AWARD NUMBER: CDMRPL-15-0-GW150188

TITLE: Therapeutic Intervention of Glial-Mediated Enhancement of Neuroinflammation in an Established Model of GWI

PRINCIPAL INVESTIGATOR: Dr. Kimberly Kelly

CONTRACTING ORGANIZATION: Centers for Disease Control and Prevention
Morgantown, WV 26505-2845

REPORT DATE: JUNE 2018

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE
JUNE 2018

2. REPORT TYPE
Annual Report

3. DATES COVERED
23 May 2017 - 22MAY 2018

4. TITLE AND SUBTITLE
Therapeutic Intervention of Glial-Mediated Enhancement of Neuroinflammation in an Established Model of GWI

5a. CONTRACT NUMBER

5b. GRANT NUMBER
CDMRPL-15-0-GW150188

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kimberly Kelly, Ph.D.

E-Mail: GUP2@cdc.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Centers for Disease Control
and Prevention
1095 Willowdale Road
Morgantown, WV 26505

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The main objectives of this project is to expand upon the current understanding of the effects of high physiological stress on nerve agent exposure to identify how the duration of corticosterone (CORT) exposure impacts the long-term endpoints of our GWI model, which glial cell types are involved in the neuroinflammatory phenotype associated with GWI, and whether cell-specific or targeted neuroinflammatory treatment can alleviate GWI symptomatology. We aim to 1) evaluate the duration of CORT exposures necessary to produce the neuroinflammatory phenotype seen in an established, clinically relevant, GWI mouse model; 2) identify the contribution of astrocytes and/or microglia in the development of enhanced neuroinflammation in our GWI model; and 3) test the efficacy of potential therapeutic interventions that target glia and reduce neuroinflammation in our GWI model.

15. SUBJECT TERMS
Gulf War Illness; Diisopropyl Fluorophosphate; Corticosterone; Lipopolysaccarides; Neuroinflammation; Neurodegeneration; Gliosis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	17	

TABLE OF CONTENTS

	Page No.
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	21
5. Changes/Problems	22
6. Products	23
7. Participants & Other Collaborating Organizations	26
8. Special Reporting Requirements	28
9. Appendices	28

1. INTRODUCTION:

The main objectives of this project is to expand upon the current understanding of the effects of high physiological stress on nerve agent exposure to identify how the duration of corticosterone (CORT) exposure impacts the long-term endpoints of our GWI model, which glial cell types are involved in the neuroinflammatory phenotype associated with GWI, and whether cell-specific or targeted neuroinflammatory treatment can alleviate GWI symptomatology. We aim to 1) evaluate the duration of CORT exposures necessary to produce the neuroinflammatory phenotype seen in an established, clinically relevant, GWI mouse model; 2) identify the contribution of astrocytes and/or microglia in the development of enhanced neuroinflammation in our GWI model; and 3) test the efficacy of potential therapeutic interventions that target glia and reduce neuroinflammation in our GWI model.

2. KEYWORDS: Provide a brief list of keywords (limit to 20 words).

Gulf War Illness; Diisopropyl Fluorophosphate; Corticosterone; Lipopolysaccarides; Neuroinflammation; Neurodegeneration; Gliosis

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Research-Specific Tasks:	Anticipated Months to complete	% Completion
Major Task 1: Obtain Protocol Approval:		
Subtask 1: Obtain NIOSH ACUC approval for animal use in the proposed project.	1-3	75%
Subtask 2: Once NIOSH ACUC approval is obtained, ACURO approval of the animal protocols will be completed.	3-6	75%
Milestone(s) Achieved: Approved Protocols	6	75%

Specific Aim 1: Expand the characterization of the established GWI model in mice		
Major Task 2: Model GWI in adult male C57BL6/J mice		
Subtask 1: Initiate GWI phenotype by exposing mice to chronic CORT treatment for either 4 or 7 day increments with DFP on day 5 or 8 respectively. Continued exposures to CORT for either 4 or 7 day increments, respectively, every other week for 30 or 90 days. [2 cohorts of mice with 5 mice/group and 1 cohort of mice with 3 mice/group X 10 groups at 2 time points (30 and 90 days)] = 260 mice]	7-12	100%
Subtask 2: Challenge mice in GWI model with LPS at 30 or 90 days. Mice will be sacrificed 6 hours after LPS exposure.	9-12	100%
Subtask 3: Sacrifice by microwave irradiation (to measure organophosphorylation) [100 mice], decapitation (to measure neuroinflammation) [100 mice], or transcardial formalin perfusion (to obtain neurohistological evidence of glial phenotype) [60 mice] and sample analysis.	12-15	100%
Milestone(s) Achieved: Determine organophosphorylation and neuroinflammatory consequences of the GWI phenotype under challenge conditions at 30 and 90 days. Determine if 30 day time point is sufficient to pilot GWI phenotype.	15-18	100%
Milesone(s) Achieved: Prepare manuscript for publication	17-20	80%

Specific Aim 2: Identify the contribution of astrocytes and/or microglia in the development of enhanced neuroinflammation in our GWI model.		
Major Task 3: Determine astrocyte contribution by modeling GWI in adult male ALDH1L1 BAC-TRAP mice	Aldh1l1 bacTRAP breeding colony is being brought out of maintenance for use to be completed this year.	

Subtask 1: Initiate GWI phenotype by exposing ALDH1L1 BAC-TRAP mice to chronic CORT treatment for either 4 or 7 day increments with DFP on day 5 or 8, respectively with continued exposure to CORT in either 4 or 7 day increments every other week for 30 days. [4 cohorts with 5 mice/group X 6 groups at 1 time point =120 mice]	2	0%
Subtask 2: Challenge the GWI phenotype with LPS. Mice within the GWI model will be treated with LPS at 30 days. Mice will be sacrificed at 6 hours after LPS.	2	
Subtask 3: Sacrifice by microwave irridation (to measure organophosphorylation) [30 mice], decapitation (TRAP of astrocyte specific mRNA for RNAseq and qPCR) [60 mice] or transcardial formalin perfusion (to obtain neurohistological evidence of glial phenotype) [30 mice] and analysis.	2	
Subtask 4: Analysis of next generation sequencing data set.	2	100%
Milestone(s) Achieved: Determine contribution of astrocytes to GWI phenotype.		
Major Task 4: Determine microglial contribution, and crosstalk with astrocytes, in the GWI model with PLX3362 in adult ALDH1L1 BAC-TRAP mice	PLX3362 treatment was done in C57 animals in a shorter 10d model.	
Subtask 1: Pretreat wildtype C57BL6/J and transgenic ALDH1L1 BAC-TRAP mice with CSF1R inhibitor (PLX3397) to deplete microglia during initial CORT and DFP exposures. Continued CORT exposures every other week for 30 days followed by LPS challenge. [4 ALDH1L1 cohorts with 5 mice/group X 6 groups at 1 time point and 3 C57BL6/J cohorts with 5 mice/group X 6 groups = 210 mice]	2	75%
Subtask 2: Sacrifice by microwave irradiation (to measure organophosphorylation) [60 mice], decapitation (TRAP of astrocyte specific mRNA for RNAseq and qPCR) [90 mice], or transcardial formalin perfusion (to obtain neurohistological evidence of glial phenotype) [60 mice] and analysis.	2	
Subtask 3: Analysis of next generation sequencing data set	2	
Milestone(s) Achieved: Determine contribution of microglia to GWI phenotype.		
Milestone(s) Achieved: Prepare manuscript for publication		
PHASE 3: Major Task 5 and 6 (subject to availability of funding)		

Specific Aim 3: Test the efficacy of potential therapeutic interventions that target glia and reduce neuroinflammation in our GWI model.		
Major Task 5: Test potential therapies at 30 days after initiation of GWI.	All to be done in 5 week paradigm; remaining treatments are planned for the coming months	
Subtask 1: Initiate GWI phenotype for 30 days. Treat with astrocyte inhibitor pentoxifylline prior to LPS challenge. [1 cohort with 5 mice per group X 12 groups X 1 time point] = 60 mice	3	0%
Subtask 2: Initiate GWI phenotype for 30 days. Treat with microglia inhibitor spironolactone prior to LPS challenge and sacrifice 6 hours later. [1 cohort with 5 mice per group X 12 groups X 1 time point] = 60 mice	3	0%
Subtask 3: Initiate GWI phenotype for 30 days. Treat with IL1aR antagonist anakinra prior to LPS challenge and sacrifice 6 hours later. [1 cohort with 5 mice per group X 12 groups X 1 time point] = 60 mice	3	0%
Subtask 3: Initiate GWI phenotype for 30 days. Treat with TNFaR antagonist etanercept prior to LPS challenge and sacrifice 6 hours later. [1 cohort with 5 mice per group X 12 groups X 1 time point] = 60 mice	3	100%
Subtask 4: Initiate GWI phenotype for 30 days. Treat with pharmacologic treatment to be obtained by previous studies prior to LPS challenge and sacrifice 6 hours later. [1 cohort with 5 mice per group X 12 groups X 1 time point] = 60 mice	3	0%
Milestone(s) Achieved: Determine best treatment option(s) to use in GWI model to be used at clinically relevant time points.		
Milestone(s) Achieved: Prepare manuscript for publication		
Major Task 6: Test best therapy option(s) at 90 and 180 days after initiation of GWI.	Replacement of long term phenotype with 5 week analysis makes this the same as Major Task 5	
Subtask 1: Initiate GWI phenotype for 90 days. Treat pharmacologically (2 best therapeutics) prior to LPS challenge. [1 cohort X 5 mice per group X 12 groups X 2 treatments] = 120 mice	3	

Subtask 2: Initiate GWI phenotype for 180 days. Treat pharmacologically prior to LPS challenge. [1 cohort X 5 mice per group X 12 groups X 2 treatments] = 120 mice	3	
Subtask 3: Sacrifice by decapitation and measure neuroinflammation at time points 90 and 180 day time points	3	
Milestone(s) Achieved: Confirm that the pharmacologic therapy chosen from the 30 day pilot studies can prevent exacerbated responses to LPS challenge at clinically relevant human time points (90 and 180 days in a mouse ~ equivalent to 20 years in a human)		
Milestone(s) Achieved: Prepare manuscript for publication.		

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Major Task 1: Obtain protocol approval.

Both IACUC and ACURO approval of animal protocols was obtained.

Major Task 2: Model GWI in adult male C57BL6/J mice.

Neuroinflammation and neurohistological analyses in male C57BL6/J mice have been completed.

Major Task 3: Determine astrocyte contribution by modeling GWI in adult male ALDH1L1 BAC-TRAP mice.

While the ALDH1L1 bacTRAP line was not able to be used, we did perform RNAseq using C57BL6/J mice. Our Aldh1l1 strain will now be able to be used and this work will be completed.

Major Task 4: Determine microglial contribution, and crosstalk with astrocytes, in the GWI model with PLX3362 in adult ALDH1L1 bacTRAP mice.

PLX3362 was used in C57BL6/J mice to gain insight into the contribution of microglia in the GWI phenotype.

Major Task 5: Test potential therapies at 30 days after initiation of GWI. And Major Task 6: Test best therapy option(s) at 90 and 180 days after initiation of GWI.

These major tasks were combined when we found that the 5 week phenotype was a good substitute for 90 and 180 day models (see 2017 annual report for details, also verbiage

was included below). One of the treatments has been completed and the others are planned for the next few months.

Results:

Neuroinflammation: Mice were treated with CORT (200 mg/L 0.6% EtOH) in the drinking water for 7 days. On the 8th day, DFP (4 mg/kg, i.p.) was administered and animals were given CORT in the drinking water for 7 days in week 3 and 5 at the end of which mice were given LPS (0.5 mg/kg, s.c.) and sacrificed by decapitation 6 hours later and prepared for qPCR analysis of inflammatory cytokines and chemokines.

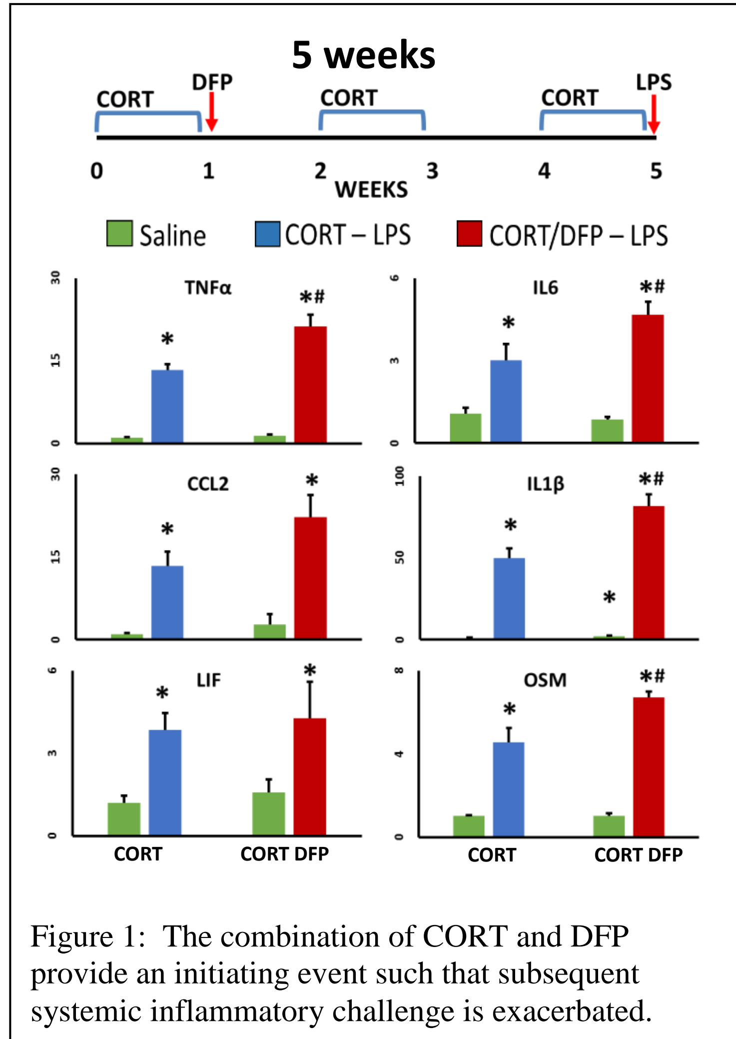


Figure 1: The combination of CORT and DFP provide an initiating event such that subsequent systemic inflammatory challenge is exacerbated.

We have found that prior chronic exposure to the stress hormone CORT can enhance the neuroinflammation caused by sarin surrogate DFP (O'Callaghan et al., 2015, Locker et al., 2017) providing evidence for combined exposures to CORT and DFP as an animal model for the initiating event producing GWI pathobiology. Here, we have expanded upon this initiating event to show that the combination of CORT and DFP produce a sensitivity to subsequent systemic inflammatory challenge (Figure 1). These data provide evidence for an animal model of Gulf War Illness that mimics the dormant phenotype that emerges when faced with an injury, infection, or illness.

Neurohistology: Mice were treated with CORT (200 mg/L 0.6% EtOH) in the drinking water for 7 days. On the 8th day, DFP (4 mg/kg, i.p.) was administered and animals were given CORT in the drinking water for 7 days in week 3 and 5 at the end of which mice were given LPS (0.5 mg/kg, s.c.) and sacrificed by transcardial formalin perfusion 24 hours later. Brains were removed and sent to FD Neurotechnologies for microglia, astrocyte, and neurodegeneration staining.

Previously we have shown acute exposure to the initiating event producing GWI pathobiology (CORT and DFP) did not produce significant glial changes in morphology or neurodegeneration (O'Callaghan et al., 2015). Here, we have used that same initiating event with a systemic inflammatory challenge at 5 weeks. While we have slides in hand for these experiments, only a subset of the microscopic images have been taken and thus an incomplete dataset is shown in the figures below.

The astrocyte response to the 5 week GWI phenotype is shown in Figure 2. The combination of CORT and DFP was able to create a pathology in which a subsequent, systemic low dose LPS challenge was able to produce astrocyte hypertrophy at 24 hours after exposure. The panels on the right depict representative control and challenged GWI phenotype treated astrocytes at high magnification to highlight the morphological difference in the astrocytes under these conditions.

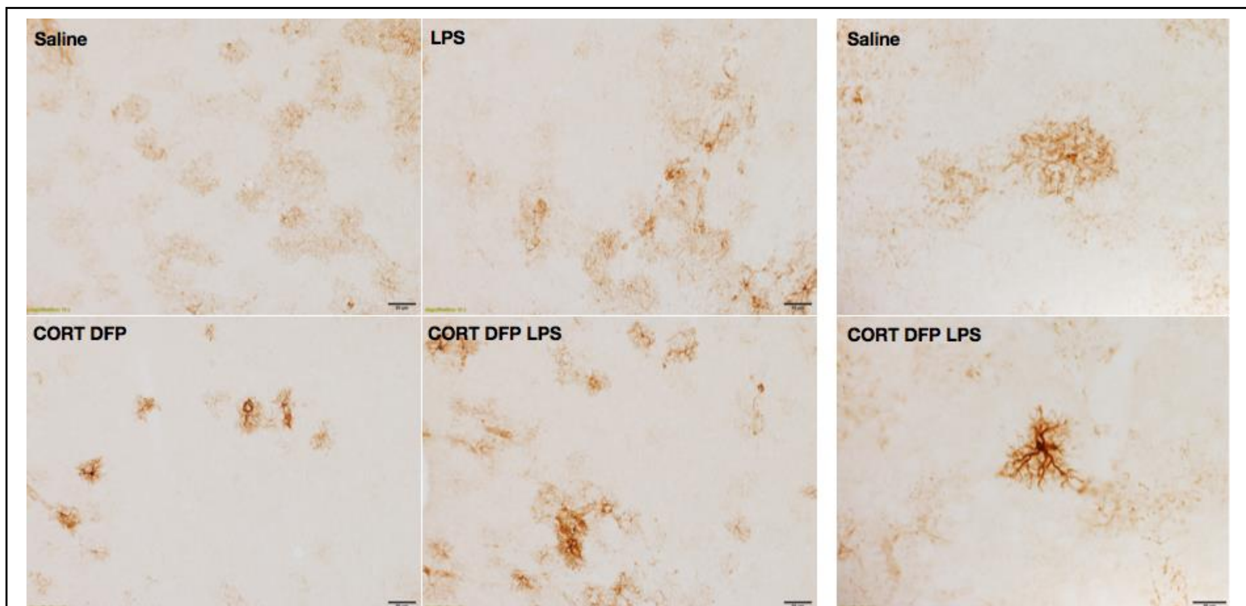


Figure 2: Astrocytes in the front Cortex appear to have increased hypertrophy (shown at 20x magnification)

Representative astrocytes (saline and CORT DFP LPS) are shown at 60x magnification in the panels to the right.

Both Silver and Fluro-Jade B neurodegeneration stains were used in this paradigm. Neither Silver (Figure 3) nor Fluoro-Jade B (Figure 4) stains showed significant changes in our model.

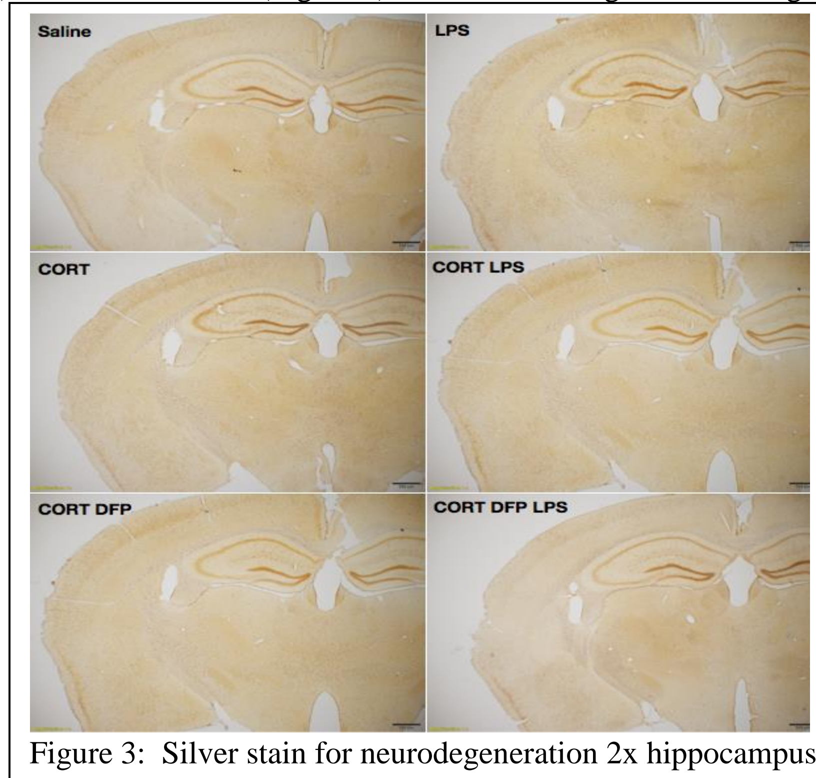


Figure 3: Silver stain for neurodegeneration 2x hippocampus.

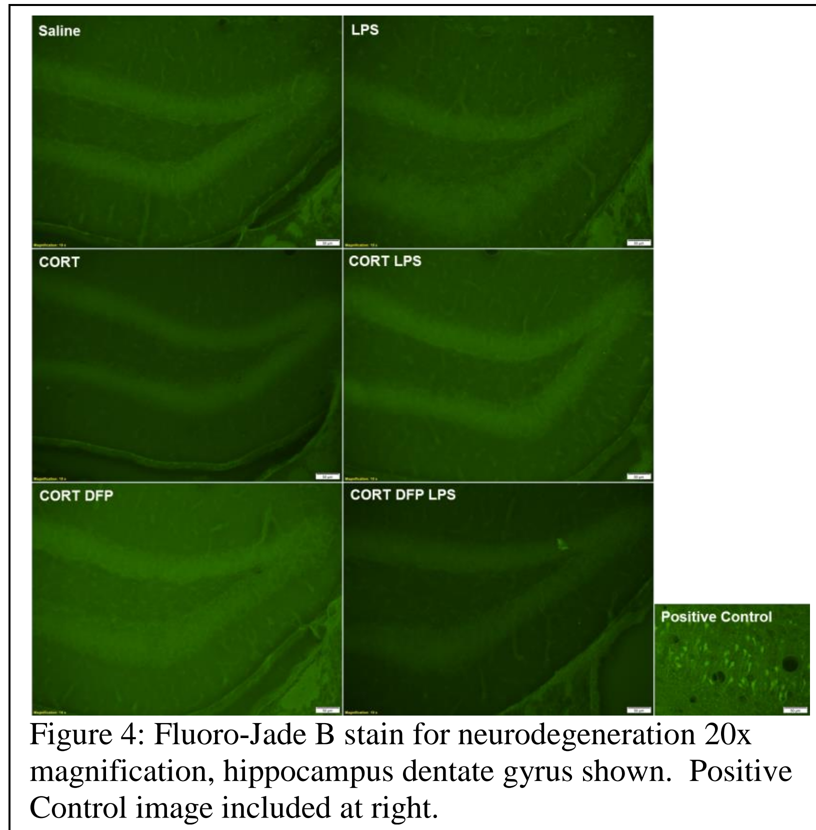


Figure 4: Fluoro-Jade B stain for neurodegeneration 20x magnification, hippocampus dentate gyrus shown. Positive Control image included at right.

Recent findings from collaborations in another GWI project have concluded that the short term time point has significant overlap with human blood samples of patients with GWI. Therefore, based on this information and the neuroinflammatory end point data shown above we have concluded that this short term animal model can be used to model the GWI phenotype (this is the milestone for task 1 in the statement of work). As such, this paradigm was used for the rest of these studies.

Microglia: Histology was done looking at IBA1 and Cd11b markers. Histological evidence of microglial morphology reveals a slight reduction in aborization of processes in both CORT + DFP and CORT + DFP + LPS exposure groups in Iba1 stained sections of cortex and hippocampus (Figure 5 and 6). Where as, when microglia were stained with Cd11b the sections show more widespread activation in cortex and hippocampus, with activation also found in substantia nigra, periaqueductal gray, brainstem, and cerebellum, areas that can effect movement, pain modulation, and other common symptoms affected by those with GWI.

Figure 5: Iba1 stained microglia in cortex

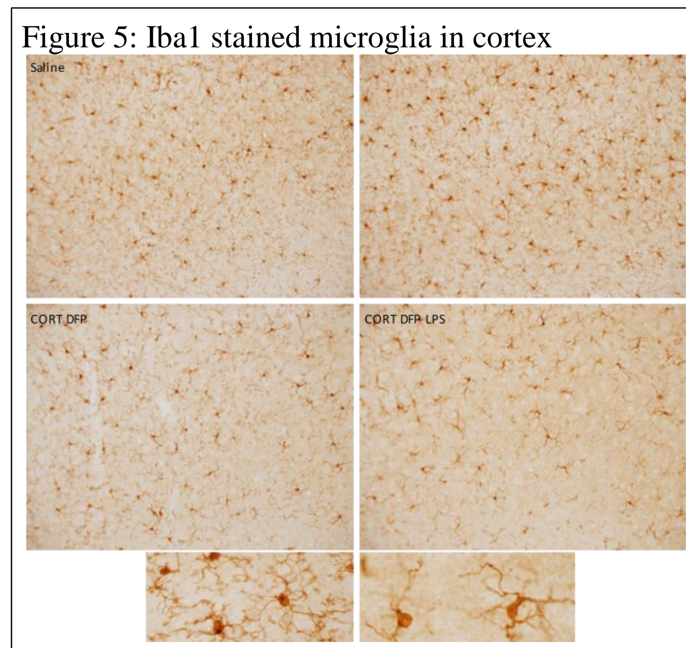
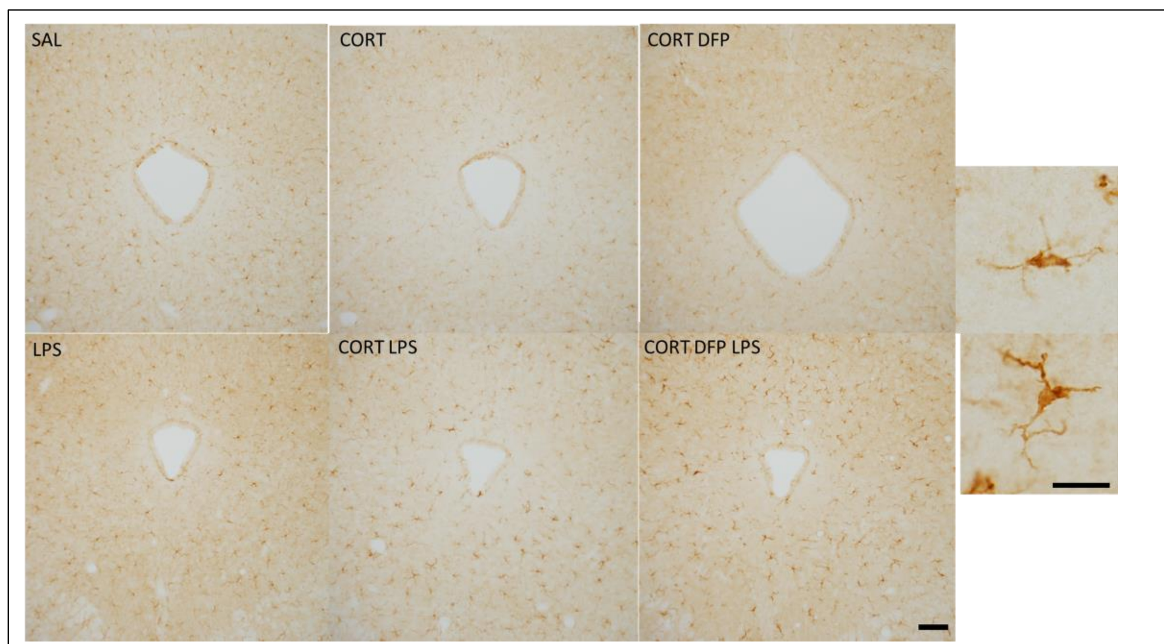
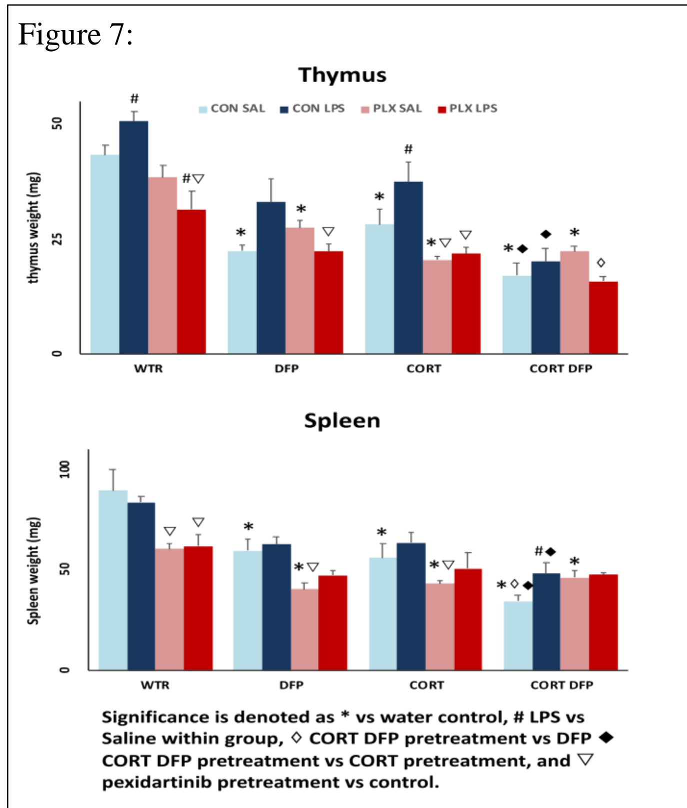


Figure 6: Iba1 stained microglia in periaqueductal gray



Pretreatment with CSFR1 inhibitor Pexidartinib (PLX) (290 mg/kg chow ad libitum over 28d), a compound that has been demonstrated to “eliminate” or reduce microglia in brain, was used to investigate the role microglia play in the pathogenesis of this neuroinflammatory disorder. Interestingly, the well documented reduction in DFP-induced mortality by CORT pretreatment was further reduced by PLX in CORT DFP (7%-36%) groups and eliminated in the DFP alone group (0%-71%).

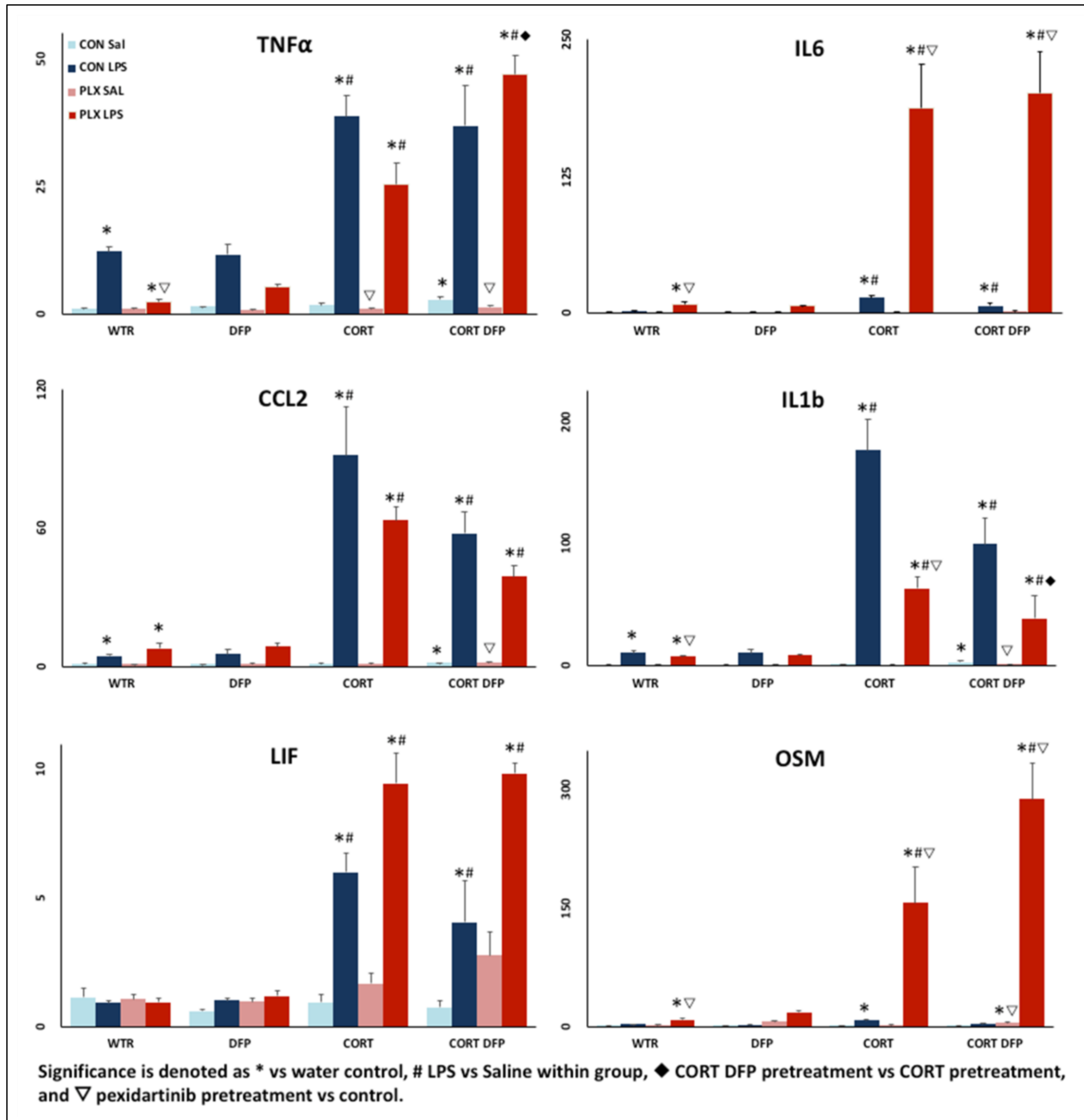
Figure 7:



Significance is denoted as * vs water control, # LPS vs Saline within group, ◊ CORT DFP pretreatment vs DFP ◆ CORT DFP pretreatment vs CORT pretreatment, and ▽ pexidartinib pretreatment vs control.

CORT pretreatment caused significant thymic involution in both control (35%) and PLX (47%) groups with PLX groups showing reduced thymic weights (11% and 27%, respectively). The further reduction of thymus and spleen weights by PLX over that of CORT may point to an additive effect by which the loss of microglia exacerbated the stressor-induced involution. This coupled with the reduction of mortality to this extent was completely unexpected and has posed many more questions as to the role microglia play in the pathogenesis of GWI. Experiments into the time frame of microglial wipe out and reintroduction are needed before meaningful evaluation of data using this paradigm can be done.

Figure 8:

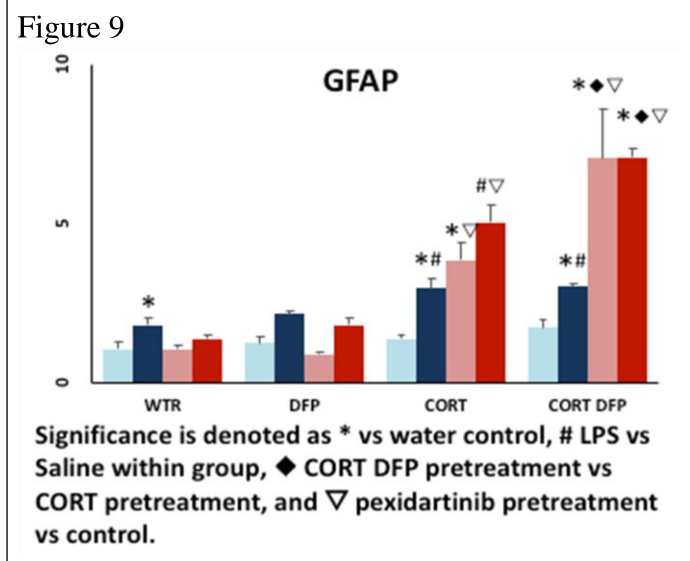


The brain cytokine/chemokine expression levels are also hard to delineate in this data set. Our hypothesis is that the extreme levels of exacerbation of IL6 and OSM specifically, point to an astrocyte response in pexidartinib pretreated animals. This is backed up by the significant increases seen in GFAP expression in pexidartinib pretreated groups that is consistent with damage or neuroinflammatory responses. It's possible that this may be due to compensation of astrocytes for the lack of microglia due to the elimination by pexidartinib or could be that microglia are important in protection against astrocyte hypertrophy.

We plan to do further analyses in which these experiments will be expanded upon to look at immunohistochemistry to look at morphological changes in astrocytes at this and later timepoints to confirm the potential astrocyte hypertrophy and to confirm the absence of microglia. We also play to look at different timing for the introduction of PLX such that an investigation as to when in the initiation of the GWI phenotype microglia are playing this role. If the microglia are

suppressed only in the initiation of the GWI phenotype does the challenge behave in the same way as if microglia are suppressed throughout the experiment. Also, if the microglia are not suppressed at the time of DFP what effect does this have on the outcome of inflammatory challenges after the fact. These questions fell outside of the scope of this project and we therefore did not go beyond this dataset for these evaluations.

Figure 9



Significance is denoted as * vs water control, # LPS vs Saline within group, ◆ CORT DFP pretreatment vs CORT pretreatment, and ∇ pexidartinib pretreatment vs control.

RNAseq analysis: RNAseq analysis was run on in the 5 week GWI phenotype using both 4 and 7 days of CORT. The inclusion of the 4 days of CORT was supposed to be an additional control for an amount of CORT that would be on board without causing the additive priming effect found when 7 days of CORT is utilized. The RNAseq analysis for the 4 days of CORT 5 week model showed a PCA plot that had no discernable differentiation in the groups and no clear outline for a definitive GWI phenotype. As can be seen in the PCA plot the CORT groups conglomerate towards the bottom left corner and no clear delineation between the CORT groups, let alone between the CORT LPS and CORT DFP LPS groups can be seen.

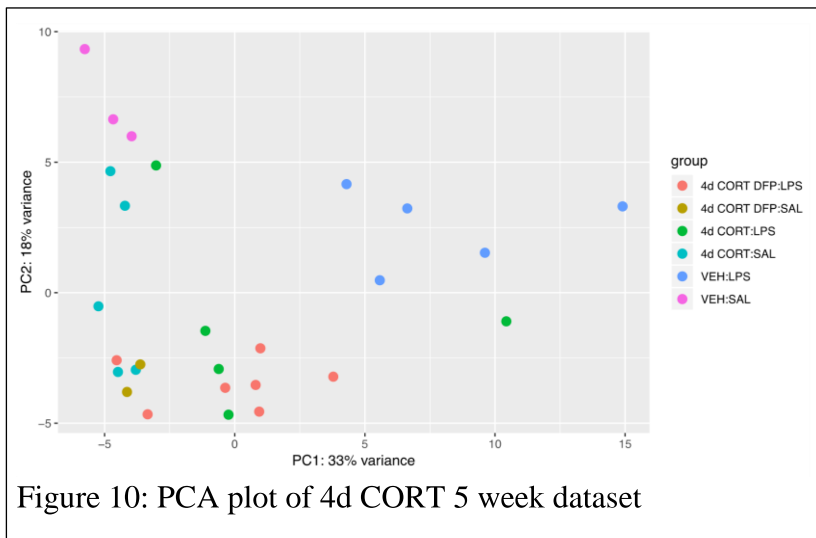
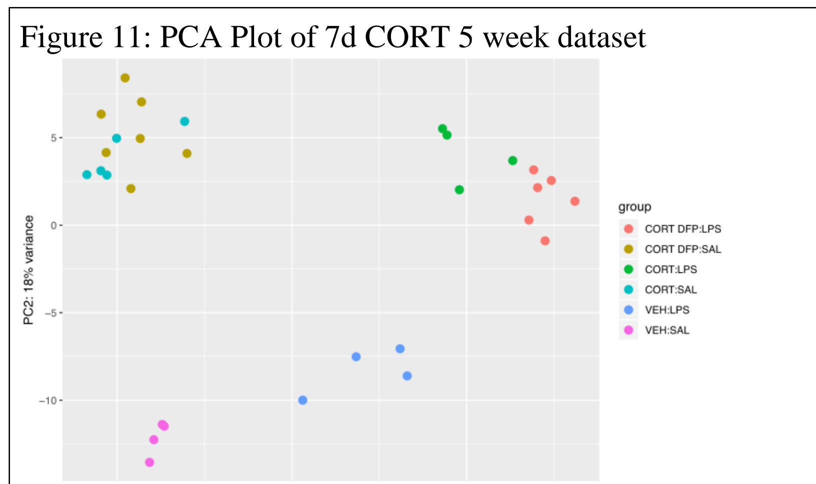


Figure 10: PCA plot of 4d CORT 5 week dataset

However, when analyzing the results of the RNAseq analysis for the 7 days of CORT groups a clear delineation between the groups can be readily identified. The clear separation between the CORT LPS and CORT DFP LPS groups shows that the DFP contributes significantly to the GWI phenotype. In fact, the lack of delineation between the CORT SAL and CORT DFP SAL groups provides further evidence for the dormant phenotype that

Figure 11: PCA Plot of 7d CORT 5 week dataset



exists with GWI in which a ‘flare up’ caused by any perturbation (illness or injury) can cause exaggerated response. Evaluation of this dataset with a cut off of $p<0.05$ and a log2FC equal to or greater than 2 or equal to or less than -2 revealed 472 differentially expressed genes (DEG), 252 of which were up regulated. 205 of these are known genes of which DAVID analysis could be performed. The top Gene Ontology terms and KEGG pathways are included in the table below.

Table 1: Top 5 “Molecular Function,” “Biological Process,” and “Cellular Compartment Gene Ontology Terms.

	Term	p-value	Genes
Molecuar Function	G-protein coupled purinergic nucleotide receptor activity	4.60E-03	Gpr34, A630033H20Rik, P2ry12
	non-membrane spanning protein tyrosine kinase activity	5.50E-03	Fgr, Itk, Ptk6, Styk1
	chemokine (C-C motif) ligand 5 binding	1.60E-02	Ccr1, Ccr5
	pantetheine hydrolase activity	1.60E-02	Vnn1, Vnn3
	alcohol dehydrogenase activity, zinc-dependent	2.40E-02	Adh1, Adh7
Biological Process	immune system process	2.80E-09	Cd180, Cd300lg, Cd74, Fgr, Itk, Nlrp10, Slamf7, Trat1, Zbp1, H2-Aa, H2-Ab1, H2-Eb1, Il31ra, Ltf, Lrmp, Serpina3g, Tlr9, Tnfrsf13b, Tnfrsf17
	immune response	4.10E-07	Cd36, Cd74, Tgtp2, Ccl12, Ccr1, Ccr5, Cxcl9, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Tlr9, Tnfsf8, Tnfrsf8
	antigen processing and presentation of exogenous peptide antigen via MHC class II	4.20E-06	Cd74, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2
	T cell costimulation	4.90E-05	Cd5, Card11, Icos, Gm1897, Spn
	antigen processing and presentation	8.10E-05	Cd74, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Raet1d
Cellulcar Compartment	external side of plasma membrane	2.10E-12	Cd19, Cd209b, Cd22, Cd36, Cd5, Cd74, Slamf7, Ccr1, Ccr5, Cxcl9, H2-Aa, H2-Ab1, Icos, Itgam, Il31ra, Il7r, Lag3, P2ry12, Raet1d, Spn, Tnfrsf13b
	MHC class II protein complex	2.10E-06	Cd74, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2
	membrane	1.00E-05	Abcc6, Abcg3, Atp2a1, Clec12a, Cd180, Cd19, Cd209a, Cd209b, Cd22, Cd300lg, Cd36, Cd5, Cd74, Fat2, Fgr, Fgd2, Fcrls, Gpr34, Nlrp10, Ptk6, Slamf7, Trat1, Tgtp2, Ush2a, Acpp, Ano7, Cacng6, Casr, Card11, Ccr1, Ccr5, Chrnb4, Crb3, Ccnb1, Cyp2e1, Degs2, Ecscr, Gdpd4, Gp9, Gbp4, H2-Aa, H2-Ab1, H2-Eb1, Icos, Itgam, Il31ra, Il7r, Loxhd1, Ly6c2, Lag3, Lrmp, Ninj2, Ncaph, Otop3, Pde6c, Kcnj15, Kcng1, Prokr1, Prss30, Prss56, Pcdh12, P2ry12, Rapsn, Rpe65, Raet1d, Sin, Styk1, Spn, Slc2a5, Slc4a1, Slco1b2, Spaca1, Tlr9, Tecrl, Trpm1, Tmprss15, Tmem72, Tmem8c, Treml1, Tnfsf8, Tnfrsf13b, Tnfrsf17, Tnfrsf8, Upk1b, Vnn1, Vnn3
	integral component of membrane	4.60E-04	Abca12, Abcc6, Abcg3, Atp2a1, Clec12a, Clec7a, Cd180, Cd19, Cd209a, Cd209b, Cd22, Cd300lg, Cd36, Cd5, Cd74, Fat2, Gpr34, A630033H20Rik, Slamf7, Trat1, Ush2a, Acpp, Ano7, Cacng6, Casr, Ccr1, Ccr5, Chrnb4, Cldn20, Crb3, Chrnb4, Cldn20, Crb3, Cyp2e1, Degs2, Ecscr, Gdpd4, Gp9, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Hsd17b13, Icos, Itgam, Il31ra, Il7r, Lag3, Lrmp, Ninj2, Olfr1033, Otop3, Kcnj15, Kcng1, Gm10825, Gm4861, Gm5127, Gm9573, Prokr1, Pcdh12, P2ry12, Sin, Styk1, Spn, Slc2a5, Slc4a1, Slco1b2, Spaca1, Spata20, Tlr9, Tecrl, trpm1, Tmprss15, Tmem72, Tmem8c, Treml1, Tnfsf8, Tnfrsf13b, Tnfrsf17, Tnfrsf8, Upk1b, Vnn1
	plasma membrane	1.30E-03	Abca12, Abcc6, Abcg3, Clec12a, Clec7a, Cd180, Cd22, Cd300lg, Cd36, Cd5, Cd74, Fat2, Fgr, Fgd2, Gpr34, Nlrp10, Slamf7, Trat1, Ush2a, Acpp, Ano7, Casr, Card11, Ccr1, Ccr5, Chrnb4, Cldn20, Clg, Crb3, Ecscr, H2-Aa, H2-Ab1, Irs3, Il7r, Ly6cr, Olfr1033, Pde6c, Kcnj15, Kcng1, Gm9573, Prokr1, Prss30, Pcdh12, P2ry12, Rapsn, Rpe65, Raet1d, Styk1, Slc2a5, Slc4a1, Slco1b2, Spata20, Tlr9, Trpm1, Tmem8c, Treml1, Vnn1, Vnn3

Table 2: Top 5 KEGG pathways

Pathway Name	p-value	Genes
Intestinal immune network for IgA production	2.40E-06	H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Icos, Tnfrsf13b, Tnfrsf17
Hematopoietic cell lineage	1.40E-05	Cd19, Cd22, Cd36, Cd5, Gp9, H2-Eb1, Itgam, Il7r
Tuberculosis	3.90E-05	Clec7a, Cd209a, Cd209b, Cd74, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Itgam, Tlr9
Cell adhesion molecules (CAMs)	1.40E-04	Cd22, Cldn20, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Icos, Itgam, Spn
Phagosome	2.00E-04	Clec7a, Cd209a, Cd209b, Cd36, H2-Aa, H2-Ab1, H2-Eb1, H2-DMb2, Itgam

Additionally, there were 33 genes upregulated and 16 genes downregulated uniquely in the CORT DFP LPS group. Of these genes, too few were known and therefore pathway and GO term analysis were not possible. A table of the genes specific to the CORT DFP LPS group is shown in Table 3.

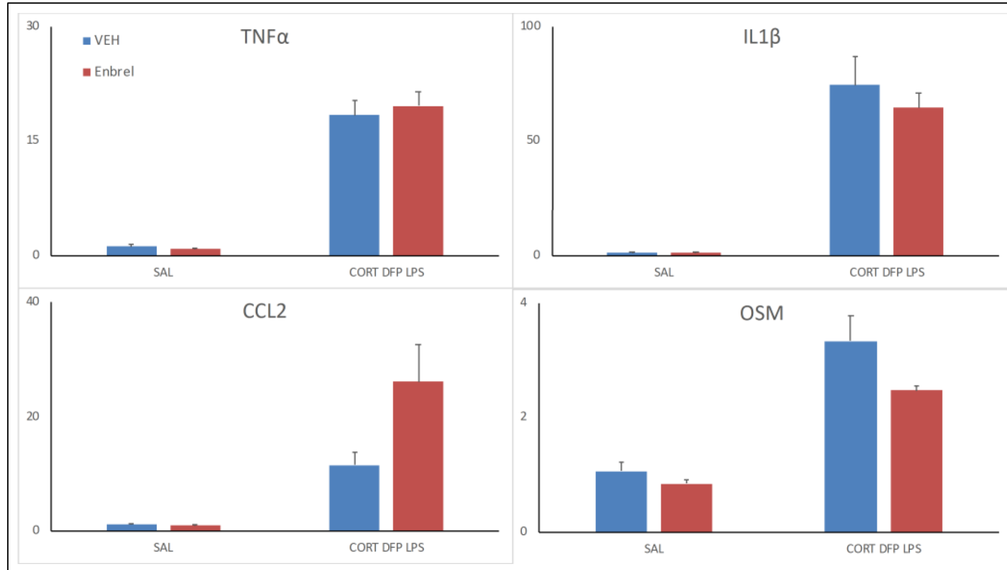
Table 3: DEGs unique to CORT DFP LPS.

upregulated	Naip7, S-adenosylmethionine_decarboxylase_246, AK199258, AK197180, Il7r, Mir8094, Mir6236, E330017L17Rik, Ak005481, AK085609, AK196308, 4933407I05Rik, Arhgap27os3, AK204572, AK214897, Tbx4, AK006138, Snora34, AK205023, AK039820, AK181773, Gm10825, Cer1, 4930482G09Rik, Crb3, Irx1, Degs2, Grap2, Ak044848, AK003327, 1700007P06Rik, Mlph, Ano7
downregulated	Cxcl11, BC030398, Gbp10, Gm14005, Serpinf2, LOC100038947, Ctsw, Oasl1, AB001425, Icam1, Fpr2, Gbp5, Lox, Htr1d, Cd14, Il1a

Importantly, the GO/KEGG analysis of this 5 week dataset gives different pathways of interest than the acute exposure, illustrating the difference between acute "toxicity" of the exposures and the long-term underlying neuroinflammatory disorder we associate with GWI.

Treatments: Treatment of the GWI phenotype with pentoxifylline, spironolactone, and anakinra are planned but have no yet been performed. Treatment with Enbrel has been done and unfortunately, did not show promising results in treatment of the GWI phenotype.

Figure 12: Enbrel treatment in the 5 week GWI phenotype.



What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Attendance at the Society for Neuroscience annual meeting, Winter Conference on Brain Research, the Military Health System Research Symposium, and Society of Toxicology annual meeting provided training and professional development in neuroscience and toxicology.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of

these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Nothing to report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Results were disseminated by attendance and presentations at the Society for Neuroscience annual meeting, Winter Conference on Brain Research, the Military Health System Research Symposium, and Society of Toxicology annual meetings

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Initially this grant included data obtained using a 30 day paradigm. It has been found that a 5 week exposure paradigm has much better results and allows for a week of CORT administration in which both DFP and LPS exposures are not directly interacting with the CORT treatment. This has developed as an excellent period to test treatment compounds, because the treatment is given at a time long enough after DFP to avoid the question of whether the initiation of the GWI phenotype has been prevented and long enough before LPS challenge to avoid the question that the treatment of interest directly impacted the mechanism of action of LPS inflammation itself. This is ideal, because: 1) we cannot treat veterans with GWI to prevent the condition that they currently have; and 2) our goal is to discover a treatment that reverses the underlying pathobiology of GWI, not treat symptoms or "flare-ups."

The use of ALDH1L1 bacTRAP animals was not possible due to breeding colony problems and IACUC approvals. Coupled with the intriguing results in the pexidartinib experiment, we were unable to finish exeriments using this strain of animals with or without the pexidartinib diet. With a new veterinarian in place, our breeding colony is already coming out of maintenance and we are approved to use the animals in our GWI paradigm. The work for these experiments will begin soon in the new year.

There was not enough time to complete all 5 treatments laid out in the SOW within the timeline due to hold ups with IACUC approvals. We did complete one to date and plan to finish the last four over the next few months.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

IACUC approval took longer than expected. Many more pilot experiments had to be planned and carried out before experiments could be performed. While the ALDH1L1 bacTRAP animals were approved for use by our IACUC, the colony was held in maintenance and no animals were produced for use. This issue has been resolved and the breeding colony is being ramped up to produce animals to use in the experiments as laid out in the specific aims.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

All protocols have been approved by the IACUC and ACURO.

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.”

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Kelly KA, Michalovicz LT, Miller JV, Rice M, Vashishtha S, Craddock TJ, Fletcher MA, Morris M, Klimas N, Miller DB, Broderick G, O'Callaghan JP: Resetting homeostasis in Gulf War Illness, a computational biology hypothesis tested in a translational mouse model. Abstract & Poster. Military Health System Research Symposium, Kissimmee, FL August 2018

Kelly KA, Miller JV, Michalovicz LT, Miller DB, O'Callaghan JP: New insights into the role microglia play in the etiology of gulf war illness. Abstract & Poster. Society of Toxicology, San Antonio, TX March 2018

Michalovicz LT, **Kelly KA**, Miller JV, Miller DB, O'Callaghan JP: Neuroinflammation and Gulf War Illness: The role of microglia in the response to in-theater toxicant exposure. Abstract & Poster. Society of Toxicology, San Antonio, TX March 2018

Michalovicz LT, **Kelly KA**, Miller JV, Miller DB, O'Callaghan JP: Microglia play a crucial role in the neuroinflammation underlying Gulf War Illness. Abstract & Poster. Society for Neuroscience, Washington DC November 2017

Kelly KA, Michalovicz LT, Miller JV, Broderick G, Craddock TJ, Miller DB, O'Callaghan JP: Resetting homeostasis in Gulf War Illness, a computational biology hypothesis tested in

a translational mouse model. Abstract & Poster. Society of Toxicology, Baltimore, MD March 2017

Kelly KA, Locker AR, Michalovicz LT, Vrana JA, Vashishtha S, Broderick G, Miller DB, O'Callaghan JP: A mouse model of Gulf War Illness reveals a primed neuroinflammatory response to subsequent systemic inflammatory challenge. Abstract & Poster. Society for Neuroscience, San Diego, CA November 2016

Michalovicz LT, Kelly KA, Locker AR, Vrana JV, Miller DB, O'Callaghan JP: Using novel ALDH1L1 bacTRAP technology to evaluate the astrocyte-specific response to Gulf War Illness-related exposures. Abstract & Poster. Society for Neuroscience, San Diego, CA November 2016.

Broderick G, Vashishtha S, Russell L, Michalovicz L, Kelly KA, Vrana JA, Locker AR, Barnes ZM, Craddock TJA, Fletcher MA, Klimas NG, Miller DB, O'Callaghan JP, Morris M: Stress potentiation of the brain's immune response to neurotoxic exposure in the field: an animal model of Gulf War Illness. Abstract & Poster. International Association for Chronic Fatigue Syndrome/Myalgeic Encephalomyelitis Research and Clinical Conference: Emerging Science and Clinical Care, Fort Lauderdale, Florida October 2016.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  Nothing to report

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

  Nothing to report
- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the

understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *biospecimen collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

<u>*Example:*</u>

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award).*

Name:	**Kimberly Kelly**
Project Role:	PI
Research Identifier (e.g. ORCID ID):	0000-0002-1146-3137
Nearest person month worked:	12
Contribution to Project:	Designed and implemented experiments, analyzed and interpreted data, preparation of abstracts/manuscripts
Funding Support:	CDC-NIOSH

Name:	James O'Callaghan
Project Role:	Co-Investigator
Research Identifier (e.g. ORCID ID):	0000-0001-8497-4598
Nearest person month worked:	3
Contribution to Project:	Designed experiments, interpreted data, preparation of abstracts/manuscripts
Funding Support:	CDC-NIOSH

Name:	Lindsay Michalovicz
Project Role:	post-doctoral fellow
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	3
Contribution to Project:	implementation of experiments, analyzed data, preparation of abstracts/manuscripts
Funding Support:	CDC-NIOSH

Name:	Brenda Billig
Project Role:	Lab manager
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	implementation of experiments
Funding Support:	CDC-NIOSH

Name:	Christopher Felton
Project Role:	laboratory technician
Research Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1
Contribution to Project:	implementation of experiments
Funding Support:	CDC-NIOSH

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to report

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. **APPENDICES:** N/A