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

PRINCIPAL INVESTIGATOR: Bita Moghaddam

CONTRACTING ORGANIZATION: Oregon Health & Science University

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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. A plethora of literature also implicates n-3 PUFA deficiency 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 propose 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—a developmentally critical period during which an individual is vulnerable to mood, psychotic and addictive disorders. We will use 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 will directly measure microglia activation in an animal model of n-3 PUFA deficiency and determine whether supplementation during early adulthood reverses this effect in correlation with behavior.

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
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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 in deficient animals before and after supplementation, and compared to adequate animals in the same age range. There are four 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" beginning after weaning, (4) animals on deficient diet that shift to adequate diet "short-term," one week before behavior testing. Behavioral testing for target date 6-12 months included open field, elevated plus maze, and delayed alternation in two of the proposed 4 groups.

Major task 4: anti-Iba1 immunohistological staining to estimate microglial number and activation. Procedures include perfusion and tissue prep after termination of behavior testing, target date 3-26 months followed by histological assessment and analyese 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. We moved from university of Pittsburgh to Oregon Health and Sciences University about a year ago. Since then we successfully established first and second generation of n-3 PUFA deficient and adequate colonies of rats in the laboratory. These animals are being used for studies in major task 3 and 4.

Major Task 3: This task is ongoing. Behavioral testing for target date 6-12 months included open filed test and elevated plus maze, novel object recognition and delayed alternation.

The elevated plus maze component has been completed. The open field, novel object recognition and delayed alternation data collection is about 50% complete, subjects are being added as breeding progresses. The collected completed 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 seconds spent in open or closed part of an elevated plus maze in animal on n-3 PUFA adequate (ADQ) and deficient (DEF) diets. Less time spent in an open arm is indicative of increased fear and anxiety. We find that ADQ diet increases anxiety as measured in this task. These data are consistent with this dietary deficiency enhancing anxiety and provides a metric for upcoming supplementation studies. While the data analysis for the other tasks are not complete, FIGURE 2 shows raw data from open field behavioral task. We have automated quantification of these tasks using SMART video tracking system (Harvard Apparatus) which speeds up the analyses and reduces potential subjective influence of manual rating by investigator.

Major task 3: This task is ongoing. We had proposed to use anti-Iba1 immunohistological staining to estimate microglial number and activation. Establishing this method and collection and analyses of the first set of data was accomplished. We are focusing on efforts on microglia activation in a key brain system that is critical for cognitive performance and stress reactivity: the midbrain dopamine containing regions of ventral tegmental area (VTA) and substantia nigra (SNc) and basal ganglia and prefrontal cortical areas that have reciprocal connections to these regions. The data generated from the initial set are depicted on FIGURE 3 and show significant microglia activation in the cohort on deficient diet as measured by reduced volume of processes.

FIGURE 1. Elevated plus maze (EPM1)

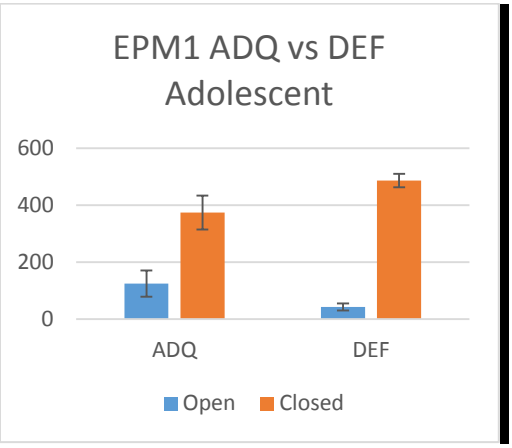


FIGURE 2. Raw open field movement data

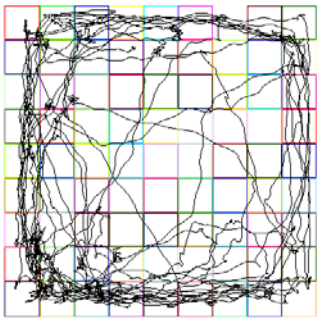
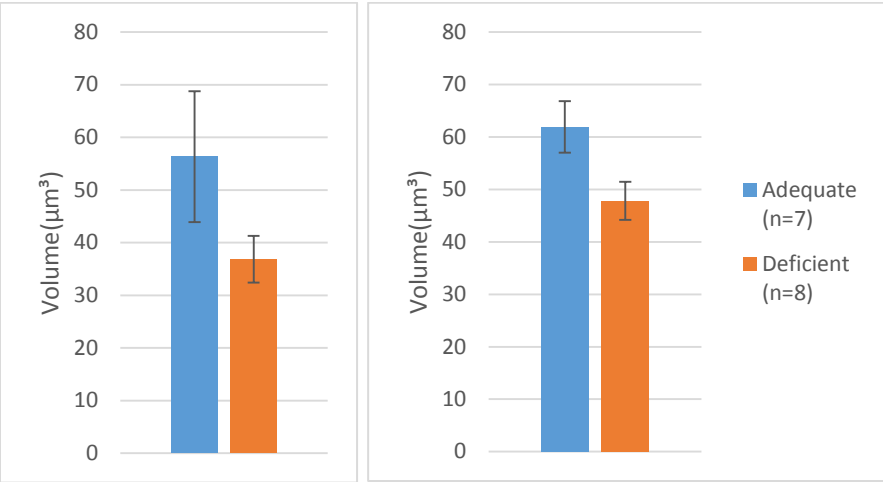


FIGURE 3. Average volume of microglia processes in VTA (lef panel) and SNc (right panels) of animals on adequate and deficient n-3 PUFA diet. The reduction in volume was significant in VTA (p=0.046) and SNc (p=0.01)



While these data using immunopreoxidase staining of microglia were promising, the huge amount of time taken to manually trace the processes (about 6-8 hours of tracing per brain region in one animal) and the concern that manual tracing is subject to inter-experimenter variability led us to put effort into validation of a more accurate and automated process. Of note, access to Advanced Microscopy Core at OHSU was key in allowing us to implement this improvement. The new method is as follows:

Fluorescent immunohistochemistry for automated microglia quantification and morphometry analysis

Background: We have optimized a protocol for fluorescent immunohistochemical visualization of microglia and automated analysis of morphology. We believe that this represents a significant improvement over our previous proposed method using immunopreoxidase staining for colorimetric visualization and manual tracing of microglia for morphometric 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.

Antibody optimization: For immunofluorescent imaging of microglia, several antibody combinations were tested at various concentrations. Two Iba1 primary antibodies were tested: Rabbit-anti-Iba1 (Wako Chemicals, #019-19741) and Mouse-anti-Iba1 (Wako Chemicals, #016-26721). Secondary antibodies tested were: 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).

We determined that the Rabbit-anti-Iba1 at a concentration of 1:1000 was the best primary antibody for microglia imaging. This primary worked well with both Goat-anti-Rabbit IgG H&L Brilliant Violet 421 and Goat-anti-Rabbit IgG H&L Alexa Fluor 488 secondary antibodies, for visualization on the blue or green channel, respectively FIGURE 4). Combination with the green secondary produced the best signal, with microglia cell bodies and processes clearly visible (FIGURE 4a and 4b)

Multichannel imaging: Multichannel imaging allows co-staining of up to four different cell types or proteins of interest. This allows simultaneous analysis of microglia and dopamine neurons, as well as association between the two cell types. It also improves upon our previous methods by facilitating determination of regions of interest. With colorimetric immunohistochemistry, bouning of regions of interest is determined by staining alternate coronal sections with TH (an indicator of dopamine cells). Iba1-stained sections are then compared to adjacent TH-stained sections in order to circumscribe midbrain regions of interest containing dopamine cells. With co-staining and multichannel imaging, regions of interest are simultaneously visualized in the tissue sections being analyzed.

For 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) allows visualization of TH-containing neurons on the red channel. Cell nuclei are also visualized on the blue channel with a DAPI stain (Vector Laboratories, H-1200). Staining cell nuclei allows quantification of all cells in an image, as well as determination of the percent of cells in a region that express Iba1 or TH (FIGURE 5)

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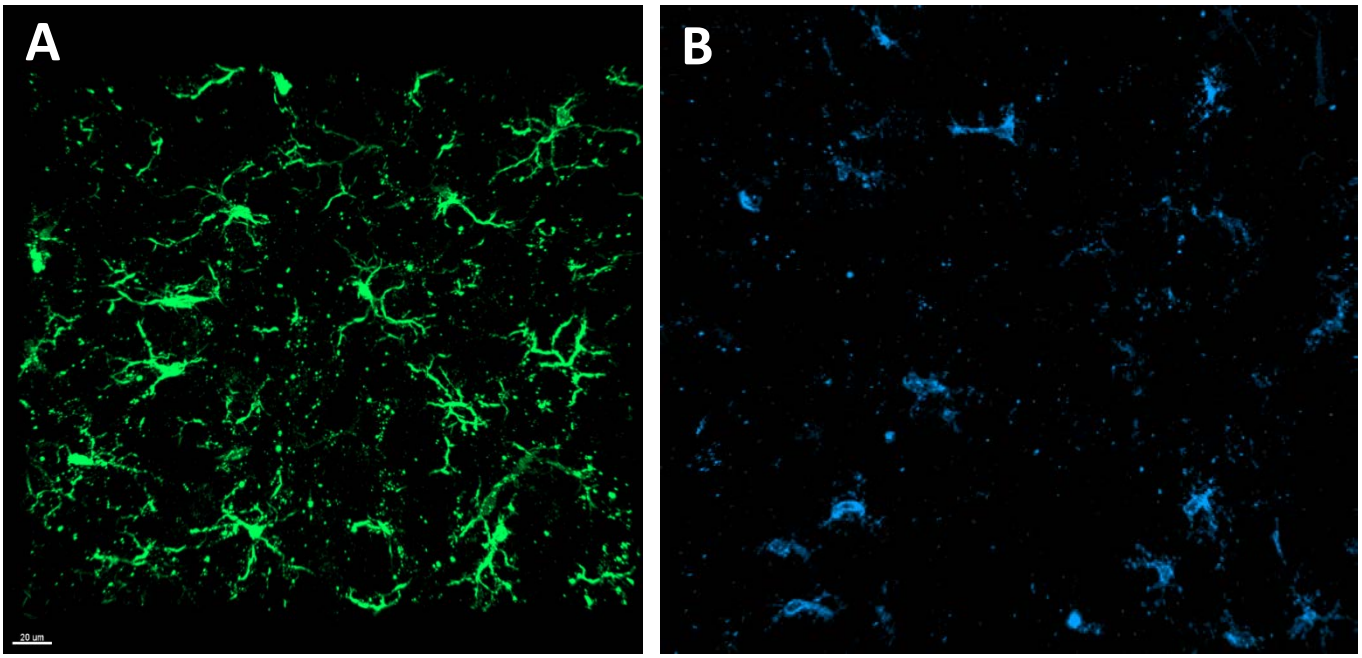

FIGURE 4 (above): Immunofluorescent imaging of microglia marker Iba1 on the Apotome microscope's green (A) and blue (B) channels.

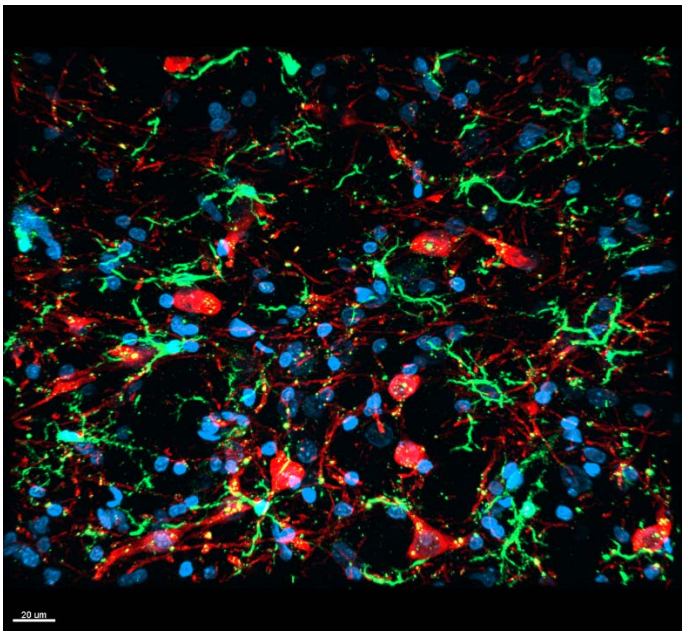

FIGURE 5 (left)
Immunofluorescent imaging of microglia (green), dopamine cells (red) and cell nuclei (blue

Image analysis: Images acquired with Zeiss ZEN imaging software are subsequently processed with Bitplane IMARIS imaging analysis software. IMARIS allows automated analysis of multiple fluorescent signals for almost all possible parameters of interest. Examples of analysis parameters include: cell number, volume, and density, as well as both proximity to and surface contact area with other cells (of the same or different type). Microglia morphometry is analyzed with the IMARIS Filament Tracer Module (FIGURE 6), allowing automatic analysis of dendrite volume, dendrite length, number of dendrite segments, number of dendrite branches, dendrite area, points, filaments, branch level, and Scholl analysis. Once analysis parameters are set, an essentially-unlimited number of images can be automatically batch-analyzed by IMARIS without supervision.

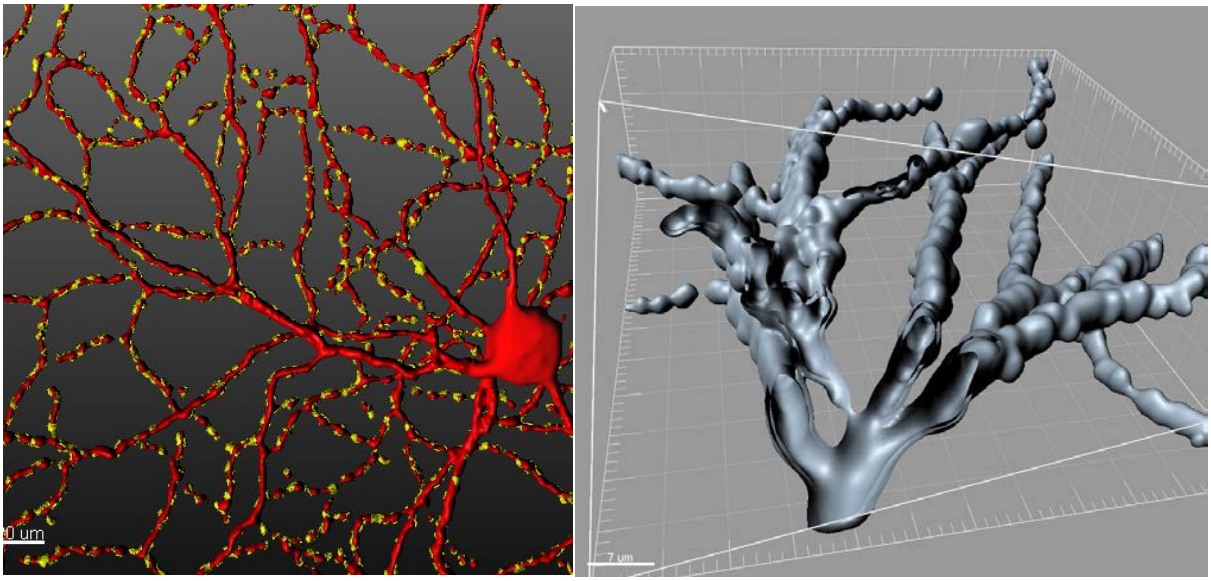

FIGURE 6: IMARIS Filament Tracer 3D modeling of dendrite morphology for automated analysis. Images from www.bitplane.com/imaris/filamenttracer.

Now that we have completed characterization of this automated assay, we will apply it for the future analysis of microglia activation in Task 4.

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

Postdoctoral training of personnel involved in the project

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?

1. Continue Major Task 2 to provide sufficient subjects complete behavioral data collection for deficient and adequate diet cohorts

2. Continue the behavioral testing in Major Task 3 involving all four groups of subjects.

3. Analyze microglia activation with the new established methods as part of Major Task 4 as tissue is generated.

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

As described above, although we were successful in using immunopreoxidase staining of microglia as proposed in Task 4 of the original application, we have characterized a new method to assess microglia activation, which we plan to use in the coming year. The new method (described above) is automated and provides a more efficient and accurate approach to quantify microglia activation in specific brain regions.

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

  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: 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 Chowhury, PhD

Project Role: Postdoctoral Researcher
Nearest person month worked: 6 calendar months
Contribution to Project: Dr. Chowhury performed the behavioral testing and initial stages of microglia measures.

Kathryn Wallin-Miller, PhD
Project Role: Postdoctoral Researcher
Nearest person month worked: 2 calendar months
Contribution to Project: Dr. Wallin-Miller has established the new method of microglia assessment and testing and processing of the tissue.

Kyle Clark
Project Role: Research Assistant
Nearest person month worked: 7 calendar months
Contribution to Project: Mr. Clark was responsible for all breeding and initial stages of tissue processing. His role has been replaced by Madeleine Allen.

Madeleine Allen
Project Role: Research Assistant
Nearest person month worked: 3 calendar months
Contribution to Project: Ms. Allen was responsible for all breeding and initial stages of tissue processing.

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

R56 MH084906 - 06A1 (Moghaddam) 07/01/17 – 06/31/18 3 calendar
NIMH/NIH $320,019
"Inhibitory Control of Prefrontal Cortex"
Anxiety is a debilitating symptom of most psychiatric disorders including PTSD, major depression, and addiction. The proposed studies aim at understanding the neuronal basis of anxiety and its impact on goal-directed behavior.
Role: PI

ENDED (closed)

3R01MH048404-26S1 (Moghaddam) 07/01/16 – 06/30/17 0 calendar
NIMH/NIH $60,000
"Neurochemical Effects of Antipsychotic Drugs Supplement"
A better understanding of the neurobiology of male versus female adolescent brain is fundamental to our understanding of the etiology of the disorder and of the design of intervention strategies.
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 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