AWARD NUMBER: W81XWH-16-1-0658

TITLE: Omega-3 Polyunsaturated Fatty Acid Status, Microglial Activation, Stress Resilience, and Cognitive Performance

PRINCIPAL INVESTIGATOR: Bita Moghaddam

CONTRACTING ORGANIZATION: Oregon Health & Science University, Portland, OR

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S)

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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13. SUPPLEMENTARY NOTES

14. ABSTRACT

It is widely reported across mammalian species that deficiency in the dietary intake of omega-3 polyunsaturated fatty acids (n-3 PUFA) negatively impacts cognitive performance and mood. n-3 PUFA deficiency has also been implicated in disorders such as ADHD, PTSD, major depressive and bipolar disorders, and schizophrenia. Defining potential neuronal mechanisms that link n-3 PUFA levels to cognitive and behavioral deficits has important implications given that the trend of the modern diet has been toward reduced n-3 PUFA intake. Here, we proposed human and rodent experiments to evaluate whether the anti-inflammation/pro-resolution effects of n-3 PUFA deficiency contribute to the adverse effects on cognitive performance and affect. In addition, these experiments focus on the expression of dietary n-3 PUFA deficiency in late adolescence/young adulthood. We will used a positron emission tomography (PET) imaging strategy in humans as a marker of activated microglia in individuals with low and high plasma n-3 PUFA. In parallel animal studies, we directly measured microglia activation in an animal model of n-3 PUFA deficiency and determined whether supplementation during early adulthood reverses this effect in correlation with behavior. We find that this dietary manipulation influences anxiety like behaviors in our rodent models in correlation with microglia activation. These findings provided a potential mechanism for n-3 PUFA regulation of brain function.

15. SUBJECT TERMS
Omega-3 fatty acids, microglia, brain inflammation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	**19b. TELEPHONE NUMBER** *(include area code)*

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1. INTRODUCTION

Background: Dietary deficiency in omega-3 polyunsaturated fatty acid (n-3 PUFA) is a common feature of the modern diet. Across mammalian species, deficiency in the intake of this essential fatty acid negatively impacts the ability to withstand stress and cognitive performance. Accordingly, recent studies in healthy civilian and military populations indicate a strong relationship between red blood cell (RBC) n-3 PUFA levels and a wide range of brain related problems including impaired cognitive performance, and increased anxiety, impulsivity and suicide. Precise brain mechanisms that underlie the behavioral detriments of n-3 PUFA deficiency and whether they can be reversed by supplementation are largely unknown. The overarching goal of this proposed work is to inform of us about specific brain mechanisms by which dietary n-3 PUFA deficiency and supplementation affects brain and behavior. The mechanistic focus will be on immune responses around neurons in brain regions that are critical for stress reactivity and cognitive performance.

Purpose (Aim2): To determine whether an animal model of n-3 PUFA deficiency is associated with brain microglia activation and whether supplementation during early adulthood reverses this effect in correlation with behavior. In an experimental animal model that mimics current western dietary n-3 PUFA deficiency, we have observed behavioral determents that suggest impaired cognitive performance and anxiety. We hypothesize that this dietary deficiency leads to an immunological insult in the brain and propose to use microglia activation as a method of quantification of this insult. Microglia are the residents of macrophage cells and are the first line of immune defense in the brain. Animals will undergo behavioral characterization before the post-mortem microglial measures. Upon establishing that there is microglia activation in brain regions of interest, we will test whether supplementation during early adulthood reverses this insult in correlation with behavior.

Scope: Establish that brain inflammation is a potential mechanism that underlies behavioral impairments in n-3 PUFA deficient diet, and quantify the impact of supplementation on reversing the inflammatory response and restoring the behavioral impairment. This has the potential to inform the clinical testing of oral and parenteral n-3 PUFA formulations as a treatment for the multitude of conditions where neuroinflammation is a focus, ranging from traumatic brain injury and multiple sclerosis to mood disorders and PTSD.

2. KEYWORDS

Omega-3 fatty acids, microglia, brain inflammation

3. ACCOMPLISHMENTS

What were the major goals of the project?

Major goals of the project (aim 2, animal study)

The major tasks listed in the approved SOW (6/2017) with listed milestones and target dates within the first 12 months are included below:

Major Task 1: Finalize and submit ACURO application

Major Task 2: Initiation and maintenance of colonies of first and second generation n-3 deficient animals (Timeline target date 2-30 months)

Major Task 3: Behavioral testing and comparison of deficient animals before and after supplementation and to adequate and deficient animals within the same age range. There are three subject groups in this Major Task: (1) animals on adequate diet, (2) animals on deficient diet that remain on that diet, (3) animals on deficient diet that shift to, and remain on, an adequate diet "long-term" diet initiated post weaning prior to initiating behavioral testing. Behavioral testing for target date 6-12 months in all three groups includes elevated plus maze, and probabilistic assessment of punishment in reward seeking actions.

Major task 4: Immunohistochemical staining to characterize neuroinflammation in all three diet groups in the following regions: ventral tegmental area (VTA), substantia nigra (SN), basolateral amygdala (BLA), dorsal and ventral striatum. Markers include known microglial activation targets Iba-1, Ki-67, CD68, and CSFR-1.
Procedures include perfusion and tissue prep after termination of behavior testing, target date 3-26 months followed by histological assessment and analyses target date 24-36 months.

What was accomplished under these goals?

Major Task 1: ACURO approval in place. Millstone achieved

Major task 2: This task has been accomplished. Following laboratory shutdown due to Covid-19, we successfully re-established the first and second generation of n-3 PUFA deficient and adequate colonies of rats in the laboratory. Second generation tested cohorts were comprised of 25 switch diet, 19 deficient diet, and 21 adequate diet animals. These cohorts are being used for studies in major task 3 and 4.

Major Task 3: This task is completed. Behavioral testing for target date 6-12 months included testing for probabilistic assessment of risk in reward seeking actions and elevated plus maze for all three groups.

1. The elevated plus maze component has been completed. The collected plus maze data in the rat age range that corresponds to late adolescence and early adulthood is shown in Figure 1 (next page). The y axis shows mean percent time spent in open, closed, and center part of an elevated plus maze in animal on n-3 PUFA adequate, deficient, and switch diets. Less time spent in an open arm is indicative of increased fear and anxiety. We find that deficient diet increases anxiety as measured in this task whereas less anxious adequate diet animals spend more time in the open arm of the maze. Switch diet animals presented a subtle decrease in time spent in closed maze arms in comparison to deficient diet animals.

The probabilistic assessment of risk in reward seeking action is complete. A total of 65 animals were trained (initiated at PND 32) and tested (beginning at PND 45) using the "seek-take" task (Figures 2,3, and 4). This task explores punishment probability learning during reward-guided actions with hopes to characterize the influence of diet and sex on this form of learning. In this behavioral paradigm, the seek action is associated with varying probability of punishment over a series of 5 blocks per session. The take action remains safe and is followed by reward delivery. We find that there are statistically significant behavioral differences and trends between adequate, deficient, and switch groups in response to risk of punishment in terms of number of trials completed and latency to seek and take. Additional sex driven differences within diet groups are also present. We find that collectively, both male and female switch diet animals completed more trials than their adequate and deficient counterparts with less sensitivity to risk of shock as determined by shorter seek latencies regardless of increasing shock probability.
Major task 4: Data collection for this task is completed and analysis is nearly finalized. We had proposed to use anti-Iba1 immunohistological staining to estimate microglial number and

activation through process length and volume tracing. We are focusing our efforts on microglial activation in a key brain system that is critical for cognitive performance and stress reactivity: VTA, SN, basolateral amygdala, dorsal and ventral striatum. Using anti-Iba1 staining, we had made the interesting and impactful observation that dietary manipulation influences microglia activation in these regions. To increase the rigor of our findings, additional microglial activation markers have been added and images currently in final stages of analysis (Figure 5). These include proliferation, phagocytosis, and colony stimulating factors such as Ki-67, CD68, and CSFR-1, respectively. Replication of previously reported process length data acquired through Iba-1 staining across these regions has been imaged and automated process tracing is currently underway for all groups across all listed regions.

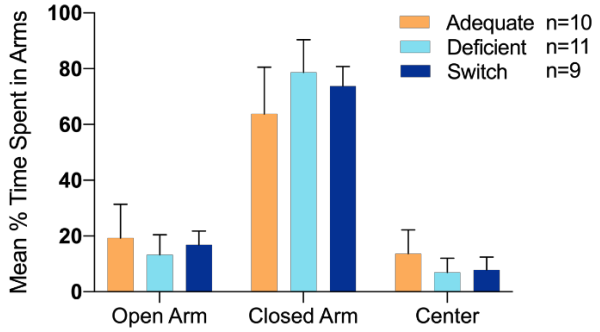

Figure 1. Elevated plus maze exploratory behavior measure in adequate, deficient, and switch animals.

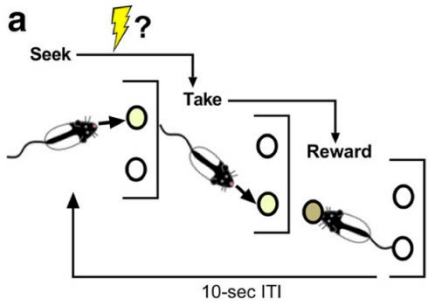

Figure 2. Probabilistic assessment of risk-taking behavior employed for behavioral testing of adequate, deficient, and switch groups (Seek-Take task).

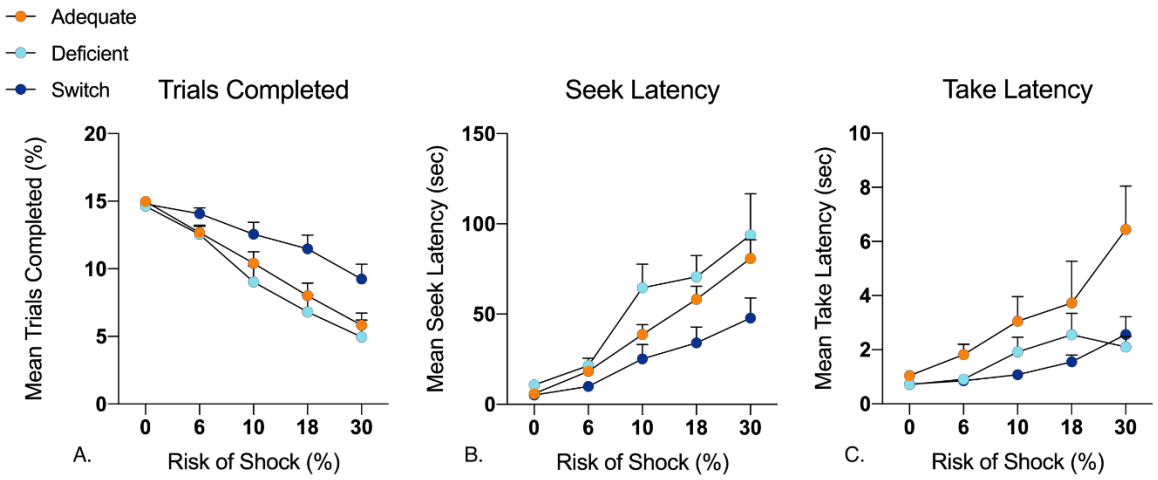

Figure 3. Assessment of risk taking in response to reward seeking actions of adequate (n=21), deficient (n=19), and switch diet animals (n=25). A. Deficient and switch diet groups exhibit statistically significant differences ($p<0.05$) in the average number of rewarded trials completed in the fourth and fifth blocks (18% and 30% risk of shock). On average, the switch diet animals completed more trials in the blocks with higher risk of shock than deficient and adequate animals. B. An increase in seek latency correlating to increased shock probability was seen for within all three animal groups. Switch diet animals display the lowest delay in action, and adequate animals demonstrate a nearly linear latency increase. However, deficient animals present an overgeneralization of shock risk as seen by a steep increase in latency at the 10% risk block, which is not observed in the adequate and switch groups. C. Switch diet and deficient

cohorts demonstrate shorter take latencies post-shock in the highest risk block (30%) when compared to adequate animals.

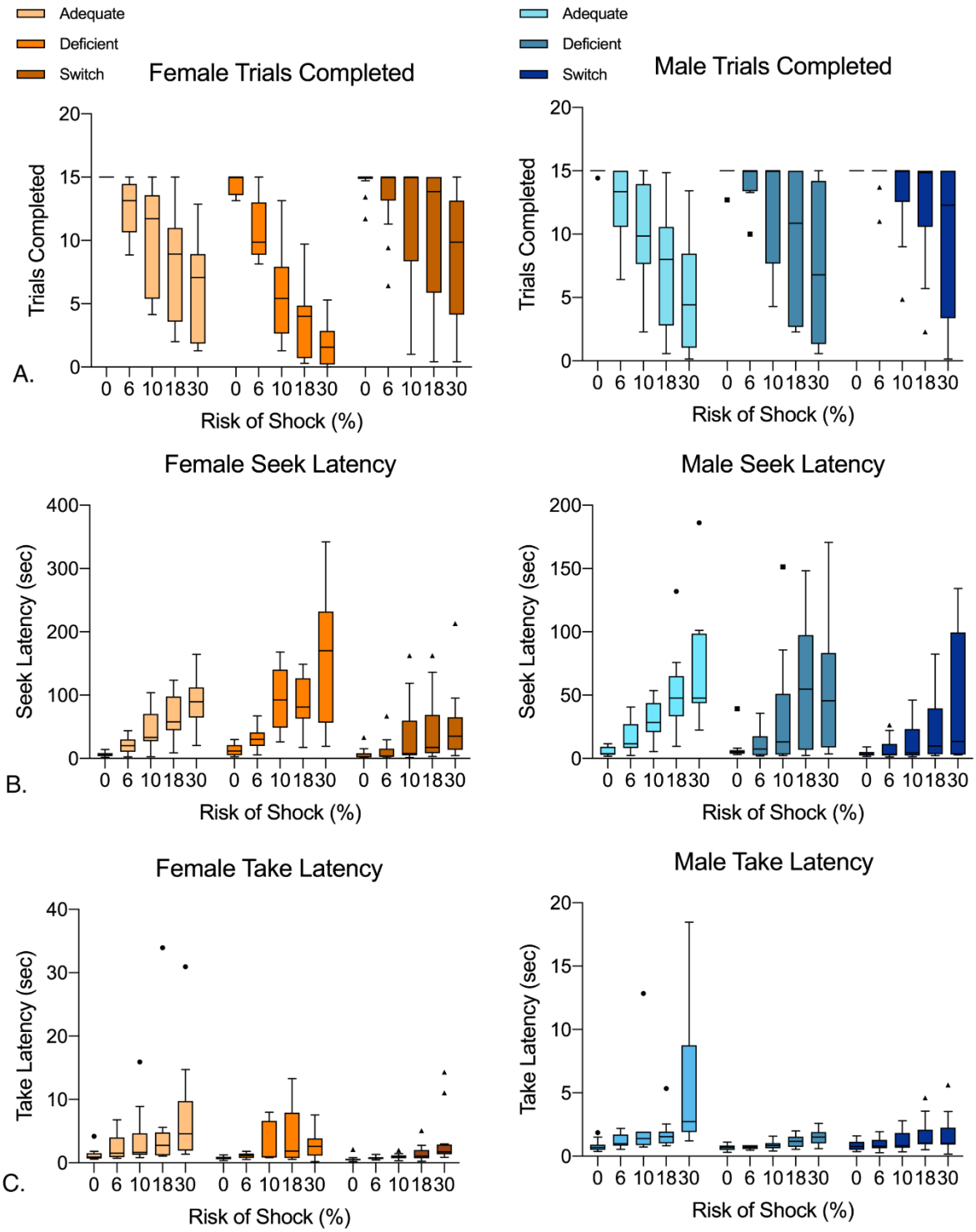

Figure 4. Comparison of performance variance by sex and diet group in seek and take latencies, along with mean trials completed (Tukey HSD test: Boxes encapsulate 25-75 percentile range. Median is depicted by middle line). Deficient females (n=9) present the highest median seek latency in anticipation

of shock at the 30% risk block, as well as the highest degree of variance amongst the adequate (n=10) and switch (n=13) female groups. Moreover, deficient females demonstrate a sharp median drop at the 10% risk block, while the median for males remains at maximum trials completed. This median maintenance is reflected in both deficient (n=10) and switch males (n=12), whereas adequate males show a decline at the 10% shock block (n=11). Switch diet males present the lowest median seek latencies in each block in comparison to both adequate (n=11) and deficient males (n=10).

Fluorescent Immunohistochemistry: For microglia quantification and morphometry and activation status analysis

Imaging**:** Immunofluorescent images are acquired with a Zeiss Apotome.2 Wide-field microscope combined with Ziess Axio Imager 2 system available at the Oregon Health and Science University Advanced Light Microscopy Core. This system is equipped with blue, green, red, and far-red lasers, allowing for multichannel imaging of up to four different antibody signals at once.

In addition to previously reported Iba-1 microglial process tracing and quantification, suppplemetory antibodies expressing proliferation (Ki-67), phagocytic activity via lysosomes (CD68), and stimulating factors (CSF-1-R) were added. Ki-67 proliferation costaining has been optomized using Mouse-anti Ki-67 (BD Pharmingen #556003 Concentrations of 1:500) and Goat-anti mouse IgG Alexa Fluor 488 (Figure 5).

Microglial Markers:

Antibody optimization**:** Several antibody combinations were tested at various concentrations for immunofluorescent imaging of microglia.

Primary Antibodies previously reported: Rabbit-anti-Iba1(Wako Chemicals, #019-19741) at concentration of 1:600 used for automated process tracing.

New supplementary microglial makers added to increase the rigor of our findings:

- Two Ki-67 primary antibodies were tested: Rabbit-Ki-67 (Abcam 16667) and Mouse-anti Ki-67 (BD Pharmingen #556003) at concentrations of 1:500. Mouse anti-Ki-67 was found to be effective at a concentration of 1:400.
- One Rabbit CSF-1R antibody was tested (Abcam 183316)- currently under optimization. Concentration of 1:400 produces unreliable results. Additional heat induced epitope retrieval (HEIR) and rat spleen positive control prove promising with antibody concentration of 1:300.
- One Rabbit-CD68 antibody was tested (Abcam 125212). CD68 concentrations are under optimization.
- One chicken-anti-Iba1 (SYSY #234-006) to allow non-conflicting co-staining of cell morphology with newly added markers. It was found to be effective at concentrations of 1:400.

Co-staining of dopamine neurons in the VTA:

A Chicken-anti-TH primary (Abcam, ab76442) combined with Goat-anti-chicken IgY H&L Alexa Fluor 594 (Abcam, ab150176) and Goat anti-chicken IgY Alexa Fluor 488 (ab150169) allow visualization of TH-containing neurons on the green and red channels.

Secondary Antibodies: Goat-anti-Rabbit IgG H&L Alexa Fluor 488 (Abcam, ab150081), Goat-anti-Mouse IgG H&L Alexa Fluor 405 (Abcam, ab175661), Goat-anti-Mouse IgG Alexa Fluor 647 (Abcam, ab150119), Donkey-anti-Mouse IgG H&L Alexa Fluor 488 (Jackson Immuno, 715-545-151), and Goat-anti-Rabbit IgG H&L Brilliant Violet 421 (Jackson Immuno, 111-675-144), Goat anti-chicken IgY (ab150169), and Goat-anti mouse IgG Alexa Fluor 488 (Abcam ab150113).

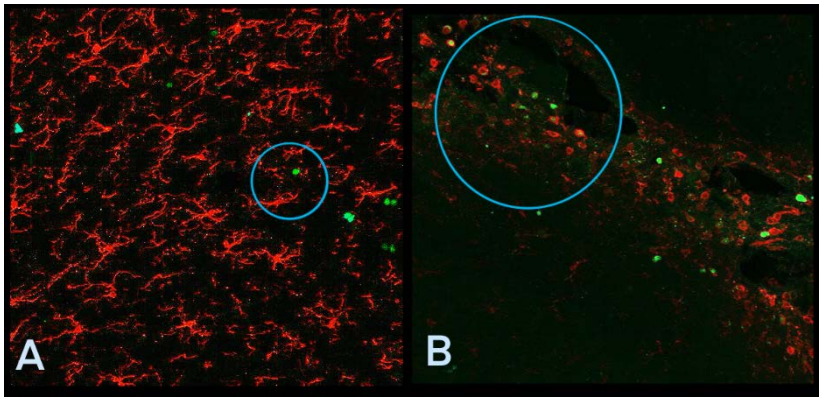

Figure 5. (A) Co-expression of Iba-1 (red) and Ki-67 (green) on microglia at 20x magnifcation of deficient diet male (PND 55) brain in the VTA. (B) Positive control of Iba-1 (red) and Ki-67 (green) co-expression located between mammillary nucleus (MM) and interpeduncular nucleus (IPN) of a standard diet, wild type male, late adolescent rat.

In summary, our previously reported findings suggest that shifting of diet after weaning mitigates the observed inflammatory microglial response induced by deficiency. This is a highly significant observation because it suggests that that microglial cell populations shift into a less activated state through means of n-3 supplementation during preadolescent/adolescent years. The inclusion of additional markers to confirm microglial activation is necessary to increase the rigor of this observation before we submit the data for publication. The implementation of Ki-67, CD68, and CSF-1R will offer a more nuanced and mechanistic characterization of our proposed shift in neuroinflammation through dietary change.

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

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Not Applicable

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?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Additional microglial activation markers have been added for further insight into the nature of potential diet induced neuroinflammation.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report

Changes that had a significant impact on expenditures

Nothing to report

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 vertebrate animals.

Not applicable

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**
  Nothing to report

  Journal publications.

  Nothing to report. Of note, a comprehensive manuscript with the data described above is in preparation. Completion of the manuscript and submission has been delayed because data collection was delayed due to pandemic shutdown of the lab.

  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?

Bita Moghaddam, PhD
Project Role: Partnering PI
Nearest person month worked from 30 Sep 2016 – 30 Apr 2018: 1 calendar month
Nearest person month worked from 1 May 2018 – 30 Apr 2019: 1 calendar month
Nearest person month worked from 1 May 2019 – 30 Apr 2020: 1 calendar month
Nearest person month worked from 1 May 2020 – 30 Oct 2020: 1 calendar month
Contribution to Project: Dr. Moghaddam supervised the project, including completion of protocols, overseeing all aspects of animal testing and data analysis.

Tara Chowdhury, PhD
Project Role: Postdoctoral Researcher
Nearest person month worked from 30 Sep 2016 – 30 Apr 2018: 6 calendar months
Nearest person month worked from 1 May 2018 – 30 Apr 2019: 1 calendar month
Contribution to Project: Dr. Chowdhury performed the behavioral testing and initial stages of microglia measures.

Kathryn Wallin-Miller, PhD
Project Role: Postdoctoral Researcher
Nearest person month worked from 30 Sep 2016 – 30 Apr 2018: 2 calendar months
Nearest person month worked from 1 May 2018 – 30 Apr 2019: 6 calendar months
Nearest person month worked from 1 May 2019 – 30 Apr 2020: 9 calendar month
Nearest person month worked from 1 May 2020 – 30 Oct 2020: 2 calendar month
Contribution to Project: Dr. Wallin-Miller has established the new method of microglia assessment and was responsible for post-processing analysis of the tissue for Major Task 3.

Kyle Clark
Project Role: Research Assistant
Nearest person month worked from 30 Sep 2016 – 30 Apr 2018: 7 calendar months
Contribution to Project: Mr. Clark was responsible for all breeding and initial stages of tissue processing.

Madeleine Allen
Project Role: Research Assistant
Nearest person month worked from 30 Sep 2016 – 30 Apr 2018: 3 calendar months
Nearest person month worked from 1 May 2018 – 30 Apr 2019: 6 calendar months
Contribution to Project: Ms. Allen was responsible for all breeding and initial stages of tissue processing.

Nicole Kahn
Project Role: Research Assistant
Nearest person month worked from 1 May 2018 – 30 Apr 2019: 6 calendar months
Nearest person month worked from 1 May 2019 – 30 Apr 2020: 7 calendar months
Contribution to Project: Ms. Kahn was responsible for all breeding (Task 2) and assisting with behavior testing and analysis (Tasks 3).

Alina Bogachuk
Project Role: Research Assistant
Nearest person month worked from 1 May 2019 – 30 Apr 2020: 2 calendar months
Nearest person month worked from 1 May 2020 – 30 Oct 2020: 6 calendar months
Contribution to Project: Ms. Bogachuk was responsible for all breeding (Task 2) and assisting with behavior testing and analysis (Tasks 3 - initial stages of tissue processing).

Aqilah McCane, PhD
Project Role: Postdoctoral Researcher
Nearest person month worked from 1 May 2020 – 30 Oct 2020: 2 calendar months
Contribution to Project: Dr. McCane contributed to running the behavioral testing (Tasks) and data analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No changes in senior/key personnel

Changes in active support for PI Bita Moghaddam:

NEW

None

Ended (closed)

(THIS AWARD)
W81XWH-16-1-0658 (Partnering PIs: Moghaddam & Narendran) 09/30/16 – 10/30/20 (NCE)
2.4 calendar

Department of Defense (DOD) – Army (PR150716P1)
"Omega-3 Polyunsaturated Fatty Acid Status, Microglial Activation, Stress Resilience and Cognitive Performance"
The project focuses on brain inflammation as a potential mechanism that underlies stress resilience and cognitive impairments in n-3 PUFA deficient rodents and humans. The proposed experiments will also focus on the expression and restoration of dietary n-3 PUFA deficiency in late adolescence/young adulthood. This is important because it is not only the age at which individuals are typically recruited into the military, but also a developmentally critical period during which an individual is vulnerable to psychiatric disorders.
Role: PI

OVERLAP

There is no overlap.

Other organizations involved as partners:

University of Pittsburgh
Pittsburgh, Pennsylvania
Partner's Contribution to the project:
Collaboration

This award involved a Partnering Award at the University of Pittsburgh, Partnering PI: Dr. Rajesh Narendran. Dr. Narendran will submit an independent Final progress report per the instructions for collaborative awards. There are no additional organizations involved as partners.

8. SPECIAL REPORTING REQUIREMENTS

Partnering PI report will be filed separately for Aim 1 (human study) by Dr. Rajesh Narendran, University of Pittsburgh, Pittsburgh, PA

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.
N/A

9. APPENDICES:

Nothing to report