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TITLE: Suppression of GWVI Toxin-Activated Microglia and Pathologies by DREADD

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Approximately one third of Veterans who served in the Gulf War later developed a chronic multi-symptom illness known as Gulf War Illness (GWI). While the exact cause is unknown, it is believed that persistent exposure to environmental toxins such as pesticides and chemical warfare agents may have interacted with combat-related stress to produce lasting neurological and psychiatric complications among this Veteran population. Neuroinflammation has been increasingly linked with psychiatric and neurological disorders and may play a role in GWI pathology. Microglia are a key mediator of neuroinflammation and the underlying goal of this project is to test the hypothesis that microglial activation acts as a causal factor to produce cognitive and psychiatric disturbances in a mouse model of GWI. In particular, this project will utilized novel Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology to inactivate microglia in our mouse model of GWI.

15. SUBJECT TERMS None listed.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER**	**19a. NAME OF RESPONSIBLE PERSON**
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Prescribed by ANSI Std. Z39.18

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1. INTRODUCTION

The overarching aim of this research initiative, generously funded by the Department of Defense (DOD), is to pioneer a groundbreaking mouse model for Gulf War Illness (GWI). Our central hypothesis posits that microglia serve as a pivotal mediator in the neuropathology of GWI. We are employing an innovative approach to realize these objectives, leveraging a Cx3Cr1-dependent designer receptor exclusively activated by a designer drug (DREADD) to suppress microglia in mice selectively. We investigated the hypothesis that microglial activation acts as a mediator in the manifestation of neuroinflammation and behavioral abnormalities after exposure to permethrin and stress. This cutting-edge methodology aims to deepen our understanding of the intricate mechanisms underlying GWI, paving the way for potential advancements in its diagnosis and treatment.

2. KEYWORDS

Gulf War Illness, microglia, permethrin, stress, pyrethroid, DREADD, learning and memory, depression, anxiety

3. ACCOMPLISHMENTS

a. Primary Goals of the Project:

Finalize Breeding of DREADD Mice and Validate DREADD-mediated microglial activation with LPS (Year 1)

-To generate a Breeding colony to produce sufficient numbers of DREADD mice necessary for all experiments.

-To use pro-inflammatory LPS administration to induce microglial activation

- To inactivate microglia via administration of clozapine-n-oxide to validate DREADD technique.

Immunohistochemistry of microglia and Animal Behavior Assessments (Year 2)

-Quantify microglial activation in a mouse model of GWI and suppression.

-To determine if microglial suppression in DREADD mice prevents spatial memory impairments in a Gulf War Illness (GWI) mouse model.

Peripheral and Neuroimmune Interaction Studies (Year 3)

-To collect brains and blood from mice exposed to neurotoxins and stress to analyze immune cell populations via transcriptomic studies

-Determine if microglial suppression in DREADD mice affects peripheral immune cell phenotypes.

Animal Behavior Assessments (Year 4)

-To determine if microglial suppression in DREADD mice prevents stress-induced psychological impairments in the GWI mouse model

- To determine differentially expressed genes in each of the relevant cell populations in the GWI mouse model

b. Accomplishments Under These goals

Gulf War Illness (GWI) is a chronic multisymptomatic disorder that disproportionately affects Gulf War veterans, and the combination of permethrin exposure with stress may have collectively and synergistically contributed to its development. However, the exact underlying pathogenic mechanisms within the brain that link permethrin and stress to the development of GWI remain unclear. In this study, we investigated the effects and the associated molecular mechanisms within the microglia-neuron interplay in a mouse model of GWI, focusing on how exposure to permethrin may act as a priming agent when followed by stress.

In Year 1, we proposed to expose mice to Gulf War toxins such as permethrin and to test the hypothesis that behavioral impairments caused by permethrin were mediated by microglial activation. In pursuit of these goals we have successfully generated a colony of Cx3Cr1-dependent DREADD mice for suppression of microglia. Additionally, we validated a novel mouse model of GWI by treating mice with 200mg/kg permethrin every day for 14 days followed by 7 days of unpredictable mild stress. Behavioral analysis of the treated mice showed a significant increase in depression like behavior as measured via forced swim test (Figure 1). We also analyzed anxiety-like behavior using the open-field test and found no significant difference between treatment groups (Figure 1). We also collected fixed brains from our GWI mice and performed immunohistochemistry to detect changes in Iba1 and CD68 to measure changes in microglial activation. We are currently in the process of performing a shell analysis to quantify microglial activation on the images generated from these brains. Additionally, we are currently performing fluorescence-activated cell sorting (FACS) using blood and brain tissue from GWI mice. Specifically, we are measuring changes in CD45, CD11b, TLR4, CD14, RAGE, CD86, MHC-II, or CD3, Ly6C, CD19.

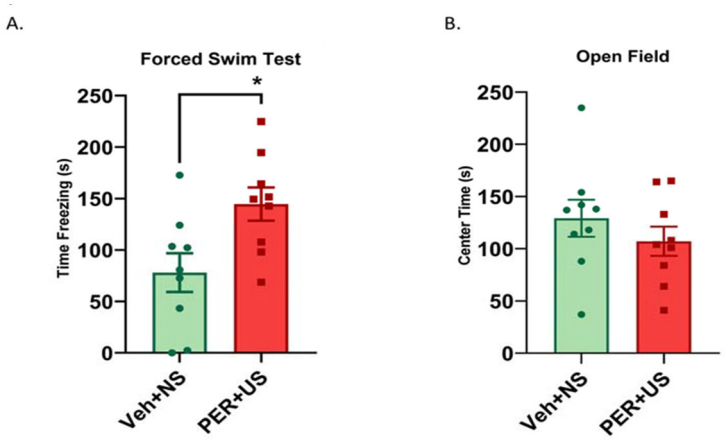


Figure 1. Behavioral changes in mouse model of GWI. Mice were exposed to permethrin (200mg/kg, i.p) for 14 days followed by 7 days of unpredictable stress. A) Mice displayed a significant increase in depressive behavior (time spent freezing) during forced swim test. B) No significant differences were detected in anxiety-like behavior as measured by time spent in the center during open field test.

In YEAR 2, we validated a novel model of Gulf War Illness in which mice were chronically exposed to permethrin for 14 days, followed by 7 days of unpredictable stress. We determined that this model was sufficient to induce a depressive behavioral phenotype as measured via forced swim test. During this reporting period we determined that our model is sufficient to induce changes in microglial activation (**figure 2).** An additional goal of the proposed studies was to generate a colony of Cx3Cr1-dependent DREADD mice for suppression of microglia. We have successfully generated and maintained this mouse colony.

During this reporting period we also sought to validate the utility of this mouse line for blunting neuroinflammation-induced behavioral changes in mice. To accomplish these goals we utilized lipopolysaccharide (LPS) to stimulate inflammation and behavioral changes in mice. Surprisingly, these studies revealed that LPS was not sufficient to induce depressive or anxiety-like behaviors in mice as assessed by forced swimming test (FST) and open field test (OFT) **(figure 3**). We then performed additional biochemical studies to determine if our Cx3Cr1-dependent DREADD line was sufficient to suppress LPS-induced changes in the secretion of pro-IL-1β and Il-1β across multiple brain regions (**figures 4 and 5).** Finally, we sought to determine if our Cx3Cr1-dependent DREADD line was sufficient to suppress the behavioral phenotype observed in our GWI model (**figure 6).** Excitingly, the use of our novel transgenic mouse line was sufficient to suppress the depressive phenotype observed in our GWI model (**figure 6**).

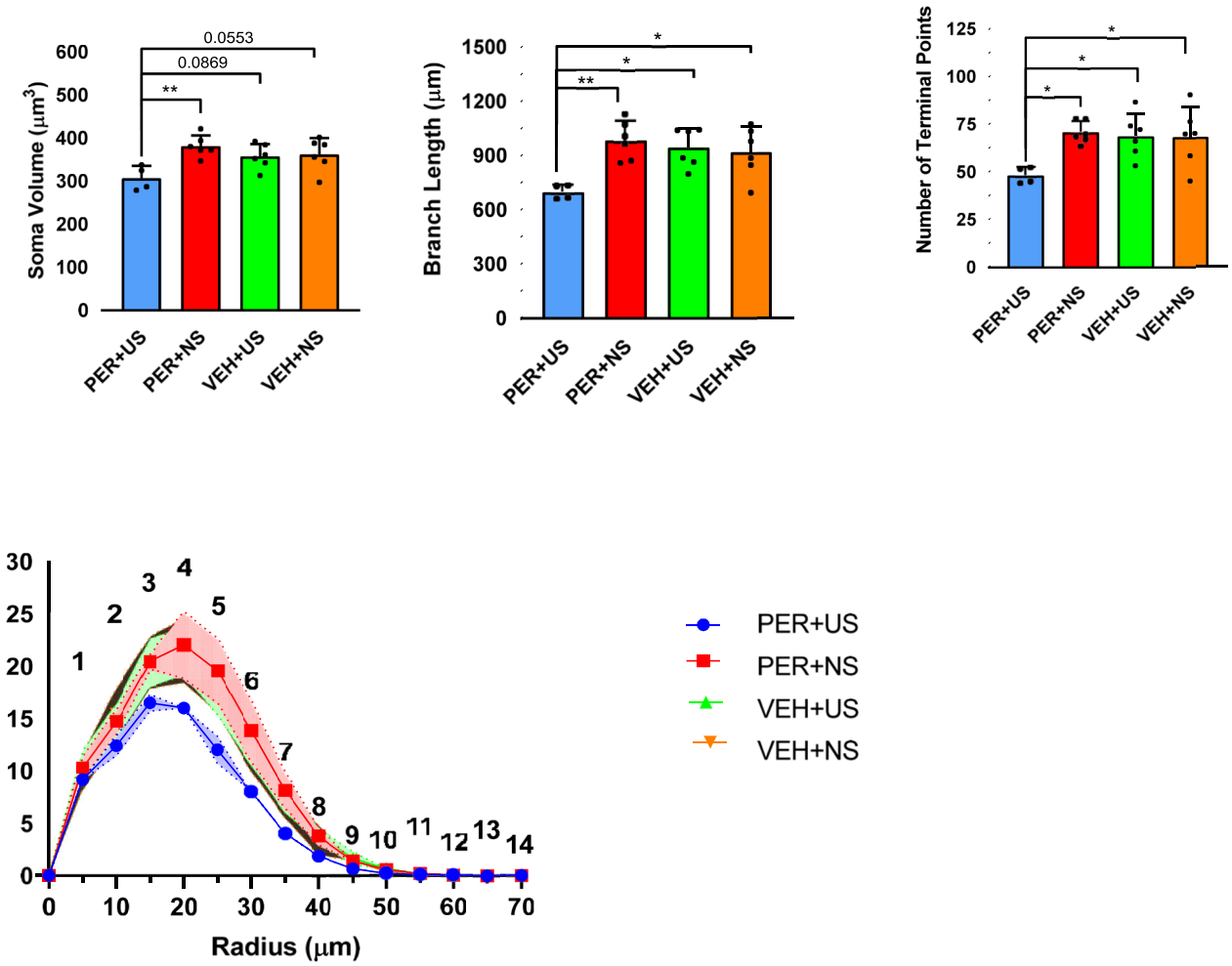


Figure 2. Exposure to permethrin followed by mild stress induced microglial activation in the hippocampus as determined via measurement of soma volume, branch length, terminal points, and sholl analysis

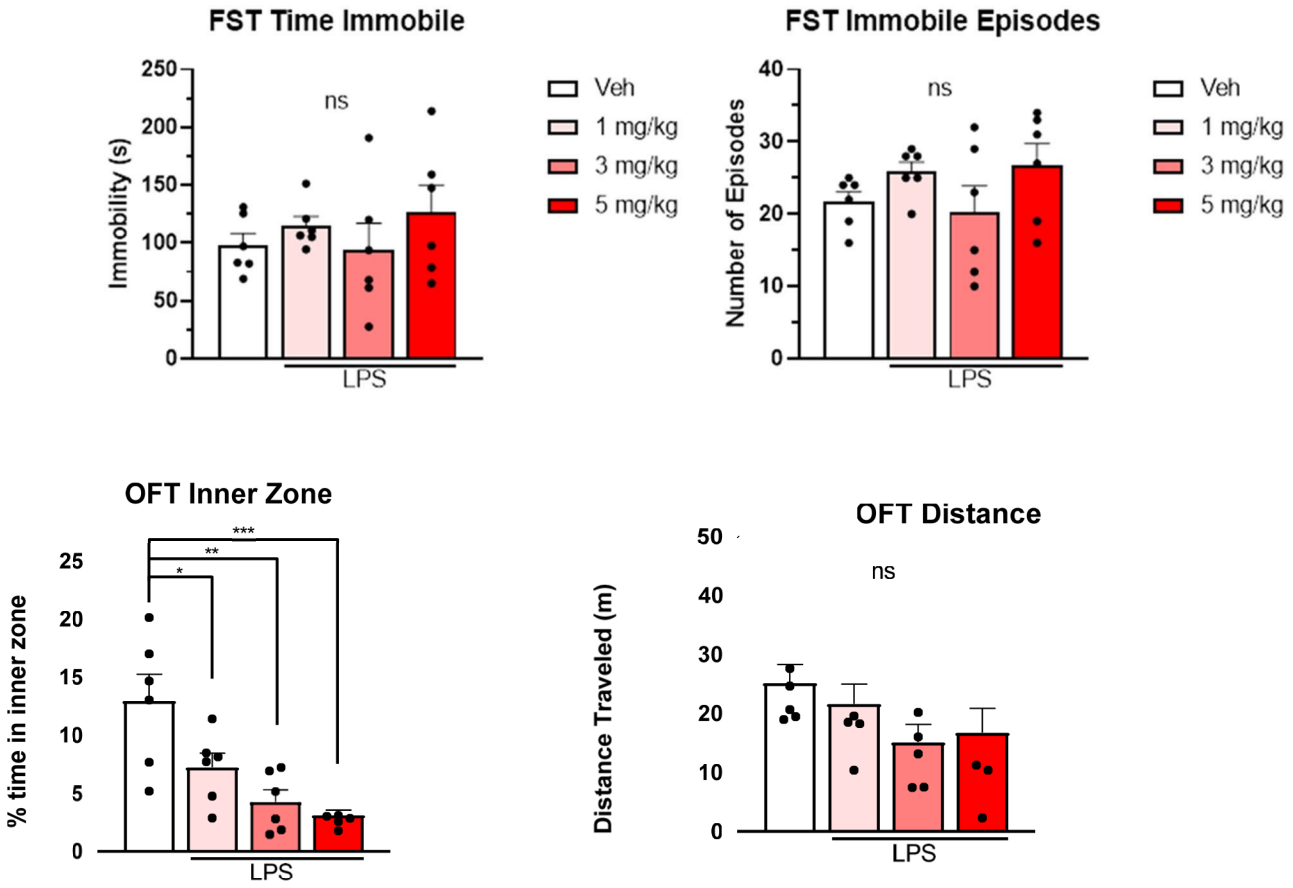


Figure 3. Administration of lipopolysaccharide (LPS) did not induce depressive or anxiety like behaviors in the forced swim test (FST) or open field test, respectively. LPS did produce a dose-dependent increase in the amount of time spent in the inner zone of the OFT, however this is opposite to what would be expected for anxiety-like behaviors.

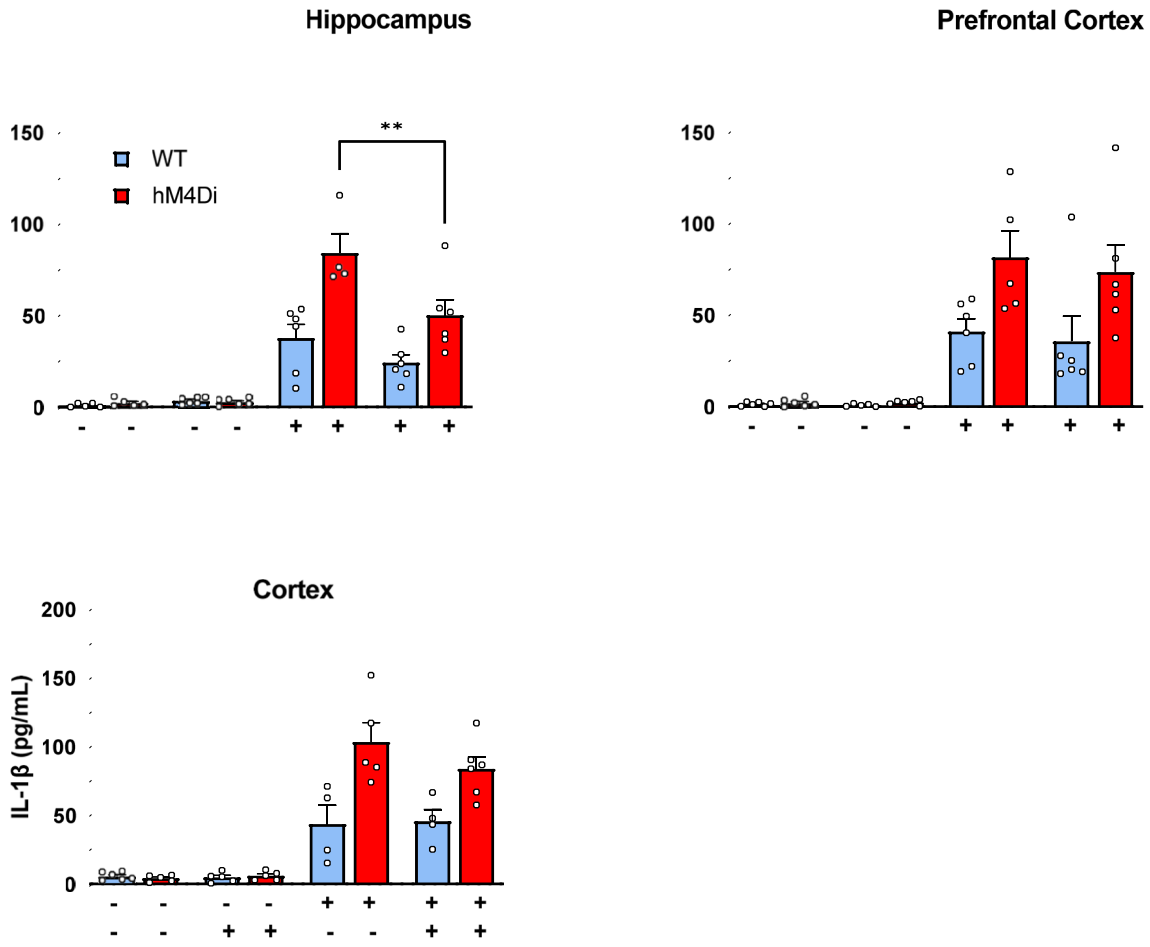


Figure 4. Activation of the DREADD inhibitory receptor hM4Di via the specific ligand JHU37160 (J60) significantly suppressed levels of IL-1β in the hippocampus, but not the prefrontal cortex or cortex as determined via ELISA.

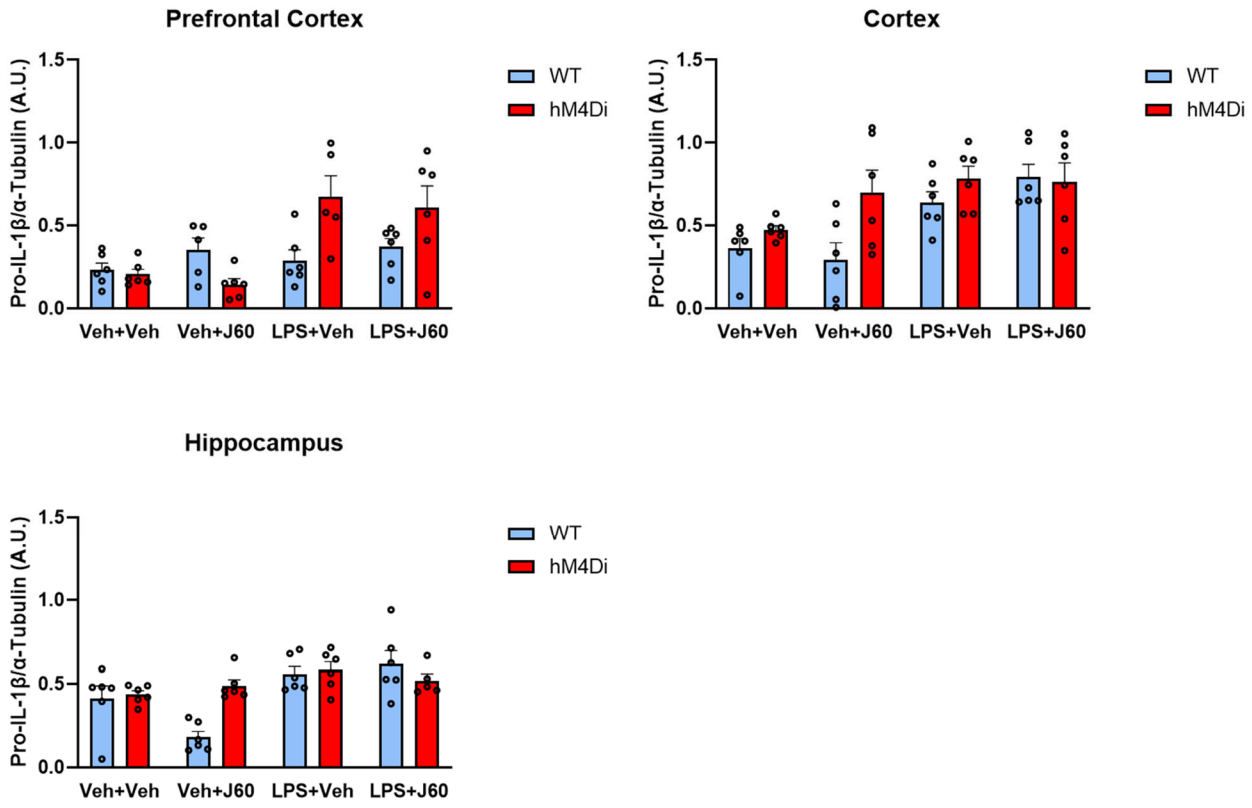


Figure 5. Activation of the DREADD inhibitory receptor hM4Di via the specific ligand JHU37160 (J60) did not suppress levels of the uncleaved Pro-IL-1β in the hippocampus, prefrontal cortex or cortex as determined via Western Blot.

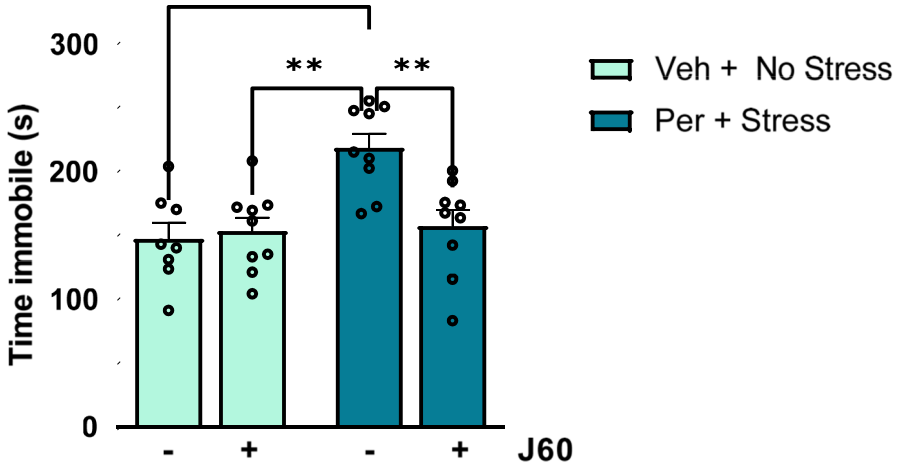


Figure 6. Behavioral changes in mouse model of GWI. Activation of the DREADD inhibitory receptor hM4Di via the specific ligand JHU37160 (J60) was sufficient to block the depressive phenotype previously observed after chronic treatment with permethrin followed by stress.

In YEAR 3, we performed additional studies to understand the role of neuro-immune interactions. Specifically, we analyzed blood from mice exposed to permethrin for 14 days followed by 7 days of unpredictable stress (our GWI model). We analyzed blood transcriptomic changes via a specific panel of immunological-relevant probes. We observed a number of immunological changes in the blood of mice exposed to our treatment. In particular, mice exposed to permethrin followed by unpredictable stress exhibited decreases in T-Cell receptor signaling, TGF-β signaling, and Th2 differentiation (**Figure 7**). When comparing specific changes in differentially expressed genes we encountered a number of interesting, but not statistically significant changes when comparing mice exposed to permethrin followed by unpredictable stress with mice treated with Vehicle and No Stress (**Figure 8),** as well as Permethrin without stress when compared to Vehicle without stress (**Figure 9**) and vehicle with stress compared to vehicle without stress (**Figure 10**). We next performed single-nuclei RNA sequencing to identify differentially expressed genes in microglia and other brain cell populations in response to our treatment. We generated a single-nuclei atlas representing the detected populations of various cell types based on their gene expression profiles (**Figure 11**). We are currently using this atlas to conduct additional bioinformatic analysis to quantify differentially expressed genes in each of the relevant cell populations.

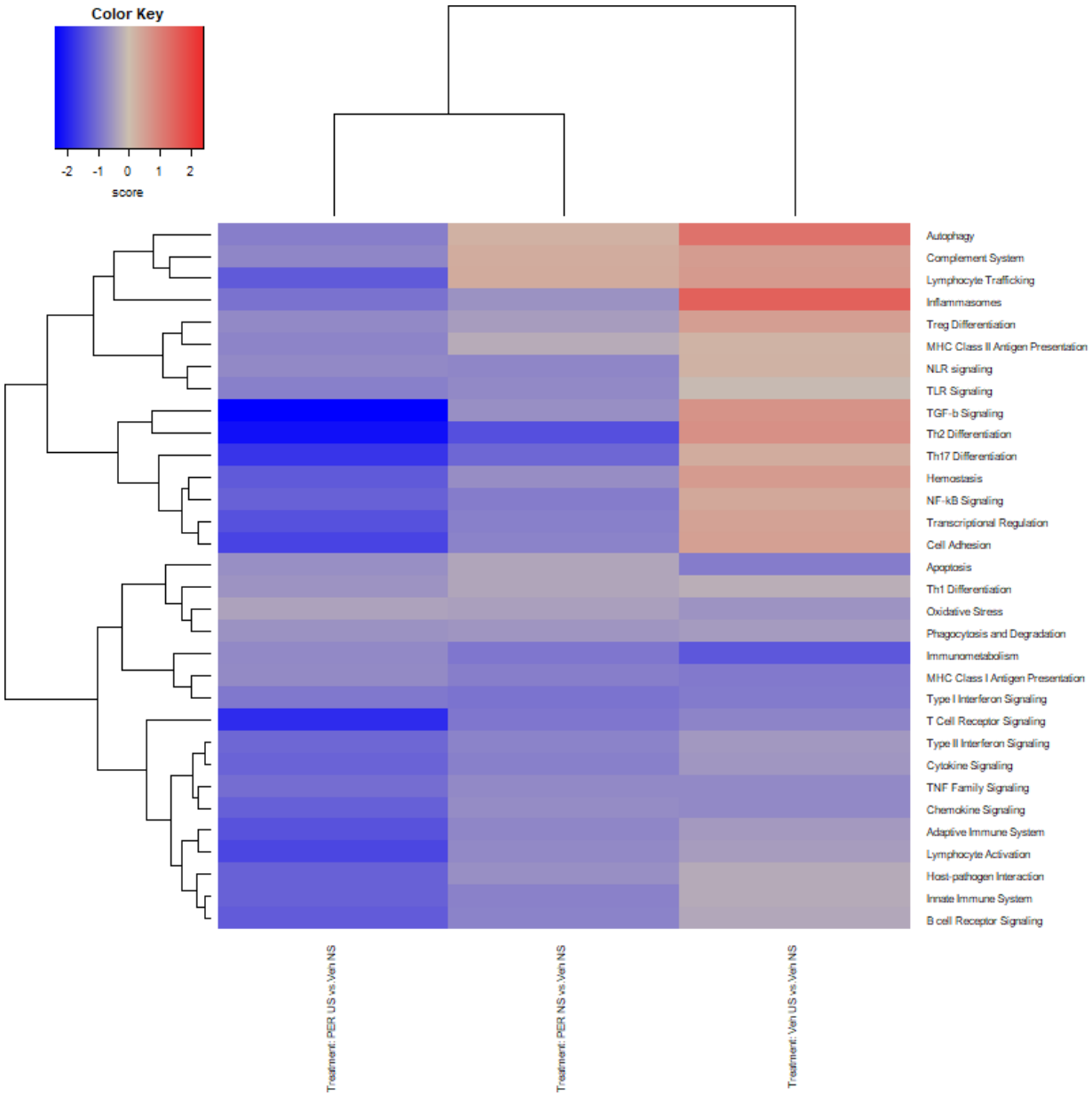


Figure 7. **Peripheral Blood Transcriptomic Changes.** Exposure to permethrin followed by unpredictable stress (PER US) lead to changes in gene expression associated with T-Cell receptor signaling, TGF-β signaling, and Th2 differentiation. Exposure to permethrin (PER NS) alone was associated with only mild changes in gene expression. Exposure to stress alone (VEH US) was associated with changes in genes relevant to inflammasome activation, autophagy, and peripheral immune cell signaling.

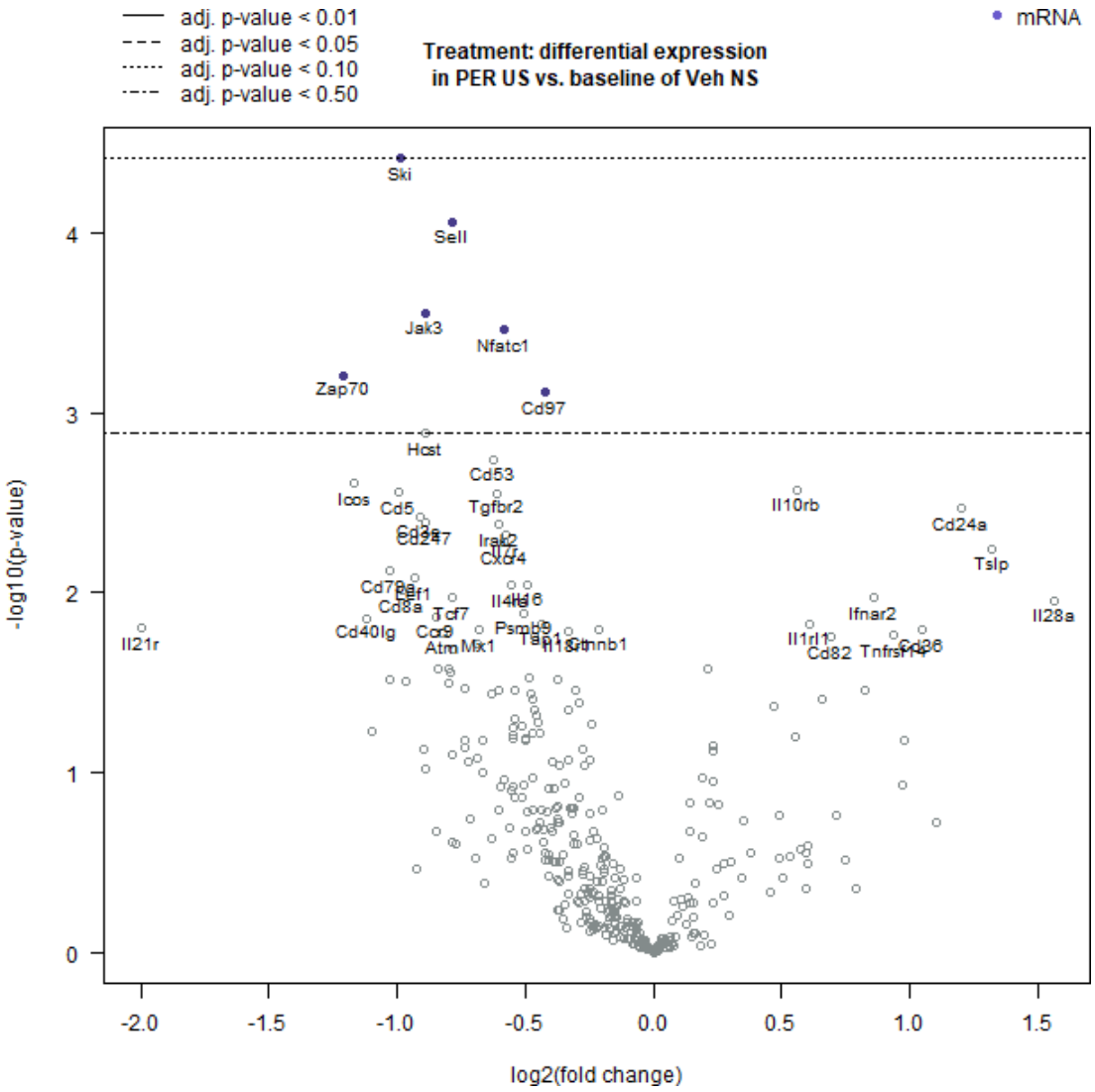


Figure 8. Genes effected by Permethin and Unpredictable Stress (PER US). We observed trending changes in the expression of Ski, Sell, Jak3, Zap70, Nfatc1, and Cd97 after exposure to permethrin followed by stress.

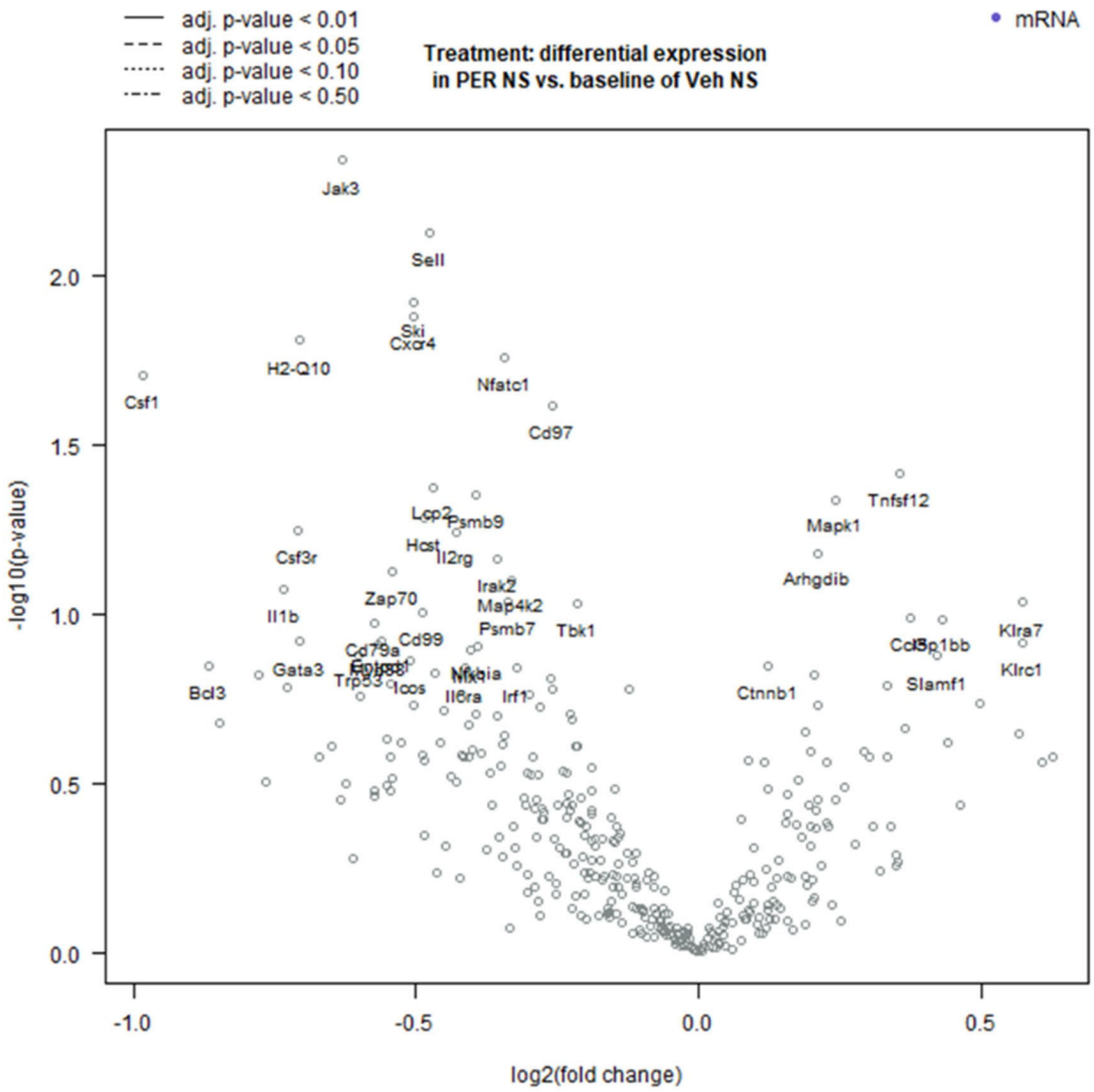


Figure 9. Genes effected by Permethin Alone (PER NS). Exposure to permethrin without subsequent stress exposure led to slight, but non-significant decreases in expression of the genes Jak3 and Sell.

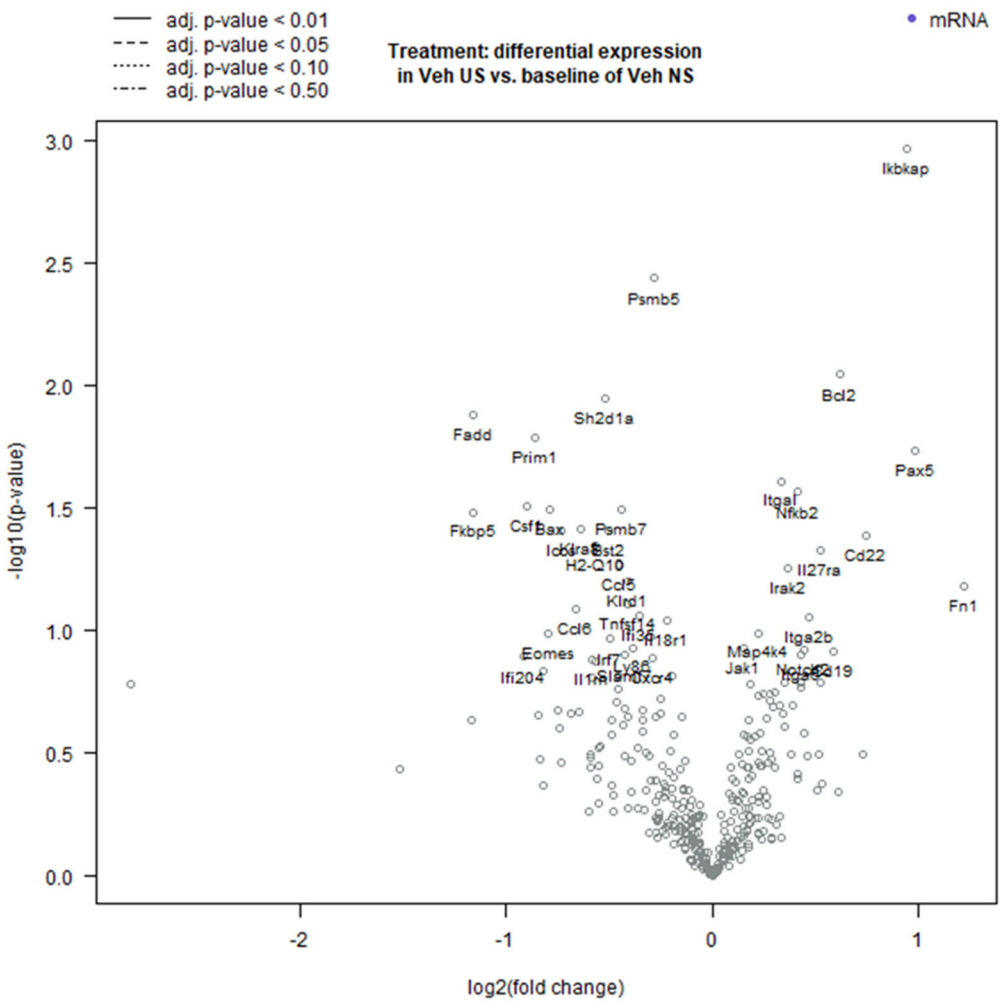


Figure 10. Differentially Expressed Genes affected by Stress Alone (VEH US). Exposure to stress alone resulted in a mild, but non-significant, increase in the expression of Ikbkap.

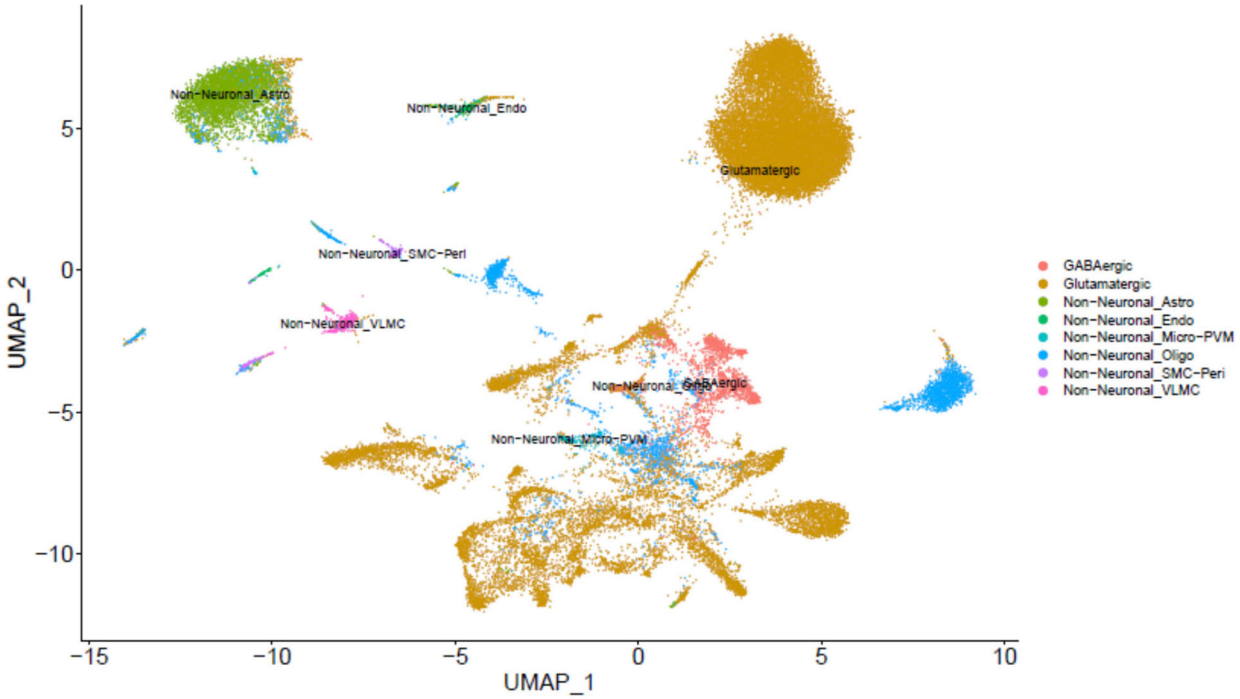


Figure 11. Single-Nuclei RNA sequencing atlas of cell types affected by our GWI treatment model. Single-nuclei RNA sequencing atlas, which will allow for further investigation of cell-type specific changes in gene expression after exposure to permethrin and stress.

In YEAR 4, to elucidate the transcriptional networks impacted within distinct microglia populations linked to depression-like behavior in mice exposed to both permethrin and stress, we conducted a single-cell RNA sequencing analysis utilizing 21,566 single nuclei collected from the hippocampal formation of mice. For bioinformatics, UniCell: Deconvolve Base (UCDBase), a pre-trained, interpretable, deep learning model, was used to deconvolve cell type fractions and predict cell identity across spatial datasets (**Figure 12**); see below. Our bioinformatics analysis identified significant alterations in permethrin (neurotoxin) /stress-associated microglia population, notably pathways related to axon development, calcium ion transport, and neurotransmission, all associated with neural synaptic plasticity (**Figure 13**). Additionally, we observed permethrin/stress-mediated changes in voltage-gated sodium channel activity, a known contributor to the pathophysiology of depression in a subset of the hippocampal pyramidal neuron of the CA1, CA2, and CA3 subregions (**Figure 14**).

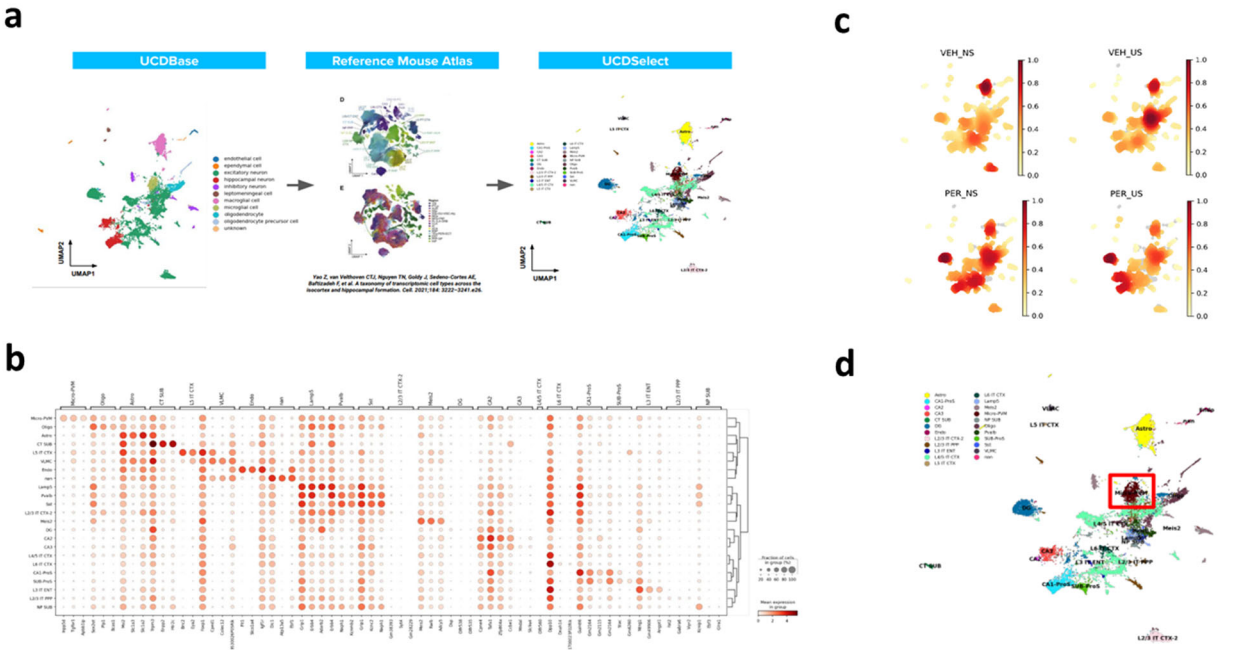
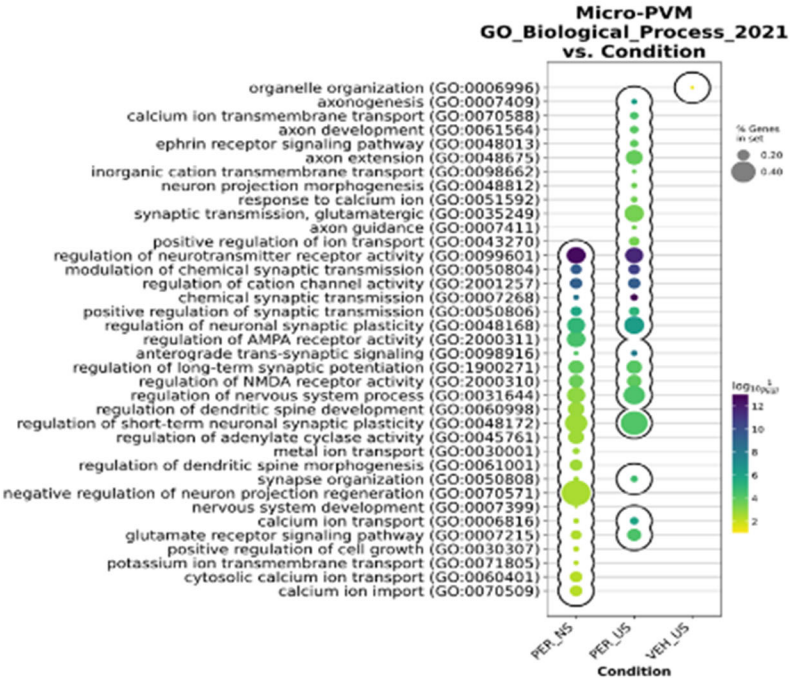


Figure 12. Characterization of Gulf War Illness-associated brain cell population through Single-Cell Sequencing. (a) UniCell Deconvolve Select (UCDSelect) was used to project annotations from a reference mouse cortex / hippocampus atlas onto novel dataset. (b) Differential expression analysis against predicted clusters to identify conserved markers specific to each putative cell annotation. (c) Cell density plots of each experimental groups (the more red, the more specific those populations are for the condition). (d) Microglial cluster 13 identity was confirmed by expression of canonical marker genes, including

a



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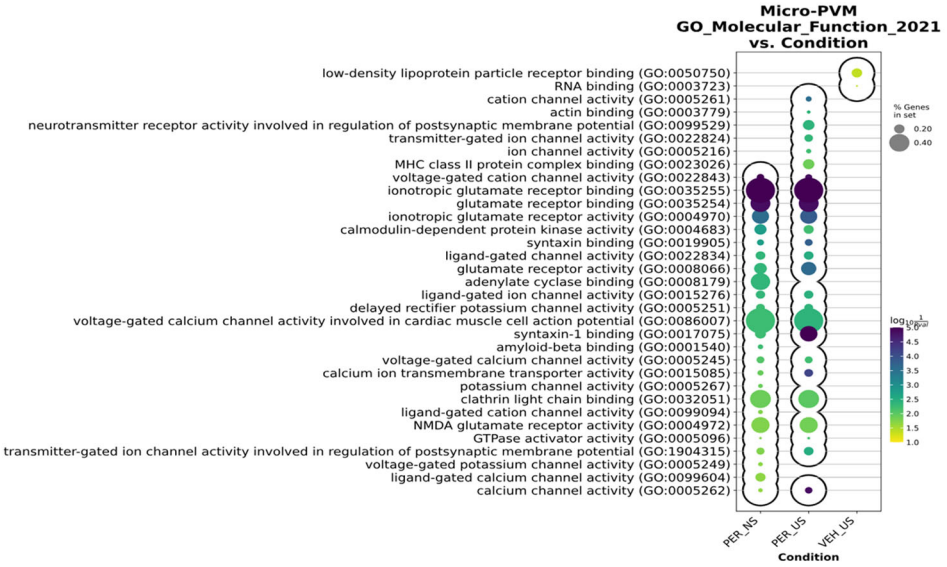

Figure 13. Bubble plots of Gene Ontology (GO) category enrichment results in microglia cell populations. (a-b) Significant alterations in permethrin/stress associated microglia population, notably pathways related to axon development, calcium ion transport, and neurotransmission, all of which are associated with neural synaptic plasticity.

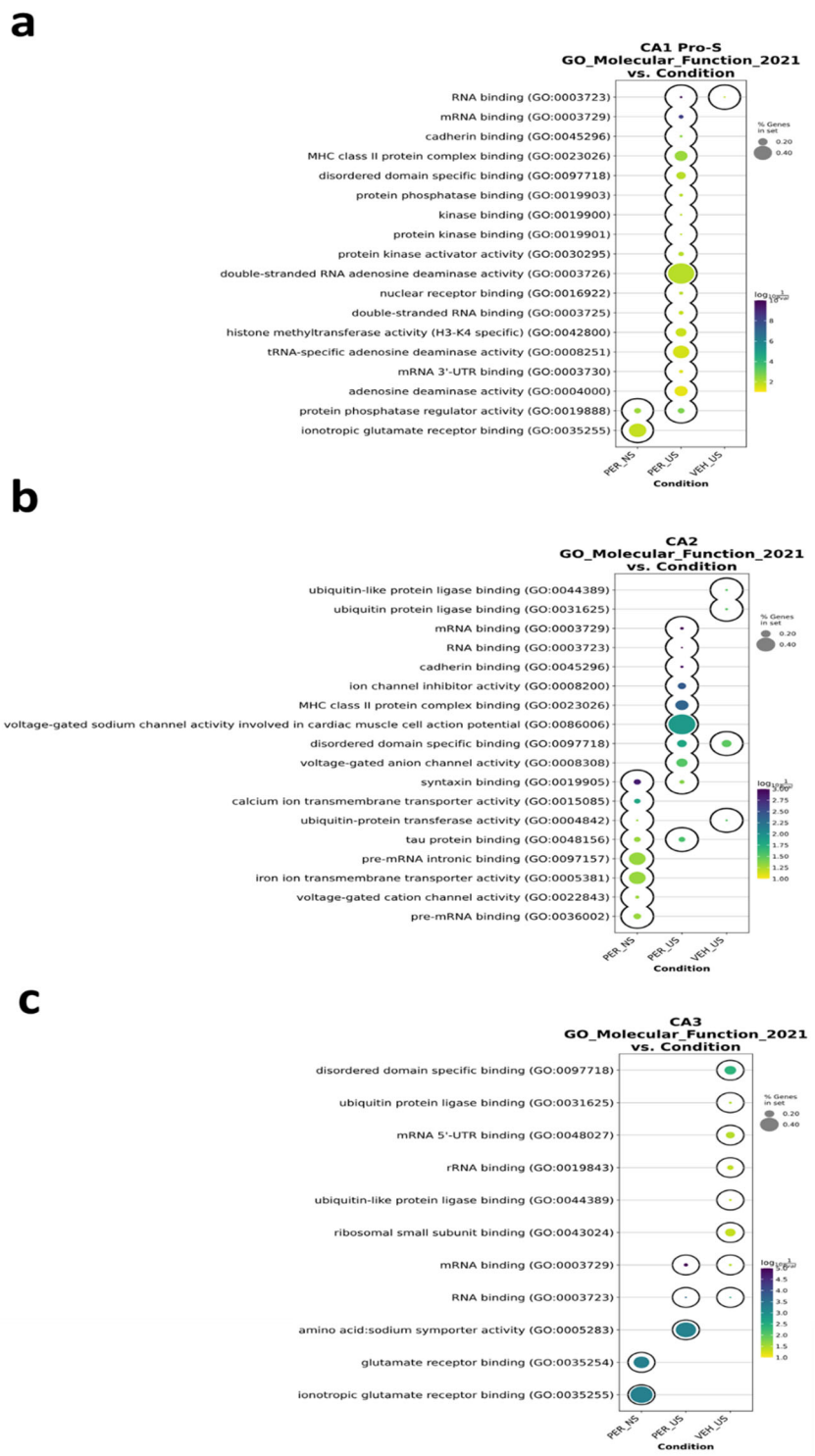

Figure 14. Bubble plots of Gene Ontology (GO) category enrichment results in neuronal cell populations. (a-c) Permethrin/stress mediated changes in voltage-gated sodium channel activity in subset of the hippocampal pyramidal neuron of the CA1(a), CA2(b) and CA3(c) subregions.

c. **Opportunities for Professional Development**
Nothing to report.

d. **Dissemination of Information to Communities of Interest**
Nothing to report.

e. **Plan for the next reporting period**
This is the final report.

4. IMPACT

a. **What was the impact on the development of the project's principal discipline(s)**
These studies support that neurotoxin exposure (permethrin) may prime toward a depression-like behavior that can be triggered by psychological stress in mice through microglial activation, resulting in alterations of neural plasticity.

b. **What was the impact on other disciplines?**
Since permethrin is a neurotoxin that is also used as a personnel care product and is commercially available, the study raise concerns about the environmental toxin for the general population

c. **What was the impact on technology transfer?**
NA

d. **What was the impact on society beyond science and technology?**
The study further emphasizes the potential impact of neurotoxin related to Gulf war veteran's deployment and possible consequences on neuroinflammatory cascaded in the brain.

5. CHANGES/PROBLEMS

a. **Changes in approach and reasons for change**
The aims we proposed are accomplished as designed.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**
NA

c. **Changes that had a significant impact on expenditures**
NA

d. **Significant changes in the use or care of human subjects**
NA

e. **Significant changes in the use or care of vertebrate animals.**
NA

f. **Significant changes in the use of biohazards and select agents**
NA

6. **PRODUCTS**

a. Journal Publications
Manuscript are under preparation

b. Books or other non-periodical, one-time publications
Nothing to Report

c. Other publications, conference papers, and presentations
Regional distribution of cellular of molecular pathways in a mouse model of Gulf War illness by Single-Cell RNA Sequencing Analysis, Yang et al 2023; presentation at "*Novel Concept in Neurodegenerative Disorders*" IABS Forum, Irvine

d. Website(s) or other Internet site(s)
NA

e. Technologies or techniques
NA

f. Inventions, patent applications, and/or licenses
NA
.

g. Other Products
NA

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a. Individuals who have worked on the project

i. Dr. Sean X Naughton, PhD
Role: Post-Doctoral Fellow
Contribution: Responsible for overall design of the project and execution of experiments.
Funding Support: 40%

ii. Kyle Trageser, BS
Role: Research Assistant
Contribution: Assisting in the execution of experiments.
Funding Support: 25%

iii. Dr. Giulio Pasinetti, MD PhD
Role: Principle Investigator
Contribution: Overall Experimental Design.
Funding Support: 15%

b. Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period
NA

c. Other Organizations involved as partners
NA

8. Special Reporting Requirements
NA

9. Appendices
Attached

Transition Plan Questionnaire

Directions: Please answer all questions that apply for each product under development. Please fill out one document per product.
This is not an application for funding; however, answers will help us understand the outcomes and products from your award.

1. After the award closes, would you be willing to periodically provide voluntary information (via email) regarding the project status (i.e. where the research is headed)? **Yes** ☑ or **No** ☐
These responses will help CDMRP demonstrate the return on its investments and will help demonstrate that the CDMRP is a responsible and successful steward of federal research funding.

2. What **conclusion(s)** does your final data support?

Gulf War Illness (GWI) is a chronic multisymptomatic disorder that disproportionately affects Gulf War veterans, the combination of permethrin exposure with stress may have collectively and synergistically contributed to its development. However, the exact underlying pathogenic mechanisms within the brain that links permethrin and stress to the development of GWI remains unclear. The principal objective of this application was to use a mouse model to establish whether inhibition of microglia prevents latent and long-term pathological and behavioral effects resulting from exposure to Gulf War toxins and psychological stress. Identifying microglia as the primary effector of these pathologies would serve as a proof of concept to target microglia reactivity as a therapeutic strategy to improve the health of veterans diagnosed with this disorder. The conclusion of the report is that neurotoxin exposure (permethrin) may prime toward a depression-like behavior that can be triggered by psychological stress in mice through microglial activation, resulting in alterations of neural plasticity.

3. Will you/have you applied for/obtained follow-on-funding for this project? **If yes**, please list (a) funding organization, (b) total budget requested/obtained, and (c) title of the funded proposal. *This information will be recorded as an outcome to this award*.

No

4. What will be **the next step(s)** for this project?

Advancing this project entails delving deeper into the exploration and validation of identified mechanisms through the application of state-of-the-art technologies like single-cell sequencing. This in-depth analysis would aim to unravel intricate details. Simultaneously, the project will focus on developing precise diagnostic tools and treatment strategies informed by the characterization of susceptible individuals. This integrated approach not only enhances our understanding of the disorder but also paves the way for more effective and tailored interventions.

5. How would you classify your **lead candidate product**? a

(a) Therapeutic (Small Molecule, Biologic, Cell/Gene Therapy): Cell/Gene Therapy
(b) Diagnostic
(c) Device
(d) Research Tool to Address a Research Bottleneck
(e) Knowledge Product (Non-material product such as a compound library, database, something that improves clinical practice, education, etc.)
(f) Other - Please Specify:

6. How does your candidate product aid the Warfighter, Veteran, Beneficiary, and/or General Population?

Following return from deployment, Gulf War Veterans did not initially present with significant health issues but this developed later in high frequency. The temporal gap between their deployment and the onset of chronic headaches, widespread pain, memory and concentration difficulties, persistent fatigue, respiratory problems, and affective psychological disorders has made it difficult for the medical community to accurately target a common underlying mechanism of the disorder. Recently, however, clinical research has detailed abnormal activity in immune systems of these Veterans. The immune system is uniquely dynamic because it is capable of retaining information about previous exposures to toxins or psychological traumas. Exposure of Veterans to toxins specific to the Gulf War may have left permanent marks on their immune systems, increasing their susceptibility to develop symptoms observed in Gulf War Veterans Illness. Because veterans with the disorder often present with cognitive disabilities, immune cells specific to the brain, microglia, may be responsible for the long term-effects of the Gulf War. . New therapeutic approaches developed for other neurological indications selectively inhibit microglia. By establishing a critical role of microglia in the onset and progression of Gulf War Veterans Illness, there could be significant evidence to test therapeutic approaches that inhibit microglia activity as a means to both prevent progression of the disorder.

7. Therapy / Product Development, Transition Strategies, and Intellectual Property

Describe the steps and relevant strategies required to move the candidate product (knowledge or tangible) to the next phase of development and/or commercialization. Please address any issues with intellectual property.

PIs are encouraged to explore the technical requirements and the current regulatory strategies involved in product development as well as to work with their organization's Technology Transfer Office (or equivalent regulatory/legal office), federal/international regulatory experts, to develop the transition plan and to explore developing relationships with industry, DoD advanced developers (e.g. USAMMDA), and/or other funding agencies to facilitate moving the product into the next phase.

The study explores the role of of a commercial neurotoxin which was employed as a pesticide in GW Veteran's deployment and is also used as a personal care product . Our studies should bring awareness not only for Veterans returning from the GW theatre but also to the general public

Award Log Number: Award Title: W81XWH-19-1-0513/Suppression of GWVI Toxin-Activated Microglia and Pathologies by DREADD
PI: Giulio Pasinetti, Icahn School of Medicine at Mount Sinai, NY **Budget:** $423,750
Topic Area: Gulf War Illness Research Program, Investigator-Initiated Focused Research Award
Mechanism: Discovery

Research Area(s): 0503, 0412 **Award Status: 08/15/2019-08/14/2023**

Study Goals:

The principal objective of this application is to use a mouse model to establish whether inhibition of microglia prevents latent and long-term pathological and behavioral effects resulting from exposure to Gulf War toxins and psychological stress. Identifying microglia as the primary effector of these pathologies would serve as a proof of concept to target microglia reactivity as a therapeutic strategy to improve the health of veterans diagnosed with this disorder.

Specific Aims:

Specific Aim 1: Validation of a Cx3Cr1-dependent DREADD for suppression and activation of murine microglia within a GWVI model system.
Specific Aim 2: Examine if suppression of cortical and hippocampal microglia following exposure to neurotoxins and chronic stress can attenuate inflammation, astrogliosis, synaptic loss, neuronal degradation in the brain, and alterations to peripheral lymphocyte immunophenotypes.
Specific Aim 3: Examine if suppression of microglia can prevent altered behavioral phenotypes observed in the model systems.

Key Accomplishments and Outcomes:

In Aim 1, we successfully generated a colony of Cx3Cr1-dependent DREADD mice to suppress microglia. Additionally, we validated a novel mouse model of GWI by treating mice with 200mg/kg permethrin every day for 14 days, followed by seven days of unpredictable mild stress. Behavioral analysis of the treated mice showed a significant increase in depression-like behavior. **In Aim 2**, we conducted a bioinformatic analysis to quantify differentially expressed genes in each relevant microglia cell population. Our bioinformatics analysis identified significant alterations in permethrin (neurotoxin) /stress-associated microglia population, notably pathways related to axon development, calcium ion transport, and neurotransmission, all associated with neural synaptic plasticity. **In Aim 3**, we sought to determine if our Cx3Cr1-dependent DREADD line was sufficient to suppress the behavioral phenotype observed in our GWI model by inhibiting microglia activation. Collectively, these studies support that neurotoxin exposure (permethrin) may prime toward a depression-like behavior that can be triggered by psychological stress in mice through microglial activation, resulting in alterations of neural plasticity. The study further emphasizes the potential impact of neurotoxin related to Gulf War veterans' deployment and possible consequences on neuroinflammatory cascaded in the brain.

Publications: Manuscript under preparation
Patents: none to date
Funding Obtained: none to date