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14. ABSTRACT

The DOD identified a strategical goal for foundational studies to identify mechanisms underlying neurological diseases including fragile X syndrome (FXS) and potential relationships to environmental/neurotoxic exposures. A correlating treatment goal included developing novel therapeutic targets for associated neurological diseases. The DOD also identified a strategic goal for foundational studies to understand correlations between nutrition and disease susceptibility. These DOD needs are juxtaposed in this application, which proposes to determine the effect of fragile X messenger ribonucleoprotein (FMRP) and postnatal diet on gut leakiness in a well-established mouse model of FXS, *Fmr1*^{KO} mice. Gastrointestinal (GI) problems are a common comorbidity in FXS. There is accumulating evidence suggesting that leaky gut syndrome causes neurological phenotypes. Although ubiquitously expressed, there is a dearth of knowledge regarding the role of FMRP outside of the brain and the mechanism of GI dysfunction in FXS. This DOD Discovery Award proposal seeks to generate novel data on GI barrier function in response to *Fmr1* genotype and diet in a mouse model of FXS. The *central hypothesis* driving this proposal is that FMRP regulates the levels of tight junction proteins in the gut barrier, and the absence of FMRP in FXS leads to leaky gut and neurological sequelae particularly in the context of soy-based diet.

15. SUBJECT TERMS
FMR1, FMRP, Fragile X, gut barrier

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INTRODUCTION

Fragile X syndrome (FXS) is a neurodevelopmental disorder clinically characterized by intellectual disability, autistic-like behaviors and seizures. FXS results from a mutation in a single gene on the X-chromosome, *FMR1*, that is associated with transcriptional silencing of the promoter and loss of fragile X messenger ribonucleoprotein (FMRP) expression. There are currently no disease specific, effective treatments. The field has primarily focused on FMRP function in the brain. This innovative application seeks to understand the role of FMRP in the gastrointestinal (GI) system and a possible role in leaky gut that could lead to brain dysfunction. GI issues are common in FXS and increasing evidence points to correlations between gut health and brain function; however, there are major gaps in understanding and treating GI complications.

The DOD is interested in identifying correlations between environmental, neurotoxic and nutritional exposures and disease susceptibility. The role of gene-environment interactions in FXS is a largely unexplored area. The scientific premise for this work is based on our past decade of research demonstrating associations between postnatal diet and altered disease comorbidities, seizure rates and biomarker outcomes in mouse and human models of FXS and autism. Of note, we found early-life exposure to single-source, soy-based diet is associated with increased risk of seizures, autism, GI problems and allergies. These data elicit the hypothesis that bioactive components of soy protein exacerbate FXS phenotypes as well as the hypothesis that gut dysfunction in FXS elicits the use of alternative feeding strategies including soy-based infant formula. Regarding the first hypothesis, soy protein is rich s phytoestrogens (plant estrogens). Accounting for body weight, infants fed soy-based infant formulas consume 6-11 times the quantity of phytoestrogens necessary to produce hormone-like effects in adults. It remains to be determined how single-source soy-based diets and/or soy phytoestrogens are associated with more severe neurological outcomes in FXS. Regarding the second hypothesis, the prevalence of GI problems in FXS and other neurodevelopmental disorders is well documented, but the mechanism is not known. Toward addressing these gaps, the objective of this DOD Discovery Award is to examine intestinal morphology and gut and brain barrier permeability as a function of *Fmr1* genotype and diet. The rationale is that tight junctions directly control barrier permeability and transport of dietary components into the blood and brain such that aberrant GI morphology in FXS could be a contributing factor to brain dysfunction.

KEYWORDS

FMR1
FMRP
Fragile X syndrome
Gut permeability

ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1 (Test FMRP Regulation of Tight Junction Proteins)

Major Task 1: Animal Approvals
- Subtask 1 : Local IACUC approval for animal work — **completed**
- Subtask 2 : USAMRMC ACURO approvals — **completed**

Major Task 2: Animal Husbandry
- Subtask 1: Breed mice ($Fmr1^{KO}$ mice and littermate controls — **in progress**

Major Task 3: Multi-Plex Assay
- Subtask 1: Isolate intestines (duodenum, jejunum, ileum, colon) from female (WT, $Fmr1^{HET}$, $Fmr1^{KO}$) and male (WT, $Fmr1^{KO}$) mice maintained on Tekla 2019; 2 months old; n=3/genotype — **sample collection in progress**
- Subtask 2: Prepare intestinal lysates, immunoprecipitated with anti-FMRP antibody, and purify associated mRNA — **will start when Subtask 1 completed**

Subtask 3: Conduct encounter Plex Set Tight Junction Panel at TRIP Laboratory
Subtask 4: Confirm top Plex Set tight junction targets by RTqPCR and western blot

Major Task 4: Protein Synthesis Assessment **plan to start after completion of Task 3**
Subtask 1: Prepare SN from postnatal mice (age 14-17 days)
Subtask 2: Incubate with Pro-Mix-L, immunoprecipitate, and visualize

Specific Aim 2 (Test GI and Brain Barrier Permeability in *Fmr1*^{KO} Mice)

Major Task 1 : Dose Mice **50% completed for n=10/cohort**
Subtask 1: Maintain breeding pair on Teklad 2019 versus casein- and soy-based matched diets to generate study cohorts: male WT and *Fmr1*^{KO}; female WT, *Fmr1*^{HET}, *Fmr1*^{KO} on each diet; 2 months old; n=3/cohort **in progress**

Major Task 2 : Assess Gut Permeability **50% completed**
Subtask 1: Fast cohorts, oral gavage with FITC-D4000, euthanize and collect tissues (blood, brain, intestine), photometric analysis of FITC-D4000

Major Task 3 : Assess Gut Morphology **sample collection in progress**
Subtask 1: Section and stain intestinal tissue
Subtask 2: Quantitate intestinal pathology by light microscopy
Subtask 3: Localization of tight junction proteins by confocal fluorescent microscopy

Major Task 4 : Assess Phytoestrogen Levels **sample collection in progress**
Subtask 1: Quantitate phytoestrogen levels in blood and brain by mass spectrometry
Subtask 2: Write manuscript and next grant

What was accomplished under the goals?
We generated and genotyped approximately 200 mice for FITC-dextran / gut permeability testing. We increased the n to 10 mice per cohort (4 diets x 5 genotypes x 10 per group) to better discern genotype specific differences. We are halfway through testing and are observing a significant increase in gut permeability with the soy-based purified ingredient diet. Additional animals will discern any genotype specific differences. We are concurrently collecting tissue samples for the Multi-Plex and Gut Morphology assays. We anticipate having all tissue collected by March of 2024.

What opportunities for training and profession development has the project provided?
The P.I. is preparing a poster for the campus ICTR Research event in January of 2024. An undergraduate student, Brynne Boeck, working on the project graduated in December of 2023 and will work in the lab in a post-bac position to help with genotyping and gut morphology analysis for 1 semester before she starts graduate work.

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?
We plan to finish the FITC-dextran testing in March of 2024 and then proceed with the remaining tasks.

IMPACT

Nothing to report. Expected impact as described in the application: A critical gap in medical care for persons with FXS is identification of early-life exposures and associated mechanisms that exacerbate disease severity. This proposal addresses the FY22 PRMRP Topic Areas of **Fragile X Syndrome** and **Nutrition Optimization**. The proposed studies will determine the effect of fragile X messenger ribonucleoprotein (FMRP) and diet on gut structure as well as gut and brain barrier leakiness in *Fmr1*^{KO} mice. The study will serve as the foundation to apply novel therapeutic and nutritional interventions to treating FXS. Positive findings could have powerful translational implications in terms of targeting intestinal tight junctions as a therapeutic strategy to reduce disease severity in FXS. There are currently no disease specific, effective treatments for FXS. Hence, it is important to examine alternate avenues including dietary therapies. The project

has potential in the field and patient care in terms of finding a potential efficacious and safe intervention (restriction of soy-based infant formula) that can be applied to newborns like the phenylketonuria (PKU) or galactosemia diets are employed for inborn errors of metabolism. The project also has potential to repurpose experimental Celiac disease drugs for FXS.

The tangible benefits of this contribution are manifold. Short-term impact includes the potential to identify a tight junction-mediated mechanism that underlies diet-induced neurological effects in *Fmr1*^{KO} mice. Soy-based infant formula is the top alternative for babies who cannot be breastfed and are fussy or allergic in response to cow milk-based formula; however, there are other alternatives if early-life exposure to single-source, soy-based diets exacerbate FXS outcomes. GI issues are a prevalent parent-reported phenotypes in FXS but have not been studied in *Fmr1*^{KO} mice. Long-term impact includes a rigorous foundation to support: (1) testing drugs that target the tight junctions of the gut epithelium on disease outcomes in FXS models, (2) implementation of newborn screening for early identification of infants with FXS who could benefit from early nutritional management, and (3) development of GI biomarkers and outcome measures that translate between preclinical and clinical FXS research. Future studies will include testing the efficacy of the experimental Celiac disease drug larazotide acetate on intestinal morphology in *Fmr1*^{KO} mice. Additional future studies will include testing diet / drug interactions. The ubiquitous use of single-source diets, predominantly grain-based chows, in research vivariums may help explain the poor translation of effective therapeutics from preclinical to clinical studies.

Patients and family members are desperate for cures and symptomatic relief for many currently untreatable conditions including FXS. Thus, there is a *critical and urgent need* to understand the role of the gut tight junctions and diet on the development of disease outcomes in FXS. In the absence of such knowledge, preventive intervention strategies will remain underemployed and novel therapeutic targets unidentified. Thus, the proposed work has impact for FXS families, the general population, civilians and military personnel.

CHANGES / PROBLEMS

Changes in approach and reasons for change:

We made two changes: (1) we switched to testing *Fmr1* mice in the FVB background versus the C57BL/6J background. We will eventually compare both strains. We made the change as the FVB in our colony were reproducing very well and the C57BL/6J were not this year. (2) We increased the n from 3 to 10 for comparison of gut permeability as a function of diet. This requires a total of 200 mice to test but will better discern diet and genotype specific differences. We are half finished with the testing and seeing a significant increase in permeability with the purified ingredient soy diet.

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

We started with Aim 2 instead of Aim 1; thus Aim 1 is delayed. We made this change as Aim 2 tests function, which is a more important questions than Aim 1, which tests structure.

Changes that had a significant impact on expenditures:

None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:

None

PRODUCTS

Nothing to report

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

Name: Cara Westmark
Project Role: P.I.
Researcher ORCHID ID: 0000-0003-3919-3279
Person Months worked: 0.96 Cal Mo.
Contribution to Project: overall coordination, training personnel, preparation of reports, FITC-dextran experiments
Funding Support: n/a

Name: Pamela Westmark
Project Role: Key Person
Researcher ORCHID ID: 0000-0001-7999-0892
Person Months worked: 2.28 Cal Mo
Contribution to Project: animal husbandry
Funding Support: n/a

Name: Brynne Boeck
Project Role: Undergraduate Student Researcher
Researcher ORCHID ID: n/a
Person Months worked: 1.2
Contribution to Project: genotyping
Funding Support: working in lab for academic credits

Has there been a change in the active support of the PD/PI(s) or senior/key personnel since the last reporting period?
New funding awarded:
P.I.: Rama Maganti
Co-P.I.: Cara Westmark, 1.08 mo effort
Key Person: Pamela Westmark, 1.44 mo effort
Agency: DOD
Title: Enhancing Sleep with a Dual Orexin Antagonist to Mitigate the Neurobehavioral Sequelae of TBI

What other organizations are involved as partners?
Nothing to report

SPECIAL REPORTING REQUIREMENTS

N/A

APPENDICES

N/A