no treatment. Exposure to anti-cholinergic drugs such as pyridostigmine bromide (PB) contributed to the development of GWI, but the mechanisms that connect the acute effects of PB with chronic dysfunction in multiple systems remain unclear. Gastrointestinal problems are frequent and debilitating chronic symptoms experienced by Gulf War veterans. The overall objective of this proposal is to understand how PB contributes to the development of functional gastrointestinal disorders in Gulf War illness. Given that the enteric nervous system (ENS) regulates gut functions, we hypothesize that that PB disrupts gut functions by creating persistent neuroinflammation within the ENS. The major activities in this reporting period include in vivo and in vitro studies to understand the acute effects of PB on the ENS, the neural control of gut functions, and the inflammatory response within the gut. Key outcomes from this reporting period include observations showing that exposure to PB creates acute and chronic changes to gut functions that include increased fecal pellet output, higher fecal fluid content, slower colonic transit, altered neuromuscular control, defective intestinal barrier function, and neurodegeneration in the ENS. Our results show that the acute exposure to PB significantly alters the anatomy and functions of the ENS. We propose that these changes contribute to the pathophysiology of GWI.					
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1. INTRODUCTION:

Gulf War Illness (GWI) is a chronic disorder characterized by a spectrum of six symptoms that include fatigue/sleep, pain, neurological/cognitive/mood, gastrointestinal (GI), respiratory and skin problems. Gut problems are over three times more common in Gulf War veterans and are a major source of low quality of life and poor health. Exposure to the anti-nerve gas drug pyridostigmine bromide (PB) is clearly linked with the development of GWI, but the exact mechanisms still remain unclear. The overall objective of this proposal is to understand how PB contributes to the development of functional gastrointestinal disorders in GWI. Our central hypothesis is that PB disrupts gut functions by creating inflammation within the branch of the nervous system that coordinates gut functions. Specifically, we hypothesize that persistent neuroinflammation is caused by chronic reactive gliosis. This study has two Specific Aims that link *in vitro* mechanistic studies with *in vivo* studies in mice to study how PB alters the integrative physiology of the enteric nervous system. Specific Aim 1 tests the hypothesis that reactive enteric gliosis driven by an acute exposure to PB causes enteric neurodegeneration and long-lasting abnormalities in gut function. Specific Aim 2 tests the hypothesis that decreasing reactive enteric gliosis with the anti-inflammatory drug, palmitoylethanolamide improves gut dysfunction driven by PB. Alterations in GI physiology influence multiple systems and directly impact behavior, metabolism and immunity. Thus, the importance of understanding the pathogenesis of persistent GI disturbances in Gulf War illness is two-fold: i) For improving the treatment of GI-specific problems and ii) For treating the broad, systemic nature of the illness as a whole.

2. **KEYWORDS:** enteric glia, enteric nervous system, gulf war illness, gastrointestinal disorders, pyridostigmine bromide, inflammation, intestinal barrier, gut, intestine, autonomic, peripheral nervous system

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Major Task 1: Characterization of neuroinflammation caused by PB in the gut and brain.

Milestone(s) Achieved: Precise timeline of neuroinflammation caused by PB in the gut and brain and specific understanding of how PB affects the neural and glial systems that regulate gut functions.

- We have completed this major task and the results were published in the FASEB Journal in 2019.

Major Task 2: Mechanistic study to define the role of glia in PB-induced neuroinflammation and gut dysfunction.

Milestone(s) Achieved: Understanding of how glial cells contribute to neuroinflammation and gut dysfunction caused by PB. Publication of at least one peer-reviewed paper.

- We have completed the studies from Major Task 2 and have published the results in the journal Neuropharmacology (Hernandez et al. 2020).

Major Task 3: Effects of reversing gliosis with PEA on gut function and neuroinflammation.

Milestone(s) Achieved: Evaluation of a potential therapeutic approach to reduce systemic neuroinflammation and restore healthy gut function in a mouse model of GWI. Publication of results in at least one peer-reviewed paper.

- We have completed the studies from Major Task 3 and have published the results in the journal Neuropharmacology (Hernandez et al. 2020).

- **What was accomplished under these goals?**

a) **Major Activities:** The major activities in this award included *in vivo* and *in vitro* studies to understand the acute and chronic effects of PB on the enteric nervous system, the neural control of gut functions, and the inflammatory response within the gut and brain in both male and female mice. In addition, experiments assessed the effects of PB on gut and brain nervous and immune systems at 5 months, the therapeutic

effects of the anti-inflammatory drug PEA, and additional neural and glial signaling mechanisms responsible for the effects of PB on gut functions.

- b) **Specific Objectives:** Specific objectives of this award were to: *i)* Assess the effects of PB on gut motility and intestinal disease *in vivo*, *ii)* Determine how exposure to PB impacts the neural control of gut functions with *ex vivo* assays, *iii)* Use functional imaging of glia and neurons in live tissue from mice exposed to PB to understand how PB affects cellular activity, *iv)* Determine the time course of neuroinflammation driven by PB in the gut and the brain and *v)* understand whether the anti-inflammatory drug PEA would provide therapeutic benefit following exposure to PB.
- c) **Significant Results/Key Outcomes:** We completed all of the remaining objectives and goals during this final period of the award. Work completed during the prior funding period showed that exposing mice to PB in a paradigm that reflects the actual exposure experienced by soldiers during the Gulf War disrupts gastrointestinal functions, and gastrointestinal and brain immune responses at 7 days and up to 30 days following exposure (see Hernandez et al., FASEB 2019). We found that PB drove these changes, in part, through effects on the enteric nervous system (ENS) that involve the development of reactive gliosis. In the final funding period, we tested the hypothesis that reducing reactive gliosis with the drug PEA improves colonic dysfunction induced by PB. To this end, we used the same paradigm of exposure to PB in mice that produced altered gut, brain, and immune responses and treated a subset of animals with PEA beginning at 2 months following exposure to PB. We assessed *in vivo* and *ex vivo* gut functions, the structure and function of the ENS, and immune responses in the gut and brain longitudinally up to 5 months following exposure to PB. Surprisingly, our results indicated that PB does not produce overt changes in GI function at 5 months. However, prior exposure to PB dramatically changed the effects of PEA and combined exposure caused major changes in gut motility, altered the excitability of enteric neurons and glia, altered enteric neurochemical coding, changed neuron and glial survival, and dysregulated cytokines and chemokines in the colon and brain. Results from this funding period were published in the journal *Neuropharmacology* – Hernandez et al. 2020 “Pyridostigmine bromide exposure creates chronic, underlying neuroimmune disruption in the gastrointestinal tract and brain that alters responses to palmitoylethanolamide in a mouse model of Gulf War Illness” PMID: 32758565. Please refer to this published article for full details. Here, we provide a brief summary of the results related to the specific objectives stated above.
- i) We began by assessing how PB, PEA, and their combination affect gut motility *in vivo* in mice. PB and PEA did not cause major changes in animal weight, colon permeability, or colonic gross anatomy that would indicate overt disease processes. Fecal pellet output and colonic bead expulsion time assays also did not indicate major changes in gut motor function driven by PB at any time assessed over a 5 month period. However, the combination of PB followed by PEA treatment caused major changes in colonic motor function in both male and female animals.
- ii) We followed these *in vitro* motility assays with *in vitro* studies of isometric muscle tension recordings to assess neuromuscular function in the colon. These experiments showed that PB affected neurogenic relaxations in the intestine in male animals, but PEA did not have major effects on contractility in either sex. Together, the *in vivo* and *in vitro* studies conducted over this period showed that exposure to PB has lasting effects that alter the impact of PEA on intestinal motor functions, and that the mechanisms underlying these effects are sex-dependent.
- iii) We conducted calcium (Ca^{2+}) imaging studies in isolated preparations of ENS to understand how exposure to PB alters gut functions through effects on cellular signaling between enteric neurons and glia. Overall, these experiments show that PEA excites enteric neurons and glia through mechanisms that involve CB1 receptors, TRPV1 channels, and intercellular signaling mediated by glial Cx43 hemichannels. However, exposure to PB shifts signaling toward mechanisms that involve PPAR-alpha, CB1, and Cx43 hemichannels. Thus, exposure to PB causes changes in enteric neuron-glia signaling mechanisms that include reducing normal signaling mediated by TRPV1 pathways and potentiating mechanisms involving neuronal endocannabinoid signaling and glial PPAR-alpha.
- iv) We assessed immunomodulatory cytokines and chemokines in the gut and the brain to understand how exposure to PB might alter immune responses in the nervous system. Interestingly, PB was not sufficient to cause major changes in cytokines/chemokines in the gut at 5 months post-exposure. PB did, however,

cause changes in IL-1a and IL-17 in the brains of male mice at 5 months post exposure but had no effect in females. The most striking effects were those caused by PEA. This drug caused profound alterations in peripheral and central immune responses that are sex-dependent. These effects did not depend on prior exposure to PB, indicating that PEA has significant immunomodulatory effects in the brain and colon.

- v) Overall, our data suggest that PEA is a poor therapeutic option for individuals with GWI. Long-lasting effects of PB are occult and are made evident by the subsequent exposure to other drugs. Changes in intercellular signaling mechanisms between enteric neurons, glia, and immune cells are affected by exposure to PB and alter the actions of unrelated drugs that are otherwise beneficial. These mechanisms could play a central role in the lasting neurological problems with individuals with GWI.

Summary: Our main results during this award show that exposure to PB promotes acute and long-term changes in nervous system and immune system function. These changes are sex-dependent and involve alterations to intercellular communication within the enteric nervous system of the intestine. Importantly, acute exposure to PB creates occult changes in neural signaling that alter the effects of other drugs such as PEA. These changes make PEA not only an ineffective therapeutic option, but also dangerous in this context. These new data provide novel insight into mechanisms that could contribute to the development of Gulf War Illness in humans. Further, our results provide important information on how sex differences contribute to the effects of PB. It also brings to light how different treatments can exacerbate previous symptoms, which is important when assessing possible treatments for this group of patients. The main outcome of our studies during this award is the identification of mechanisms that likely contributed to the ongoing health problems experienced by Gulf War veterans.

Other achievements:

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

- Dr. Siomara Hernandez-Rivera, Ph.D. was the postdoctoral research associate assigned to this project. Dr. Hernandez-Rivera is an underrepresented minority woman who comes from a disadvantaged background and working on this project gave her the opportunity to gain essential technical and professional skills that are important in her development as a scientist. In addition, working on this project has allowed Dr. Hernandez-Rivera to broaden her knowledge base and explore a new area of research. Dr. Hernandez-Rivera has learned many new techniques to accomplish the experimental goals in this project. For example, Dr. Hernandez-Rivera has received training in live cell imaging and is now proficient at calcium imaging experiments in the enteric nervous system. In addition, Dr. Hernandez-Rivera has received technical training in mouse models of intestinal inflammation, ex vivo and in vivo measures of motility and barrier function, and immunohistochemical studies of the enteric nervous system. While working on this project Dr. Hernandez-Rivera received one-on-one mentorship from Dr. Gulbransen (PI) in technical, conceptual, and career development areas. Dr. Hernandez-Rivera and Dr. Gulbransen had scheduled weekly meeting to discuss broad project and professional development goals and Dr. Gulbransen and Dr. Hernandez-Rivera had daily conversations regarding more detailed aspects of her research. Dr. Hernandez-Rivera has been integrated within a core group of researchers at Michigan State University who focus on gut research (the "MSU gut group") and her interactions within this group have allowed her to network and broaden her knowledge base. These will be important skills as she develops into an independent researcher. Dr. Hernandez-Rivera presents her work regularly at the Gulbransen lab's weekly lab meeting and receives feedback on her work and presentation skills. She is currently receiving mentorship on presenting her data in written format and Dr. Gulbransen is mentoring her in the upcoming manuscripts preparation. Dr. Hernandez-Rivera presented her research at the Great Lakes Glia meeting (September 30, 2019) and at Experimental Biology (April 6-9, 2019) receiving an award as Best poster presentation of a Postdoctoral fellow. Dr. Hernandez-Rivera was able to visit (February 19-22, 2019) Dr. Carmen Hinojosa-Laborde's research laboratory in the U.S. Army Institute of Surgical Research receiving advices on the possible career opportunities as an independent researcher working on a military base helping military efforts to care for the combat wounded.

- **How were the results disseminated to communities of interest?**

- Data from this project was presented at multiple scientific meetings including Experimental Biology, the Great Lakes Glia meeting, and the American Neurogastroenterology and Motility meeting. In addition, results were published in three separate manuscripts in the FASEB journal, Neuropharmacology, and Scientific Reports (see publication list below).
- **What do you plan to do during the next reporting period to accomplish the goals?**
- NA – This is the final technical report and no additional reporting periods follow.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
- Our results suggest novel mechanisms of disease progression driven by acute drug exposure in the intestine. Also, our results suggest sex difference in response to the exposure of PB treatment. These findings could significantly impact the current usage of similar drugs and also the current theories of Gulf War Illness disease pathogenesis.
- **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?**
- Our current results suggest novel mechanisms of disease pathogenesis in Gulf War Illness. These findings could uncover new therapeutic targets that could improve the quality of life of veterans suffering from Gulf War Illness.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
- None since last period.
- **Actual or anticipated problems or delays and actions or plans to resolve them**
- None since last period.
- **Changes that had a significant impact on expenditures**
- None since last period.
- **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**
- NA
- **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:

- **Publications, conference papers, and presentations**
 - Great Lake Glia Meeting, Traverse City, MI September 29 – October 1, 2019
 - Experimental Biology, Orlando, FL April 6-9, 2019
- **Journal publications**
 - Gastrointestinal neuroimmune disruption in a mouse model of Gulf War illness. Hernandez S, Fried DE, Grubisic V, McClain JL, Gulbransen BD. **FASEB J**. 2019 May;33(5):6168-6184. DOI: 10.1096/fj.201802572R. PMID: 30789759.
 - Pyridostigmine bromide exposure creates chronic, underlying neuroimmune disruption in the gastrointestinal tract and brain that alters responses to palmitoylethanolamide in a mouse model of Gulf War Illness. Hernandez S, Morales-Soto W, Grubisic V, Fried D, Gulbransen BD. **Neuropharmacology**. 2020 Aug 3 DOI: 10.1016/j.neuropharm.2020.108264 PMID: 32758565
 - Effects of a high fat diet on gut microbiome dysbiosis in a mouse model of Gulf War Illness. Angoa-Perez M, Zagorac B, Francescutti DM, Winters AD, Greenberg JM, Ahmad MM, Manning SD, Gulbransen BD, Theis KR, Kuhn DM. **Scientific Reports**. 2020 Jun 12;10(1):9529. DOI: 10.1038/s41598-020-66833-w PMID: 32533086
- **Books or other non-periodical, one-time publications**
 - Nothing to Report
- **Other publications, conference papers, and presentations**
 - Nothing to Report
- **Website(s) or other Internet site(s)**
 - Nothing to Report
- **Technologies or techniques**
 - Nothing to Report
- **Inventions, patent applications, and/or licenses**
 - Nothing to Report

- Other Products

- Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- What individuals have worked on the project?

Name:	Brian D. Gulbransen, Ph.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	orcid.org/0000-0003-1145-3227
Nearest person month worked:	1.2
Contribution to Project:	Oversees all aspects of the project. Provides critical review of data and coordinates the efforts of staff. Provides mentorship for the postdoctoral trainee (Dr. Hernandez-Rivera) and oversees the work of the technician (Dr. Fried). Contributes to writing manuscripts, composing figures, and the presentation of results.
Funding Support:	<p>2R01 DK103723 (Gulbransen) 05/01/2019-06/30/2025 NIH/NIDDK <i>Regulation of enteric motor neurocircuits by enteric glia in health and disease</i> The major goals of this project are to understand how specialized interactions between enteric glia and neurons regulate motility and how alterations in those mechanisms contribute to disease. Role: PI</p> <p>R01 DK120862 (Gulbransen) 03/01/2019 – 02/28/2023 NIH <i>Enteric glia and visceral pain</i> Major goals: To study how enteric glia contribute to the generation of visceral pain through interactions with nociceptors and immune cells. Role: PI</p> <p>Research Fellowship Award (Grubisic) 01/01/2019 – 12/31/2021 Crohn's and Colitis Foundation (CCF) <i>The role of enteroglial adenosine A2BRs in functional recovery after colitis.</i> Major goals: To study the role of glial adenosine 2B receptors in colonic inflammation. Role: Mentor</p> <p>W81XWH1610631, Department of Defense (Gulbransen) 09/15/2016-09/14/2020</p>

	<p><i>Pyridostigmine bromide, the enteric nervous system and functional gastrointestinal disorders in Gulf War illness.</i></p> <p>Major goals: To study how the anti-nerve gas drug pyridostigmine bromide contributes to functional gastrointestinal disorders in Gulf War illness through effects on the enteric nervous system.</p> <p>Role: PI</p> <p>MSU Foundation Endowment (Gulbransen) 08/15/2017 - 08/14/2022 MSU Foundation</p> <p>Foundation endowment Role: Endowed Professor</p>
Name:	Siomara Hernandez-Rivera, Ph.D.
Project Role:	Postdoc
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Dr. Hernandez-Rivera has performed all the in vivo and in vitro experiments mention before in the section 3: accomplishment.
Funding Support:	None (supported by this grant)
Name:	David Fried
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Mr. Fried provides technical support for all ex vivo measurements of gut motility including assisting with experiments and data analysis.
Funding Support:	None (supported by this grant and RO1 DK103723)

- **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 course of this award, the PI established a collaboration with Dr. Donald Kuhn at Wayne State University who is also funded to study mechanisms in Gulf War Illness. This collaborative efforts produced a paper in Scientific Reports (2020).

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:** NA
- **QUAD CHARTS:** NA

9. APPENDICES:

- Nothing to report