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TITLE: Exaggerated Cap-Dependent Translation as a Mechanism for Corticostriatal Dysfunction in Fragile X Syndrome Model Mice

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14. ABSTRACT

We hypothesize that excessive cap-dependent translation is a causative factor in FXS. To test this hypothesis, we will 1) determine cortico-striatal synaptic composition, function, and plasticity in FXS model mice, and 2) determine whether altered cortico-striatal synaptic plasticity and repetitive/perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors. Our studies will provide information concerning whether increased eIF4E-eIF4G interactions are a *biological risk factor* for ASD. Our studies should also provide important information concerning the role of upregulated cap-dependent translation in FXS, and could link FXS mechanistically at the level of cap-dependent translational control to TSC, and autistic patients with *PTEN, CYFIP1* and *EIF4E* mutations. Moreover, the results of these studies would provide information for the design and use of compounds to *therapeutically target* eIF4E-eIF4G interactions and eIF4A for treating patients with FXS and other ASDs.

15. SUBJECT TERMS
autism spectrum disorder, cap-dependent translation, eIF4E, repetitive behaviors, perseverative behaviors, social behaviors

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Introduction

Our overall hypothesis is that repetitive and perseverative behaviors exhibited by fragile X syndrome (FXS) patients that can be recapitulated in FXS model mice are caused by exaggerated eIF4E-mediated translation at cortico-striatal synapses. To test this hypothesis we proposed two specific aims: **1) to determine cortico-striatal synaptic composition, function and plasticity in FXS model mice and 2) to determine whether altered cortico-striatal synaptic plasticity and repetitive/ perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors.** Using a multi-disciplinary approach that takes advantage of the strength of the Klann and Bagni laboratories, we proposed to determine the cortico-striatal synaptic composition, function, and plasticity in FXS model mice using biochemical, molecular, proteomic, gene-targeting and electrophysiological approaches. Then, we will determine whether altered cortico-striatal synaptic composition and function, as well as repetitive and perseverative behaviors displayed by the FXS model mice are reversed by cap-dependent translation inhibitors. The studies will determine whether excessive cap-dependent translation, via increased eIF4E-eIF4G interactions, triggers changes in synaptic composition and function, as well as abnormal behaviors that are associated with individuals with FXS.

Key Words

fragile X syndrome (FXS), eIF4E, synaptic plasticity, cortico-striatal synapses, repetitive behaviors, perseverative behaviors, cap-dependent translation

Accomplishments

Herein I will describe the research accomplishments associated with each task and subtask that was outlined in the approved Statement of Work.

Major goals of project

Major task 1 in the Statement of Work was to determine the cortico-striatal synaptic composition, function, and plasticity in FXS model mice. These experiments were to be completed in years 1 and 2. Major task 2 in the Statement of Work was to determine whether altered cortico-striatal synaptic plasticity and repetitive/ perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors. These experiments were to be completed in year 3.

Accomplishments under the major goals

The first item in major task 1 was to obtain regulatory approval for the use of mice by New York University (NYU) IACUC Committee and USAMRMC Office of Research Protections. The animal protocol was approved the NYU IACUC and Committee as well as the USAMRMC Office of Research Protections.

The first subtask in the Statement of Work was for my lab to determine measure fEPSPs, AMPA receptor- and NMDA receptor-mediated fEPSCs in FXS model mice and their wild-type littermates. These experiments have begun and data sets should be collected in the next two months.

The second and third subtasks are being done in the Bagni lab and the progress on these subtasks should be described in her report.

The fourth subtask was to isolate the PSD-95 interactome from the cortical, hippocampal, and cortico-striatal regions of the Fmr1 knockout/PSD-95^{TAP} mice and PSD-95^{TAP} control mice at different developmental stages. This subtask is being done in the Bagni lab and the progress should be described in her report.

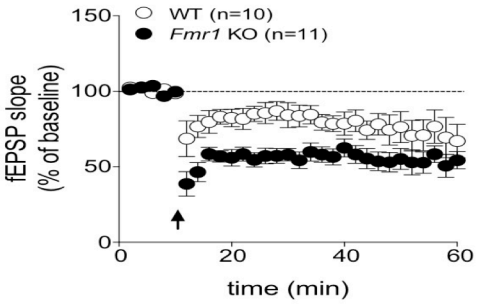

Figure 1. Cortico-striatal LTD is enhanced in FXS model mice. LTD was induced in cortico-striatal slices from wild-type (WT) and *Fmr1* knockout (KO) mice with one train of high-frequency stimulation (indicated by the arrow) of 100 Hz.

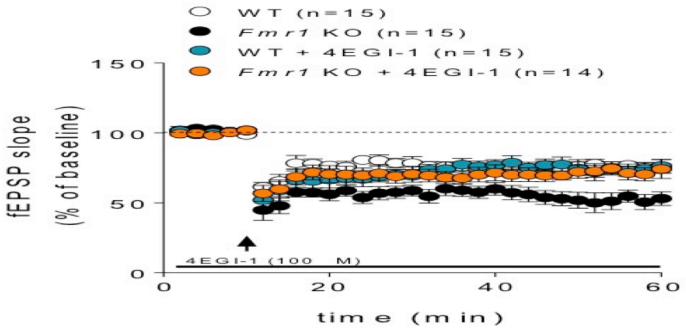

Figure 2. Enhanced cortico-striatal LTD in FXS model mice is normalized by 4EGI-1. LTD was induced in cortico-striatal slices from wild-type (WT) and *Fmr1* knockout (KO) mice with one train of high-frequency stimulation (indicated by the arrow) of 100 Hz in the presence of either vehicle or 100 μM 4EGI-1.

The fifth subtask was for my lab to measure LTD at cortico-striatal synapses in FXS model mice and their wild-type littermates. We have found that FXS model mice exhibit enhanced LTD at cortico-striatal slices (Figure 1). These findings are similar to the enhanced LTD that we observed previously in eIF4E transgenic mice that overexpress eIF4E (Santini et al., 2013). We, and others, have reported previously that FXS model mice have increased eIF4E-eIF4G interactions (Sharma et al., 2010; Ronesi et al., 2012). Therefore, we asked whether blocking eIF4E-eIF4G interactions with 4EGI-1 could reduce the enhanced LTD in FXS model mice as it does in eIF4E transgenic mice (Santini et al., 2013). Our data indicate that 4EGI-1 normalizes the enhanced LTD in cortico-striatal slices in FXS model mice (Figure 2). Taken together these findings are consistent with the notion that excessive eIF4E-dependent translation results in altered synaptic plasticity at cortico-striatal synapses in FXS model mice.

The sixth subtask was to generate D2R-eGFP/ *Fmr1* knockout and D1R-tomato/*Fmr1* knockout mice. These mice were generated and we conducted experiments that are described below.

The seventh subtask was to conduct the protein composition analysis of the PSD-95 interactome from Fmr1 knockout/PSD-95^{TAP} mice and PSD-95^{TAP} control mice at two different developmental stages. This subtask is being done in the Bagni lab and the progress should be described in her report.

The eighth subtask is to measure synaptic function and LTD in the *Fmr1* knockout/D2R-eGFP and *Fmr1* knockout/D1R-tomato mice. We have done these experiments and have found that the *Fmr1* knockout/D2R-eGFP mice have enhanced LTD (Figure 3) as do the *Fmr1* knockout/D1R-tomato mice (data not shown). These findings are important because they indicate that the enhanced LTD in FXS model mice (Figure 1) is not impacted by also expressing eGFP in D2R-containing neurons or tomato in D1R-containing neurons. We continue to conduct whole-patch recordings from D2R-containing neurons in the *Fmr1* knockout/D2R-eGFP mice to determine whether they exhibited enhanced LTD. We continue to conduct whole-patch recordings from D1R-containing neurons in tghe *Fmr1* knockout/D1R-tomato mice to determine whether they exhibited enhanced LTD.

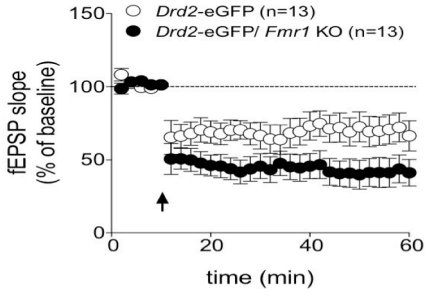

Figure 3. Cortico-striatal LTD is enhanced in Drd2-eGFP/*Fmr1* knockout mice. LTD was induced in cortico-striatal slices from Drd2-eGFP and Drd2-eGFP/*Fmr1* knockout (KO) mice with one train of high-frequency stimulation (indicated by the arrow) of 100 Hz.

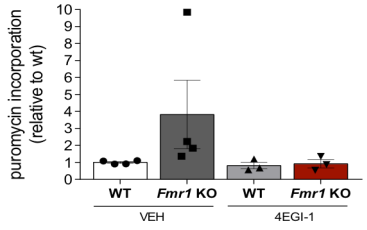

Figure 4. Increased protein synthesis in the dorsal lateral *Fmr1* knockout mice. SUnSET, which measures incorporation of puromycin into newly synthesized proteins, was utilized to demonstrate increased protein synthesis in the dorsal lateral striatum of *Fmr1* knockout mice. 4EGI-1 blocked the increase in protein synthesis in the *Fmr1* knockout mice.

Although not proposed explicitly in the original application, we have utilized *Fmr1* knockout mice to determine whether there is increased protein synthesis in the dorsal lateral striatum and the *Fmr1* knockout/D2R-eGFP and *Fmr1* knockout/D1R-tomato mice to determine the whether any increase in protein synthesis is cell type-specific. Using the SUnSET technique, we have found that the Fmr1 knockout mice have a robust increase in *de novo* protein synthesis (Figure 4). Moreover, the increase in protein synthesis in the *Fmr1* knockout mice was blocked by 4EGI-1. Thus, inhibiting eIF4E-eIF4G interactions can normalize the excessive protein synthesis in the dorsal striatum of *Fmr1* knockout mice.

We proceeded to use FUNCAT in *Fmr1* knockout/D1R-tomato mice to visualize *de novo* protein synthesis in D1R-containing medium spiny neurons in the dorsal lateral striatum. We found that D1R-containing medium spiny neurons exhibit robust increases in *de novo* protein synthesis, which was blocked by 4EGI-1 (Figure 5). Thus, the increase in *de novo* protein synthesis in the dorsal lateral striatum of *Fmr1* knockout mice is due to in part to increases in D1R-containing medium spiny neurons. We also conducted FUNCAT experiments in *Fmr1* knockout/D2R-eGFP mice. We also observed an increase in *de novo* protein synthesis in the D2R-containing medium spiny neurons (Figure 6), but it was not as robust as the increases in the D1R-containing medium spiny neurons (Figure 5).

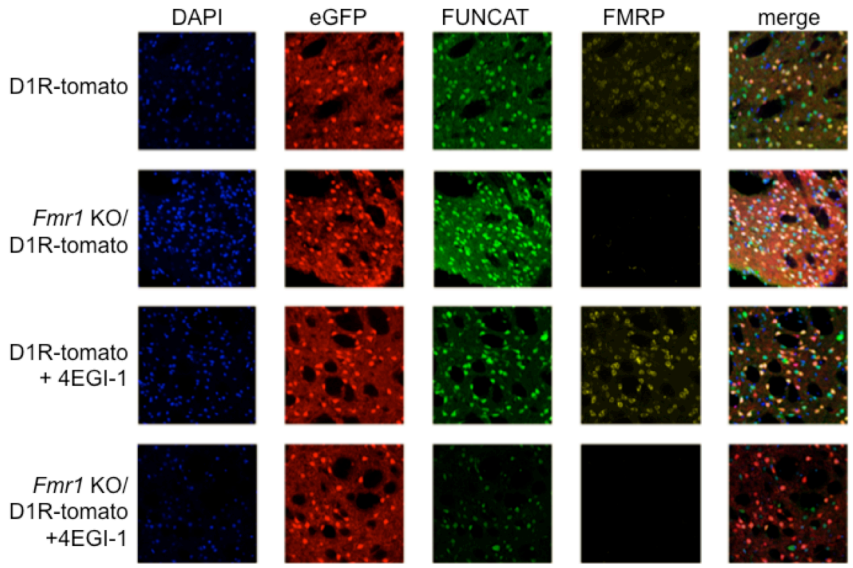

Figure 5. Increased protein synthesis in D1R-containing medium spiny neurons in the dorsal lateral *Fmr1* knockout mice. FUNCAT, which measures incorporation of a methionine analogue into newly synthesized proteins, was utilized to demonstrate increased protein synthesis in D1R-containing medium spiny neurons the dorsal lateral striatum of *Fmr1* knockout mice. 4EGI-1 blocked the increase in protein synthesis in the *Fmr1* knockout mice.

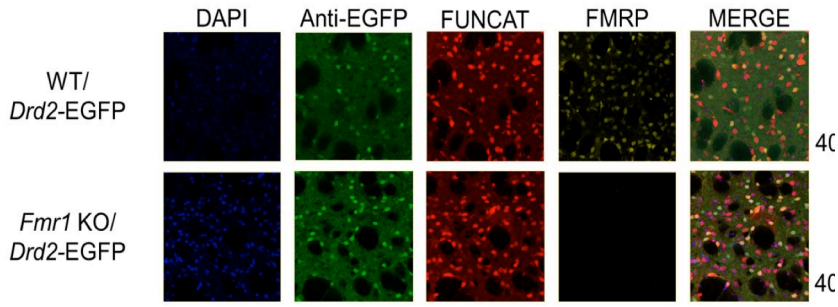

Figure 6. FUNCAT analysis of D2R-containing medium spiny neurons in FXS model mice. Immunofluorescence detection of *de novo* protein synthesis in the dorsal lateral striatum. Increased *de novo* translation is observed in *Fmr1* KO/Drd2EGFP (FUNCAT red) mice compared to wild-type (WT) littermates.

The ninth and tenth subtask in major task 1 was to generate cell type-specific deletion of *Fmr1* in D1R- and D2R-containing neurons and examine the synaptic function in the dorsal lateral striatum of these mice. We have generated these mice and are currently examining their synaptic function as well as their behavior. We have found that that *Fmr1/Drd2-Cre* mice exhibit increased self-grooming (Figure 7) and marble-burying behavior (Figure 8). These ASD-like behavioral abnormalities are accompanied by an increase in locomotor activity in the *Fmr1/Drd2-Cre* mice (Figure 9),

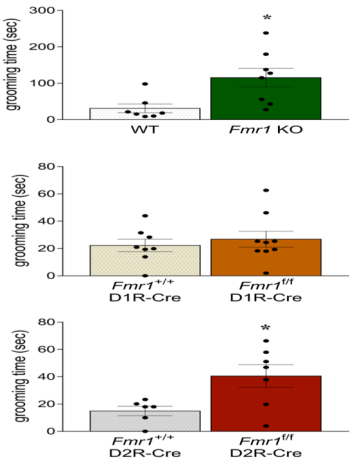

Figure 7. Self-grooming behavior of FXS model mice. *Fmr1* KO and *and Fmr1/D2R-Cre*, but not *Fmr1/D1R-Cre* mice, show an increased self-grooming behavior compared to their wild-type (WT) littermates. Data are mean ± SEM, n=7-9 mice. * p< 0.05 with a two-tailed Students t-test for unpaired data.

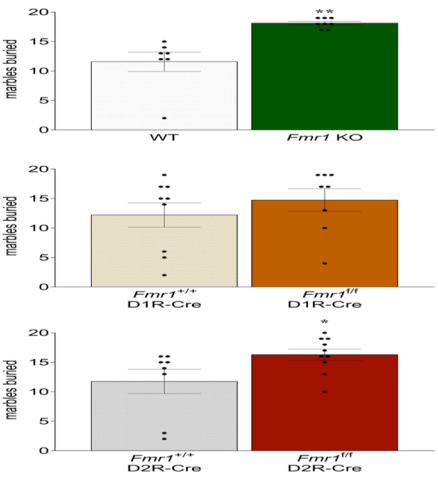

Figure 8. Digging behavior of FXS model mice in the marble-burying test. *Fmr1* KO and *Fmr1/D2R-Cre*, but not *Fmr1/D1R-Cre* mice, bury more marbles than their wild-type (WT) littermates. Data are mean ± SEM, n=7-9 mice. **p<0.01 and * p< 0.05 with a two-tailed Students *t*-test for unpaired data.

which suggest alterations in striatal synaptic function /plasticity Conversely, our preliminary findings indicate that the *Fmr1/Drd1-Cre* mice exhibit motor activity impairments (Figure 7), suggesting that the lack of *Fmr1* affects the inhibition of motor output of the indirect pathway primarily by reducing the control on the striatonigral pathway.

We are currently examining the synaptic function in the D1R/*Fmr1* and D2R/*Fmr1* knockout mice and predict that there will be alterations consistent with increased repetitive behaviors.

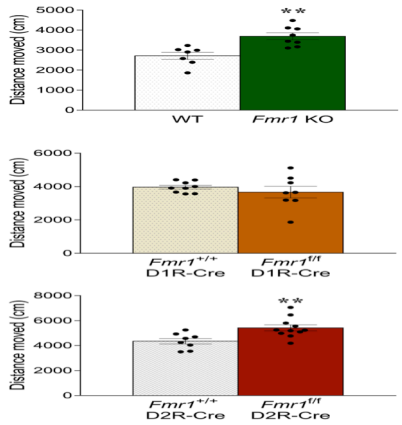

Figure 9. Locomotor activity of FXS model mice in an open field arena. *Fmr1* KO and *and Fmr1/D2R-Cre*, but not *Fmr1/D1R-Cre* mice, exhibit increased locomotor activity compared to their wild-type (WT) littermates. Data are mean ± SEM, n=7-9 mice. **p<0.01; * p< 0.05 with a two-tailed Students *t*-test for unpaired data.

The eleventh subtask was to validate (protein levels, subcellular localization in vitro and ex vivo) selected dysregulated proteins in the *Fmr1* knockout mice at two developmental stages. This subtask is being done in the Bagni lab and the progress should be described in her report.

Major task 2 was to determine whether altered cortico-striatal synaptic plasticity and repetitive/ perseverative behaviors displayed by FXS model mice are reversed by novel cap-dependent translation inhibitors. There are three subtasks: 1) to determine whether inhibiting eIF4E and eIF4A can reverse altered cortico-striatal synaptic plasticity in FXS model mice (Klann lab), 2) to determine whether inhibitors of eIF4E and eIF4A have a (positive) effect on the cortico-striatal PSD-95 interactome protein composition, modification, and localization (Bagni lab) and 3) to determine whether ICV infusion of inhibitors of eIF4E and eIF4A can reverse repetitive and perseverative behaviors exhibited by FXS model mice (Bagni lab).

As mentioned above, we have found that 4EGI-1 reduces 1) the enhanced mGluR-LTD in *Fmr1* knockout mice (Figure 2), 2) increased protein synthesis in dorsal striatum of *Fmr1* knockout mice (Figure 4), and 3) increased protein synthesis in D1R-containing medium spiny neurons in *Fmr1* knockout mice (Figure 5). We will continue working on subtasks 1 and 3 in the final year and foresee no problems in completing the experiments.

In addition to these accomplishments, the Klann and Bagni labs completed a study focused on the role altered eIF4E signaling in the hippocampus of FXS model mice. These studies were the basis for this application. To summarize, we found treating FXS model mice with 4EGI-1 reversed defects in hippocampus-dependent memory and dendritic spine morphology. We also found that 4EGI-1 normalized enhanced hippocampal mGluR-LTD and upregulated Rac1–p21-activated kinase (PAK)–

cofilin signaling, altered actin dynamics, and dysregulated CYFIP1/eIF4E and CYFIP1/Rac1 interactions in FXS mice. Our findings are consistent with the idea that an imbalance of protein synthesis and actin dynamics contributes to pathophysiology in FXS mice. These findings will be published in *Science Signaling* on November 7, 2017 (Santini et al. (2017) *Sci. Signal.* 10: eaan0665). Funding from the CDMRP and DoD was acknowledged in this publication.

Summary of accomplishments

- Demonstration that FXS model mice display enhanced LTD at cortico-striatal synapses
- Demonstration that enhanced cortico-striatal LTD in FXS model can be normalized by inhibiting eIF4E-eIF4G interactions with 4EGI-1
- Demonstration that *Fmr1* knockout/D2R-eGFP mice have enhanced LTD
- Preliminary data demonstrating that *Fmr1* knockout/D1R-tomato mice have enhanced LTD
- Demonstration that *Fmr1* knockout mice have increased protein synthesis in the dorsal lateral striatum
- Demonstration that *Fmr1* knockout mice have increased protein synthesis in D1R- and D2R-containing medium spiny neurons, and that the increased protein synthesis is more robust in the D1R-containing neurons
- Demonstration that *Fmr1/Drd1-Cre* mice exhibit repetitive behaviors and increase locomotor activity. In contrast, Fmr1/Drd2-Cre mice exhibit impaired locomotor activity.
- Publication in *Science Signaling* demonstrating that 4EGI-1 can reverse a number of hippocampal phenotypes displayed by FXS model mice.

Opportunities for training and professional development

Nothing to report.

Dissemination of results to communities of interest

Nothing to report.

Plan on what to do during next reporting period to accomplish the goals

We will continue to conduct the experiments as outlined in the statement of work. We have made good progress toward accomplishing our goals in the first year of work and foresee no problems as we continue on in year 3.

Impact

Our overall hypothesis is that repetitive and perseverative behaviors exhibited by FXS patients that are recapitulated in FXS model mice are caused by exaggerated eIF4E-mediated translation at cortico-striatal synapses. In the short-term, if our hypothesis is correct, the results would directly demonstrate that excessive cap-dependent translation, via increased eIF4E-eIF4G interactions, triggers changes in synaptic composition and function, as well as abnormal behaviors consistent with humans with FXS. The long-term impact of these studies is that they should provide a mechanistic link between FXS patients and other ASD patients that carry mutations for example in the genes that encode eIF4E, tuberous sclerosis complex 1 and 2 (TSC1 and 2) and phosphatase and tensin homolog (PTEN). Moreover, the results of these studies should also provide valuable information for the design and use of compounds to therapeutically target the translational machinery for treating patients with FXS. This

research is timely because, unfortunately, clinical trials based with metabotropic glutamate receptor 5 (mGluR5) antagonists aimed at ameliorating increased mGluR5-dependent protein synthesis in FXS, have not been successful. Thus, there is an urgent need to identify other molecular pathways that underlie ID and ASD in FXS that can be targeted by novel compounds. Finally, there is great interest in the pharmaceutical industry in developing drugs that target translational control proteins for treating various types of cancers. It is meaningful to highlight that the FMRP/CYFIP1/eIF4E pathway is directly related to the metastatic processes and that FXS patients have a decreased risk of cancer incidence. Overall, the results of our studies should provide a foundation for the evaluation of new drugs that target translational control proteins for treating FXS as they become available.

Changes/Problems

We foresee no changes in approach in year 3. We do not anticipate either problems or delays. There will be no changes that impact expenditures.

Products

There have been no publications from the Klann lab directly based on this work thus far. However, a paper from the Klann and Bagni labs will be published in *Science Signaling* on November 7, 2017 in which we demonstrated that 4EGI-1 can reverse a number of hippocampal phenotypes displayed by FXS model mice (Santini et al. (2017) *Sci. Signal.* 10: eaan0665). Funding from the CDMRP has been acknowledged in this publication. The Klann lab currently is preparing a manuscript for submission on the studies described herein.

The preliminary data generated in the first three years of this work has been included in the following presentations during the past three years:

1) Invited speaker, Simons Foundation SFARI Investigators Meeting, New York, NY
2) Invited speaker, Society for Neuroscience meeting, Speaker for Symposium entitled "Dysregulation of Mechanistic Target of Rapamycin Signaling in Mouse Models of Autism", Chicago, IL
3) Seminar speaker, Brain Research Institute, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA
4) Plenary speaker, Postdoctoral Research Symposium, Emory University School of Medicine
5) Invited speaker, Gordon Research Conference entitled "Fragile X and Autism-related Disorders: Unlocking Complex Disorders with Basic Research", Mt. Snow Resort, West Dover, VT
6) Seminar speaker, Neuroscience Graduate Program, Uniformed Services University of the Health Sciences, Bethesda, MD
7) Invited speaker, First International SYNGAP1 Conference, Houston, TX
8) Seminar speaker, Neuroscience Graduate Program, University of Illinois at Urbana-Champaign, Champaign, IL
9) Seminar speaker, George Washington Institute for Neuroscience/Children's National Center for Neuroscience, George Washington University School of Medicine/Children's National Health System, Washington, DC
10) Seminar speaker, Neuroscience Institute, Medical University of South Carolina, Charleston, SC
11) Keynote speaker, Grand Opening of Grand Rapids Research Center, Department of Pediatrics and Human Development, College of Human Medicine, Michigan State University, Grand Rapids, MI
12) Invited speaker, International Fragile X and Neurodevelopmental Disorders Workshop, Saint-Alexis-des-Monts, Quebec, Canada
13) Invited Speaker, Winter Conference on Brain Research, Symposium entitled "Treatments for Intellectual Disability and Autism Targeting Protein Synthesis: The Prospects for Individualized Treatments", Whistler, British Columbia, Canada
14) Seminar speaker, Academic Health Center Duluth Seminar Series, University of Minnesota Medical School and College of Pharmacy, Duluth, MN

15) Invited speaker, Gordon Research Conference entitled "Fragile X and Autism-related Disorders: Convergence and Divergence Between Fragile X and Autism Spectrum Disorders", Lucca (Barga), Italy
16) Invited speaker, National Centre for Biological Sciences, Bangalore, India

Participants & Other Collaborating Organizations

Name: Eric Klann
Project Role: Principal Investigator
Person months worked: 2 cal mos
Contribution to Project: Design and supervise experiments, interpret data.

Name: Francesco Longo
Project Role: Postdoctoral Fellow
Person months worked: 12 cal mos
Contribution to Project: Design and perform experiments, analyze data.

Name: Anna Vorobyeva
Project Role: Postdoctoral Fellow
Person months worked: 12 cal mos
Contribution to Project: Design and perform experiments, analyze data

Name: Claudia Bagni
Project Role: Collaborating Principal Investigator
Dr. Bagni will submit an independent report with the requested information.

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