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TITLE: Assessment of Glutamatergic Neurosystem in Fragile X Syndrome for Targeted Therapy

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13. SUPPLEMENTARY NOTES

14. ABSTRACT The purpose of the proposed research is to examine the role of mGluR5 expression in the brain in relation to behavioral symptoms including anxiety, learning, memory and locomotor activity in adults with Fragile X syndrome and genetically-modified mice (FMR1 Knock Out) towards developing an improved neurobiological model of the disorder. To this end, the study will also evaluate the outcomes of therapeutic drugs in FMR1 Knock Out mice targeting mGluR5 to inhibit or enhance glutamate induced signaling. DTI and MEG will be used examine disruptions in structural and functional brain connectivity. Preliminary findings show no group differences of mGluR5 expression or in learning, memory, or general motor performance behaviors in the mice as a function of gender or diagnostic group, paving the way for examining modulations in mGluR5 expression and associated behavioral changes with gender or progression of disease. For human studies we have set-up working protocols for neuroimaging, acclimation, and clinical testing, and completed data collection (PET, MRI, DTI and MEG) with two control subjects. Three patients with Fragile X have been also been scheduled. In the human data, uptake of [^{18}F]FPEB shows regional correspondences with those seen in mouse data and is consistent with the existing literature.

15. SUBJECT TERMS
FXS, mGluR5, FMR1, PET, MRI, DTI, MEG, humans, moue models

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In the face of recent failures of mGluR-based clinical trials in FXS, which may be potentially related to dosing and drug tolerance issues, this project persists in its search for a better understanding of the mGluR mechanism in the hope of positive treatment outcomes for this condition. Specifically, the proposed multimodal approach (PET (positron emission tomography), MRI/DTI (magnetic resonance imaging/diffusion tensor imaging), MEG (magnetoencephalography)) incorporating both human patients and mouse models with FXS, to identify structural and functional networks of impacted brain regions, along with longitudinal tracking of the progression of the disorder as well as response to the drugs targeting the MGluR5 system presents a powerful opportunity to drill deeper into the underlying mechanism for a fuller understanding of Fragile X syndrome.
The proposal aims to (a) find correlates between regional mGluR5 expression in the brain and different behavioral measures including anxiety, learning, memory and locomotor activity using genetically-modified mice (FMR1 Knock Out); (b) use PET imaging to identify affected brain areas in adults between age of 18 and 50 years with Fragile X syndrome, and correlates in genetically-modified mice; (c) use DTI and MEG to examine disruptions in structural and functional connections within the network of impaired brain areas; (d) evaluate the outcomes of therapeutic drugs in FMR1 Knock Out mice targeting mGluR5 to inhibit or enhance glutamate induced signaling to balance neurotransmission. The results of the proposed work hold tremendous promise for the identification of neuroimaging biomarkers for the design and evaluation of treatments, and, at a more basic level, a deeper understanding of the impaired glutamatergic signaling system. Finding translatable similarities between glutamatergic neurotransmission in FXS mice and human Fragile X Syndrome will help guide future clinical trials toward more successful outcomes and effective drugs for treatments.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

FXS, mGluR5, FMR1, PET, MRI, DTI, MEG, mouse model, humans

3. ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major tasks for Aim 1 a:
1. Local IACUC Approval: Estimated date 8/1/2017; Obtained 7/28/2017
2. ACURO Approval: Estimated timeline of 2 months; Obtained in 3+ months (10/6/2017) creating 3+ months delay in starting the studies.
3. Behavioral studies in FMR1 and control mice were planned to start according the SOW in 3 months' time line but they started 3+ month later (1/29/-2/2/2018) because of the delay in the ACURO approval. We could not order the mice before all the permits were approved. Also the Jax laboratory could not provide 10 fragile X mice from the same litter so we had to divide the order into two shipments; we had six mice in the first batch and eight mice in the second batch. The control mice were also matched in age and group size to the FXS mice. This resulted in the group size being increased by four mice relative to the initial target of 10 mice per group. However, in the course of the studies we lost a few mice and anticipate having 10 mice in each group (male FXS and control and female FXS and control), as proposed, for the final studies.
4. PET imaging studies for each shipment were conducted according to the estimated plan in the SOW, starting on February 6, 2018.
5. Repeated behavioral and PET imaging studies have been conducted in all eight groups but due to the initial start delay of 3+ months to get the ACURO approval, the age dependent studies have been delayed by the same duration; hence, the last behavioral and PET imaging studies will be done in June and the immunohistochemical studies will be done in July 2018 whereas the time frame for them in the original SOW was March 2018.

Major tasks for Aim 1 b:
1. Estimated time for the local IRB approval was one month (8/1/2017); It was obtained 11/2/2018 creating a 4+ month delay in submitting the protocol to HRPO.
2. HRPO Approval: Estimated time line was 2 months; Obtained 2/13/2018. An overall 7.5 month delay in starting the recruitment and evaluation of subjects for the studies.
3. PET, MRI, DTI, MEG studies + behavioral testing: Estimated time line to start the studies was 3 months. Due to the delays in obtaining the necessary approvals to start the studies, we are now in this phase. Two control subjects have completed PET, MRI, and DTI studies. Three patients have been interviewed and are scheduled for participation. The study is in good standing despite the initial approval-related delays.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Accomplishments:

1. The biggest challenge and most important accomplishment in this first year has been in obtaining the required approvals for the proposed studies in animals and especially with human involving the use of vulnerable subjects viz. patients with Fragile X syndrome.

2. Concerning Specific Aim 1a, we have conducted preliminary behavioral and PET imaging studies in 14 male and 14 female FMR1 mice as well as 14 male and 14 female base mice.

3. Behavioral imaging studies included studies of learning and performance using Morris water maze, learning and locomotor activity using rotarod, anxiety using elevated plus maze and aggression and social dominance using tube dominance test and open field test to investigate movement.
In the studies of learning and memory we did not find significant group differences in the mice as a function of age or diagnostic status (control vs FXS) in the early development, 4-6 weeks of age. However, the learning curve started to show reversal in male FXS mice at age 20 weeks. No significant changes were observed in general motor performance but male FXS mice were significantly more active in open field test than male control or female FXS mice. This activity however leveled off by the age of 20 weeks. The results at baseline testing in the behavioral studies were similar in the male and female groups providing an excellent plateau to investigate possible modulation induced by progression of the disease and differences between the sexes. Appendix 1 shows a summary of the results in the baseline studies conducted at the age of 4-6 weeks.

4. PET imaging studies of expression of metabotropic glutamate 5 receptor in the brain using [^{18}]FPEB as the imaging ligand showed some variability between the different mice groups (male/female; FXS/base) even at baseline testing conducted at 6-7 weeks of age. The accumulation of [^{18}F]FPEB was highest in female base mice followed by male FXS mice, female FXS mice and male base mice. However the variability in the binding values within each group was too large to yield significant differences between the groups which is important since we do not expect such differences in early years before development of symptoms. Appendix 2 shows summary results.

5. Specific aim 1b: This part of the project has suffered a significant delay as noted earlier, due to the human subjects' approval process for vulnerable populations. Regardless, we have actively engaged with various Fragile X organizations, locally and nationally, as part of our recruitment and education activities. We are working with developmental disability clinics on the east coast to identify qualified subjects from their database. We have been collaborating closely with patient families to identify strategies and session timings to optimize procedural compliance during imaging and ensure optimal data quality.

6. We have set-up working protocols for neuroimaging, acclimation, and clinical testing, and completed data collection (PET, MRI, DTI and MEG) with two control subjects (Appendix 3). Three patients with Fragile X have been enrolled in the study and scheduled for testing and imaging.

7. The simultaneous PET-MR acquisition allows for clear co-registration between the two modalities, and fitting of time-activity curves to different brain regions. The PET data from our sample subject reveal distinct brain regions of the [^{18}F]FPEB tracer uptake, selective to mGluR5 expression in these areas relative to that in the cerebellum (reference region of low uptake). The areas consist of the insula, putamen, caudate, thalamus, and temporal lobe, which dovetail well with areas implicated in the neurobiology of FXS (see Fig 1 in Appendix 3)

8. Structural MRI analysis: We examined cortical thickness and subcortical/cerebellar volumes in a sample control subject; these measures in patient data will help put our results in the context of existing literature reporting regional structural differences in individuals with FXS. We focused on thickness differences in superior temporal, superior frontal, lateral occipital, pars opercularis, caudal ACC and insula areas (see Appendix 3, Table 1) and volume differences in caudate, palladium, amygdala and thalamus (see Appendix 3, Table 2) in keeping with the FXS literature.

9. Diffusion Weighted Imaging (DWI): White matter abnormalities that have been reported in humans with FXS in frontostriatal pathways as well as in parietal sensory-motor tracts. To this end, we examined white matter integrity, specifically fractional anisotropy (FA) and mean diffusivity (MD) in select tracts implicated in FXS: superior longitudinal fasciculus (SLF), inferior longitudinal fasciculus (ILF) and uncinated fasciculus (UNC) (see Appendix 3, Table 3).

10. Resting State Magnetoencephalography (rs-MEG) data was analyzed for functional connectivity using Amplitude Envelope Correlations (AEC) method. Given the sensory issues in FXS, we focused on connectivity between the nodes (M1, S1, SMA, SP) within the sensorimotor network (see Appendix 3, Table 4) and the functional connectivity of these nodes to other brain regions implicated in FXS towards developing a more complete neurobiological model of FXS with improved explanatory power of its symptomatology

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Nothing to Report.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of

these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

A write-up of the study will be appear in the Fraxa Foundation newsletter as part of our education outreach and recruitment efforts for families afflicted with FXS. Social media of the National FXS Organization has also posted announcements about our study which have attracted a fair number of inquiries.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We plan to step up enrollment, especially of patient participants, through the cooperation of local FXS networks and outpatient clinics, in an extended geographic area including RI, ME, NH,VT, NY, CT and NJ. We are working closely with the Radioactive Tracer Production staff at MGH to increase the number of production slots for [^{18}F]FPEB needed for this study to provide participants with more timeslots. We have obtained approval for expanding the age range of our subjects to improve our patient enrollment prospects recruitment, given the rarity of this disorder. We aim to complete data collection with a subset of patients and matched controls to allow for statistical group comparisons of structural and functional brain differences towards identification of biomarkers that may be used to test drugs targeting MGluR5 for controlling glutamate signaling. Longitudinal analysis of mouse data will guide our predictions and analysis of the human data.

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?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Too early to Report

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Too early to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Too early to Report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

1. Obtained local IRB approval for expanded the age range of participants to help meet recruitment goal for patients

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As mentioned in several parts of this report, the delay in obtaining all the necessary approvals directly hindered the start the experiments.
Concerning the preclinical studies the milestones of the specific aim 1a were to be obtained in 13 months from the beginning of the project. We were able to initiate these studies starting only from the approval date 10/6/2017.
Time point 13 months was also to start the studies for the specific aim 2. In order to gain time we will start the studies for aim 2 when the experimental studies for aim 1a are completed and the comparative data analyses is ongoing.
Concerning human studies we aim to increase the frequency to of the studies so that we will have a subject in every other week.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

The only significant impact on expenditures in this first year of the grant year was the delay in hiring of qualified study staff due to delay in obtaining the necessary approvals to start the studies.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

1. Expanded the age range of the participants to 18-50 years as explained above.

2. Families will be given the option to stay with patients during the scan, provided they meet with imaging safety requirements

Significant changes in use or care of vertebrate animals

No changes in the approved protocols.

Significant changes in use of biohazards and/or select agents

No changes.

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**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report at this stage.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report at this stage.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report at this stage.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report at this stage.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Multi-animal simultaneous imaging:
In order to be able to conduct all the planned imaging studies we have developed a technique for simultaneous imaging of up to 4 mice. The imaging ligand is produced at an offsite facility and radioactivity decay limits its time dependent usage. To get the most out of the radioligand we developed a cradle to hold 2 mice side by side and we can also load them above each other enabling simultaneous data acquisition in even 4 mice. The data reconstruction and management is still done individually. This technique is now used by several other investigators when doing imaging studies in mice.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report at this stage.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

Nothing to Report at this stage.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	Anna-Liisa Brownell "no change"
Project Role:	
Research Identifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	
Name:	Maria Mody "no change"
Project Role:	
Research Identifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	
Project Role:	Michael Whalen "no change"
Research Identifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	
Name:	Aijun Zhu
Project Role:	
Research Itendifier:	
Nearest person month worked:	1 month; left1/31/2018
Contribution to the Project:	
Funding Support:	
Name:	Sevda Lule
Project Role:	Technician
Research Idendifier:	
Nearest person month worked:	12
Contribution to the Project:	Performed behavioral studies

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Anna-Liisa Brownell's funding from NIH/Collagen Medical, LLC; Contract HHSN268201400044C; was closed on February 28, 2018.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

9. APPENDICES: attached

Appendix 1. Behavioral Studies in Mouse Models

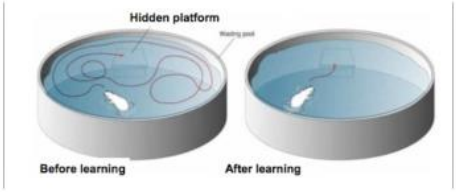
Behavioral Tests

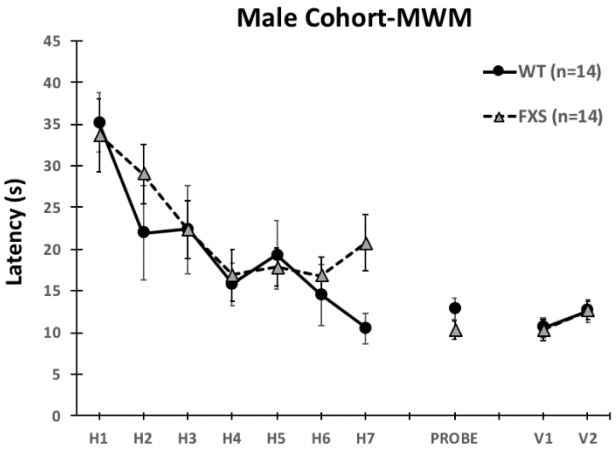
- Morris Water Maze (MWM)
- Rotarod
- Elevated Plus Maze (EPM)
- Open Field Test (OFT)
- Tube Dominance Test

- Group1: Male (n=6+8), 4-6 weeks old.
- Group2: Female (n=6+8), 4-6 weeks old.

Morris Water Maze (MWM)

- It is widely used in behavioral neuroscience to study spatial learning and memory.





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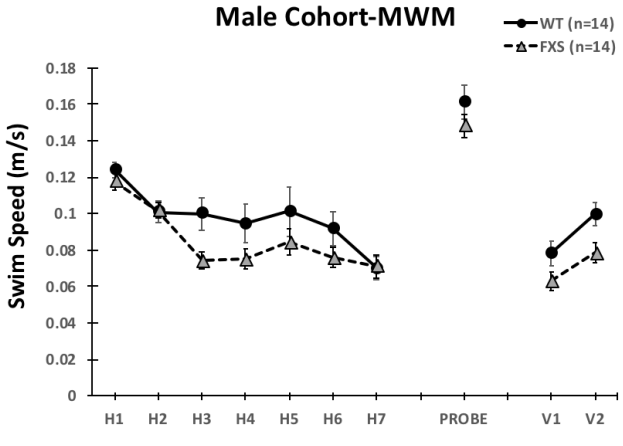

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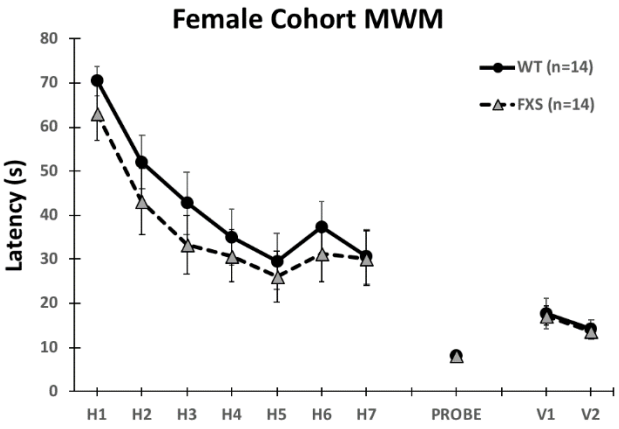

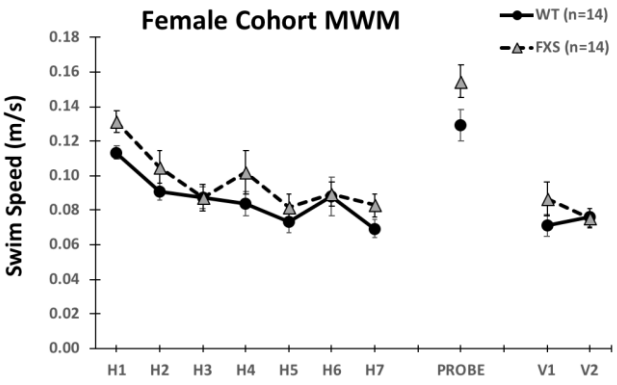

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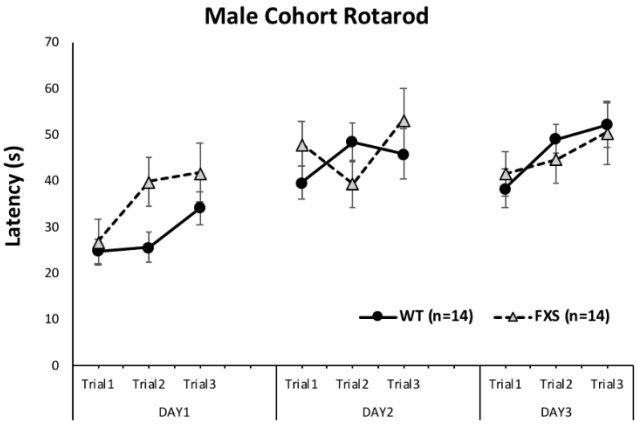
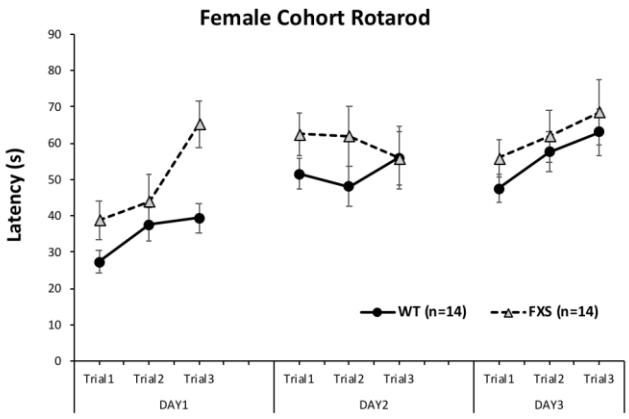
Rotarod

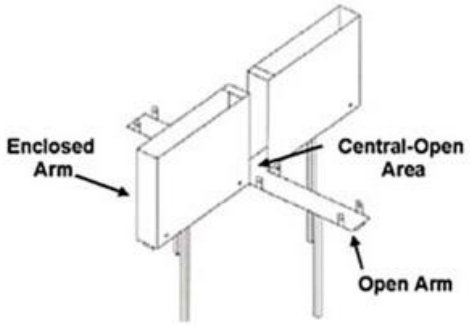
- The test is widely used to generally assess motor performance in rodents. The test measures the rodent's ability to maintain itself on a rod that turns at accelerating speeds.

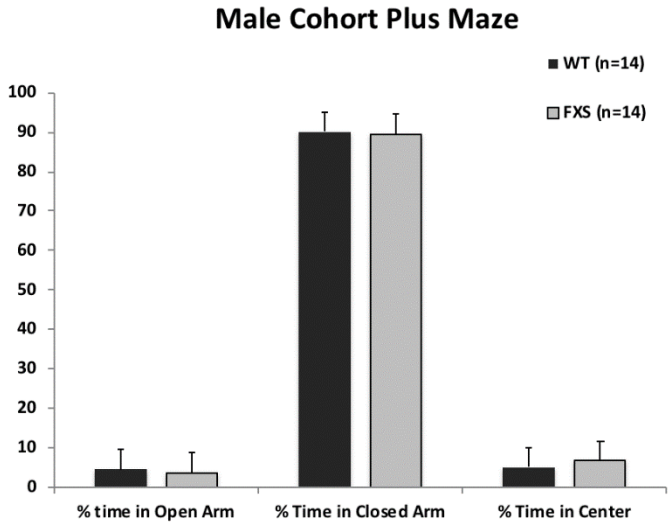


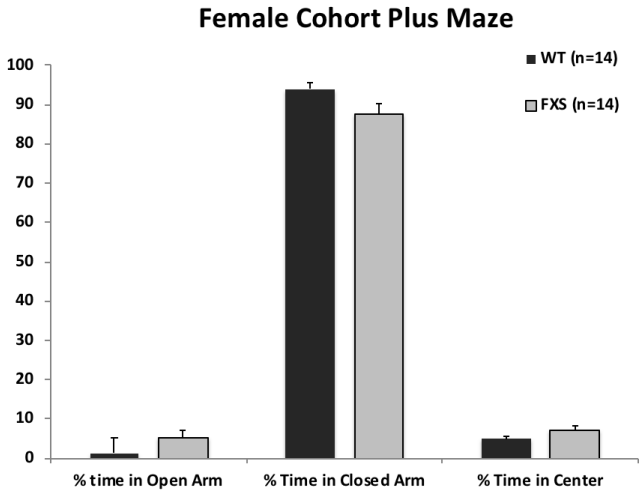


Elevated Plus Maze

- The test measures the anxiety level in rodents.

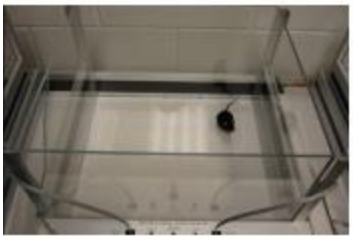


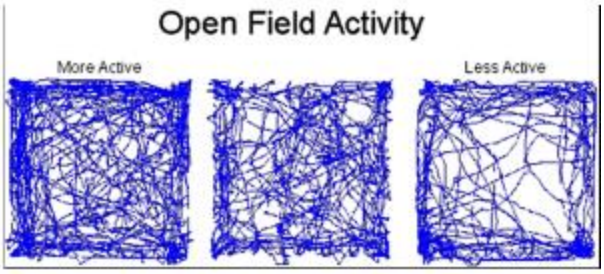


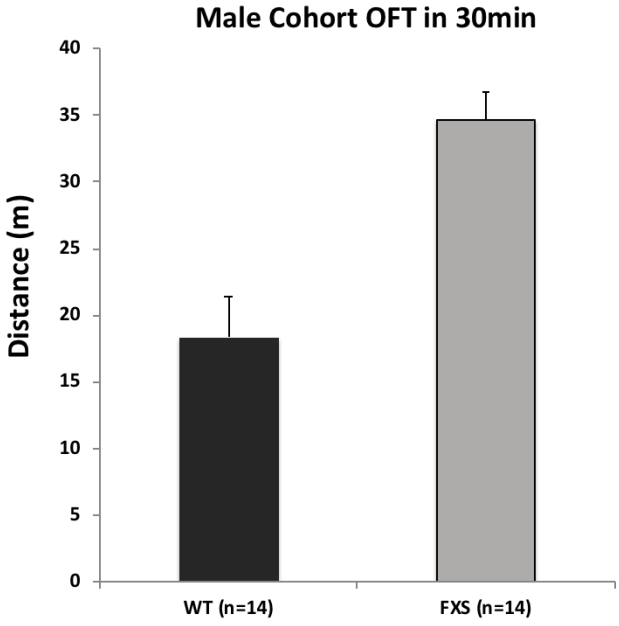


Open Field Test (OFT)

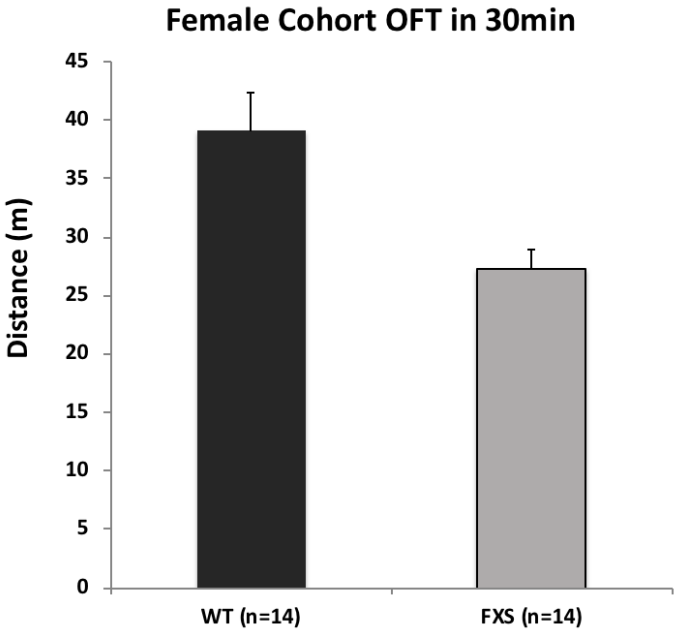
- The test is used to assay general locomotor activity levels and willingness to explore in rodents.







P=0.0002

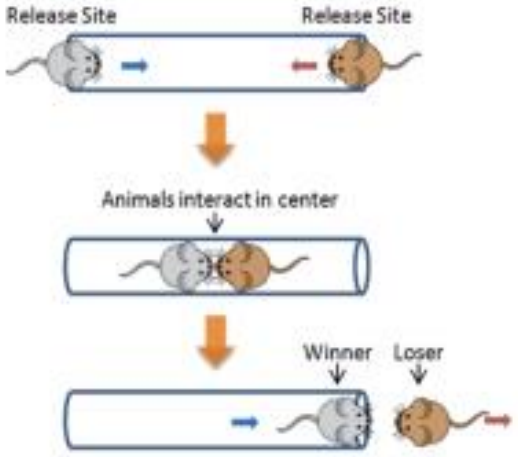


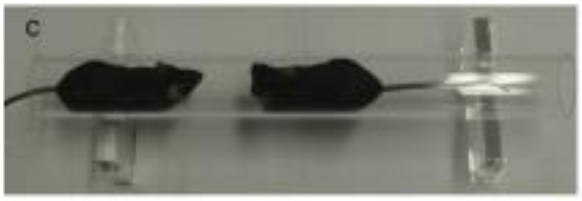
P=0.0031

Tube Dominance Test

- The test assesses cognition in rodents, particularly social dominance through the measurement of aggression.

- In this task, **test** and stimulus mice were released simultaneously into opposite ends of a 30 cm long clear plexiglass tube raised 2.0 cm above the bench surface. The tube diameter (3.2 cm) aimed to ensure that mice could not turn around or move past each other. Typically, one mouse will exert dominance and force the other to back out of the tube, which is thought to reflect a tendency for aggression. The trial concluded when one mouse placed his forepaws out of the tube.

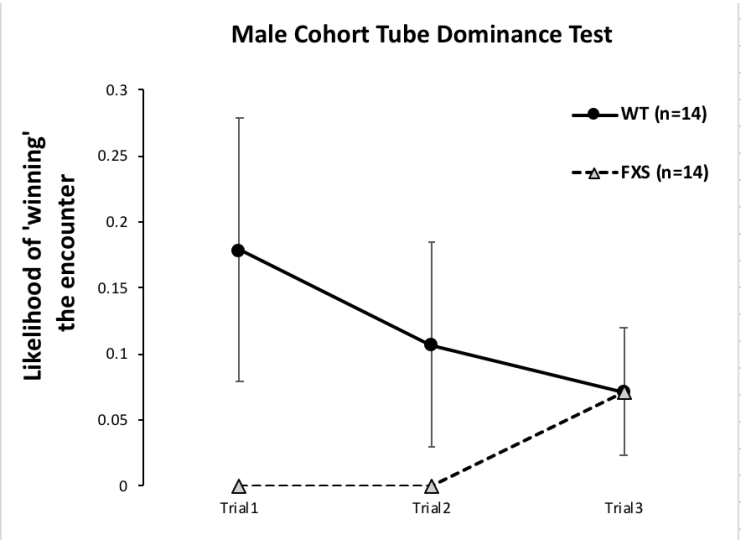




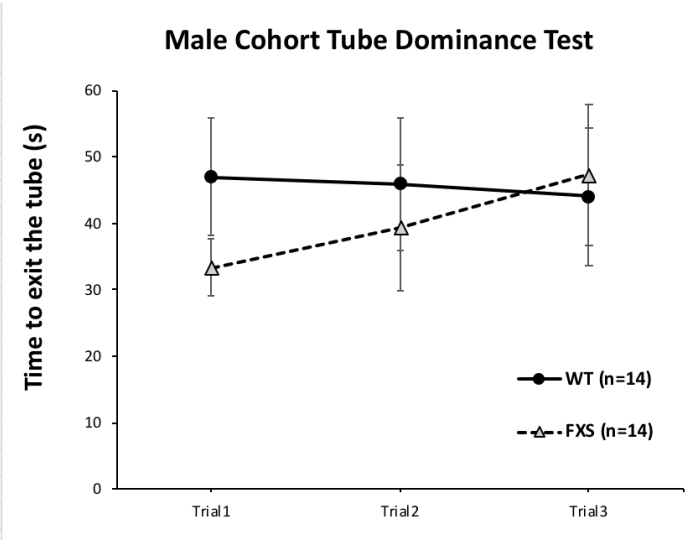
Winner = 1 (control mouse forced out of tube)

Loser = 0 (Test mouse forced out of tube)

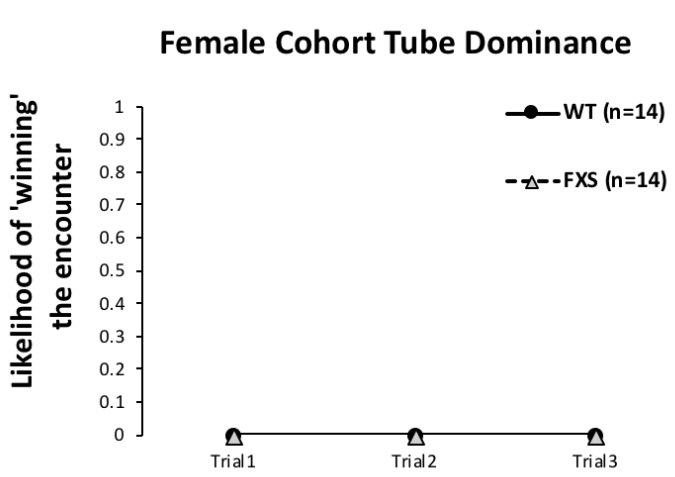
Neutral = 0.5 (Neither mouse forced out of tube after 120s)



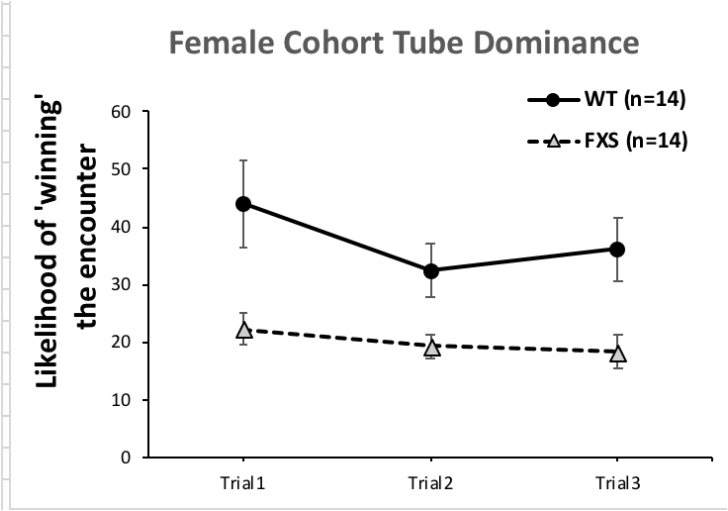
P=0.214



p=0.618


Female Cohort Tube Dominance
Likelihood of 'winning' the encounter
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT (n=14)
FXS (n=14)
Trial 1
Trial 2
Trial 3

P=1


Female Cohort Tube Dominance
Likelihood of 'winning' the encounter
60
50
40
30
20
10
0
WT (n=14)
FXS (n=14)
Trial 1
Trial 2
Trial 3

p=0.002

Appendix 2. Preliminary PET Imaging Studies in Mouse Models

After completion of behavioral studies in each of the eight groups of mice including male and female FXS and control mice we conducted PET imaging studies on mGluR5 expression using 3-[^{18}F] fluoro-5-(2-pyridinylethynyl) benzonitrile [^{18}F]FPEB as the imaging ligand (Wang et al, 2007).

For imaging studies, mice were anesthetized with isoflurane/nitrous oxide/oxygen (1-1.5% isoflurane at 1 L/min flow) and placed on the imaging table. Catheterization of tail vein was done for the administration of the radiolabeled ligands. Once the animal was adjusted to the scanner, a CT imaging was done before administration of the radioactive ligand to obtain high resolution anatomical information and data for attenuation correction. Radiolabeled ligand, (0.2mCi, specific activity of 1900mCi/μmol for [^{18}F]FPEB) was injected into the tail vein. Dynamic volumetric imaging data were acquired for 60min after administration of the ligand. Imaging data were corrected for uniformity, sensitivity, scatter, attenuation, and decay. PET images were reconstructed using maximum likelihood iteration method (MLEM) with 30 iterations. The regions of interest (ROI)s including striatum, frontal, parietal and temporal cortex, thalamus, hippocampus, cerebellum and heart area are drawn on all coronal and axial levels, visualized in the fused CT-PET images (Brownell et al, 2015). Activity per unit volume, percent activity of injected dose and the ligand concentration were calculated.

All the baseline studies have been completed and part of the longitudinal follow up studies. The final changes of mGluR5 modulation in longitudinal studies will be determined after completion of all the imaging studies.

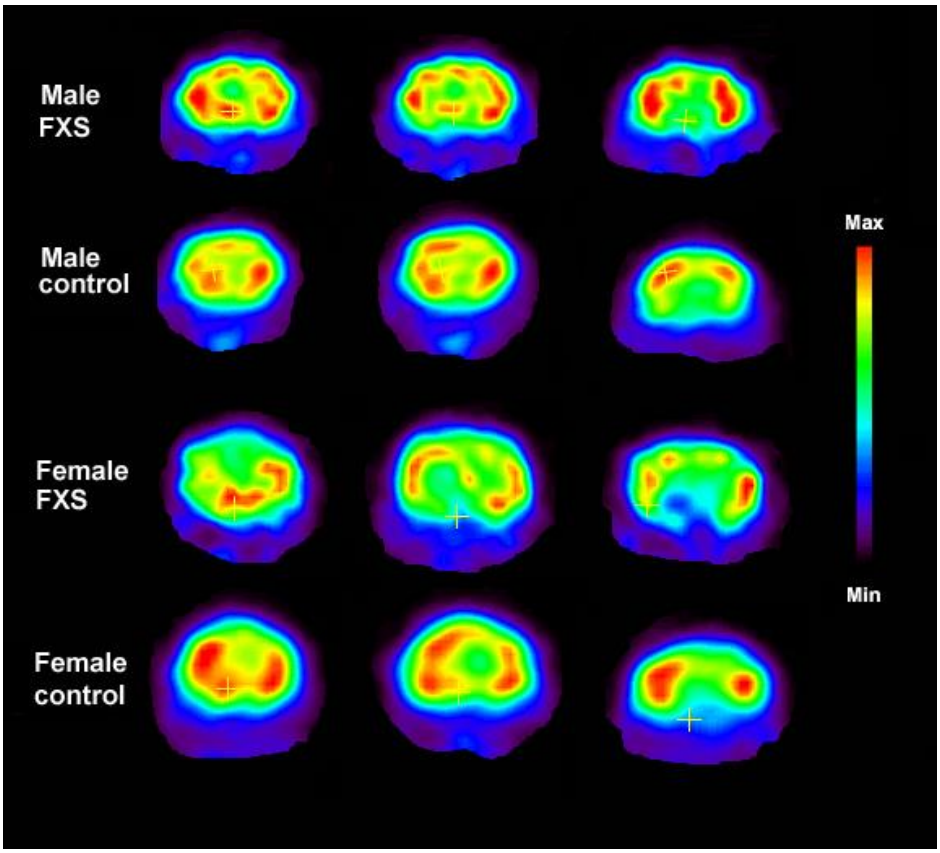


Figure 1. PET imaging studies of mGluR5 expression of 3 coronal levels in male FXS and control mice as well as in female FXS and control mice at the age between 6-7 weeks. These are the baseline studies and every mouse will we longitudinally compared to its own baseline study to obtain individual data that will be grouped for the statistical analyses. Data from the selected brain areas (known to be related to specific behavioral data)

will be compared with the data obtained from the human subjects to investigate modulation of the functional network in brain.

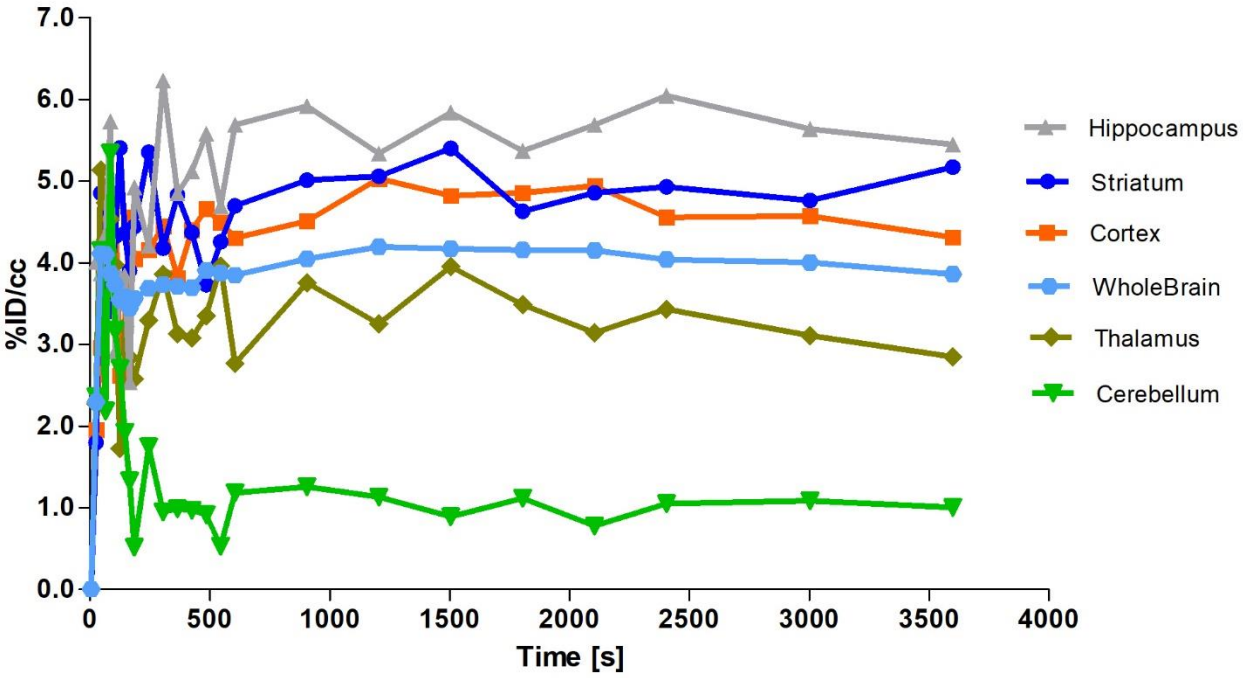


Figure 2. An example of time-activity distribution of [^{18}F]FPEB in different brain areas in a male FXS mouse.

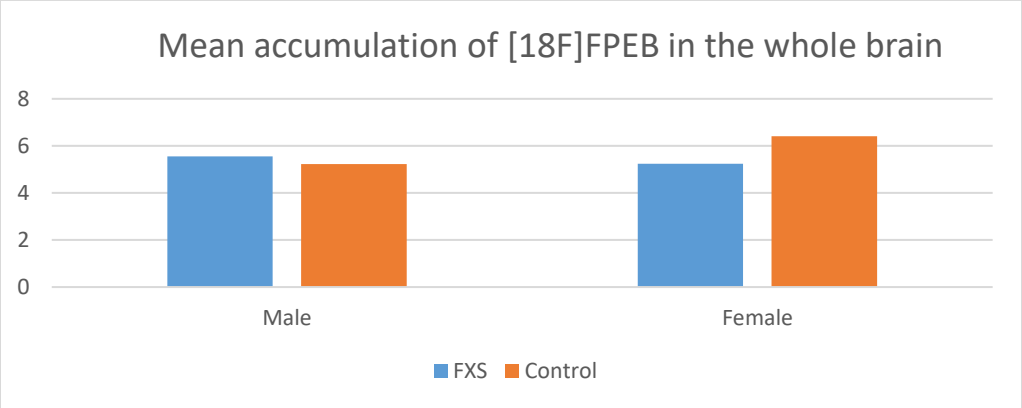


Figure 3. At the age of 6-7 weeks FXS mice are supposed to be non-symptomatic and these preliminary studies show that there is no significant differences on mGluR5 expression between the groups at that early age.

Appendix 3. PET, MRI, DTI and MEG Studies in Human Subjects

1. PET studies.

PET data were acquired simultaneously with MRI data. For PET imaging ([^{18}F]FPEB) was synthesized following published methods to achieve high specific activity (85 GBq/μmol, 2.3 Ci/μmol), using a synthesis protocol approved under RDRC guidelines.
Once the subject was settled in the imaging bay, anatomical MR scout images were acquired. Then, 5 mCi of [^{18}F]FPEB was delivered intravenously and PET acquisition was started and continued for 90min. Emission data were acquired and stored in list-mode format. The head attenuation map was obtained using a recently implemented MR-based attenuation correction method based on the data obtained from a dual-echo ultra-short echo time (DUTE) sequence.
The images were reconstructed using the Ordinary Poisson Ordered Subset Expectation Maximization (OP-OSEM) 3D algorithm from prompt and random coincidences, normalization, attenuation and scatter coincidences sinograms based on 16 subsets and 6 iterations. The reconstructed volume consists of 153 slices with 256×256 pixels (1.25×1.25×1.25mm3).
MPRAGE images were co-registered with the Montreal Neurological Institute Brain Atlas to determine ROIs including caudate, putamen, thalamus, insula, occipital, temporal, frontal and parietal lobe, and cerebellum were drawn. Obtained map was then co-registered to PET data on distribution of [^{18}F]FPEB. Further analyses of the PET data on [^{18}F]FPEB function will be done by calculating the Logan distribution volume ratio (DVR), with pons or cerebellum data as the tissue input function[4] and also by the standardized uptake value (SUV) on which the two groups will be compared.

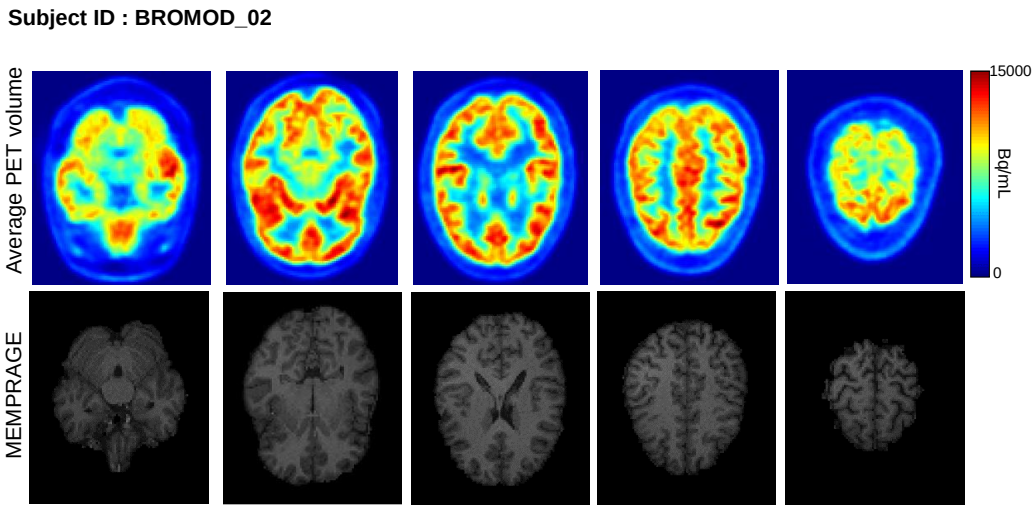


Figure 1. (upper row) Distribution of [^{18}F]FPEB is shown in axial view in a control subject. Imaging data is acquired for 90 min. (lower row) MR images are acquired simultaneously with PET data and they verify the anatomical uptake areas.

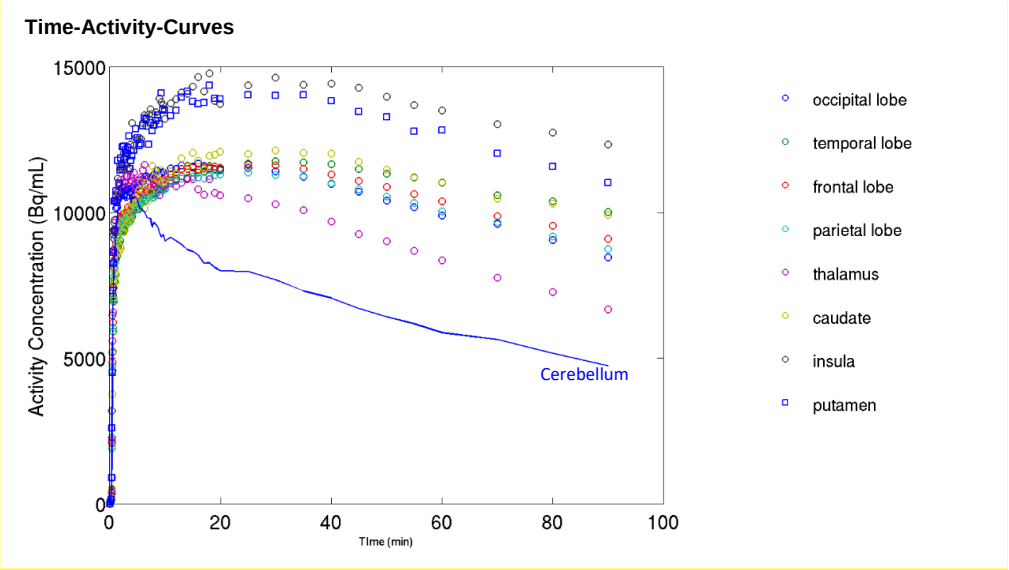


Figure 2. Time activity distribution of [^{18}F]FPEB in a human subject processed from the selected brain areas known to be affected in FXS. The main difference of these curves compared to mouse data is obvious expression of mGluR5 in the cerebellum.

2. MRI Studies

Morphometric analysis was carried out with the FreeSurfer software (http://surfer.nmr.mgh.harvard.edu ; Fischl et al. 1999, 2000, 2004). The procedure included: motion correction, intensity normalization, Talairach registration, skull stripping, segmentation of subcortical white matter, tessellation of the GM/white matter (WM) boundary, automated topology correction, and surface deformation. We used a 10 mm (full-width at half-maximum) Gaussian kernel to smooth maps. FreeSurfer creates a surface 3D model of the cortex using intensity and continuity information and automatically parcellates the cortex into 34 gyral-based regions-of-interest (ROIs) per hemisphere, according to the Desikan-Killiany atlas. For each of the 68 cortical parcellations, FreeSurfer calculates the average cortical thickness, among other measures. We focus on thickness differences in superior temporal, superior frontal, lateral occipital, pars opercularis, caudal ACC and insula areas, in keeping with the existing literature on FXS.

Table 1: Cortical Thickness of select cortical areas implicated in FXS in a control subject

	LH	RH
lateral occipital	2.071	2.150
superior temporal	2.905	2.974
superior frontal	2.799	2.871
pars opercularis	2.639	2.496
caudal ACC	2.796	2.443
Insula	3.121	3.013

Subcortical volumes were calculated with FreeSurfer's automated procedure for volumetric measures. Each voxel in the normalized brain volume was assigned to one of 40 labels, using a probabilistic atlas obtained from

a manually labeled training set [Fischl et al, 2002). The labels we used for further analysis were the caudate nucleus, pallidum, thalamus, and amygdala (left and right hemisphere).

Table 2: Volumes of select subcortical regions implicated in FXS in a control subject

	LH	RH
caudate	4223.6	4270.8
pallidum	2010.4	2073.4
amygdala	1739.5	1578.4
thalamus	8113.0	8662.9

3. DWI (Diffusion Weighted Imaging)

As mentioned above, we performed automated segmentation and cortical parcellation of T1-w data using Freesurfer version 5.3.0. The standard Freesurfer "recon-all" processing stream was used, which provides surfaces and morphometry data for each subject in addition to gray and white matter segmentations. This information was also subsequently used to restrict tractography analysis to white matter. Tensor fitting and tractography were performed within Freesurfer (TRACULA version 1.56),using the ball-and-stick model. Below, we present white matter integrity, specifically fractional anisotropy (FA) and mean diffusivity (MD) in select tracts implicated in FXS: superior longitudinal fasciculus (SLF), inferior longitudinal fasciculus (ILF) and uncinate fasciculus (UNC)

Table 3: Fractional Anisotropy (FA) and Mean Diffusivity (MD) of select white matter tracts implicated in FXS in a control subject

	FA	MD
superior longitudinal fasciculus	.3705	.00005
inferior longitudinal fasciculus	.3670	.00050
uncinate fasciculus	.32352	.00055

4. MEG (Magnetic Encephalography)

Below we present resting state connectivity results in the alpha band for one control participant (right-handed), based on a subset of left hemisphere ROIs in the sensorimotor network (M1_lh, S1_lh, SMA_lh, SP_lh) and three control nodes, one within the sensorimotor network in the opposite hemisphere (SP_rh) and two outside the network (viz., V1_lh and V1_rh).

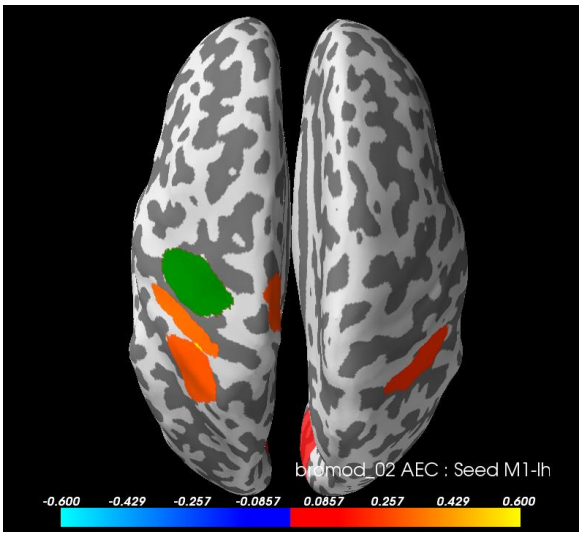
The pre-processed raw data was bandpass filtered with high-pass cutoff of 8 Hz and low-pass cutoff of 13 Hz to obtain alpha band activity. The alpha band was selected given its role in movement-related processing in the brain. Additional analyses will include beta band activity insofar as motor-related processes have been associated with oscillatory power in both the alpha and beta frequency bands over sensorimotor regions in both humans and nonhuman primates (Sanes & Donoghue, 1993; McFarland et al., 2000; deLange et al, 2008), as well as whole brain source connectivity analysis. A segment of continuous data with a length of 4 minutes was used to obtain time series data in source space. The beginning 10 seconds of the data were excluded to avoid artifacts induced by filtering.

For each label, the extracted time course data underwent Hilbert transformation and the amplitude envelope data were taken from the absolute value of the analytic signal from the Hilbert transformation. The amplitude envelope data were then orthogonalized between each pair of the labels in two directions (X orthogonal to Y and Y orthogonal to X)

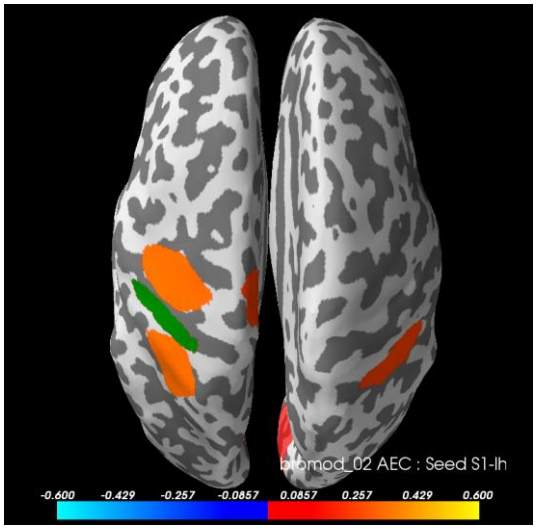
The orthogonalized envelope data was low-pass filtered at 0.5Hz and down sampled at 1Hz before being used to calculate pair-wise correlations. Absolute values of correlation coefficients of the two directions were averaged to obtain the final correlation coefficients (i.e., functional connectivity index) for each pair as presented in table below.

Table 4: Amplitude Envelope Correlations (AEC) between a subset of ROIs within and outside the sensorimotor network in a control subject.

AEC_ave	M1-lh	SMA-lh	S1-lh	SP-lh	SP-rh	V1-lh	V1-rh
M1-lh	1	0.262149609	0.31441373	0.28405551	0.215301985	0.09657023	0.09023876
SMA-lh	0.26214961	1	0.24059574	0.30862471	0.275519738	0.12167293	0.135179
S1-lh	0.31441373	0.240595744	1	0.32807407	0.243870296	0.04445071	0.0796952
SP-lh	0.28405551	0.308624705	0.32807407	1	0.344383208	0.10737838	0.1852459
SP-rh	0.21530198	0.275519738	0.2438703	0.34438321	1	0.10360557	0.14351477
V1-lh	0.09657023	0.121672926	0.04445071	0.10737838	0.103605573	1	0.59900493
V1-rh	0.09023876	0.135179005	0.0796952	0.1852459	0.143514773	0.59900493	1



AEC between M1 (green seed) and select nodes within and outside sensorimotor network



AEC between S1 (green seed) and select nodes within and outside sensorimotor network

References:

Brookes, M.J., Woolrich, M.W., Barnes, G.R., 2012. Measuring functional connectivity in MEG: a multivariate approach insensitive to linear source leakage. NeuroImage 63, 910–920

Brownell AL, Kuruppu D, Kil KE, Jokivarsi K, Poutiainen P, Zhu A, Maxwell M. PET imaging studies show enhanced expression of mGluR5 and inflammatory response during progressive degeneration in ALS mouse model expressing SOD1-G93A gene. J Neuroinflammation. 2015 Nov 24;12:217. doi: 10.1186/s12974-015-0439-9. PMID:2659763

Colclough, G.L., Brookes, M.J., Smith, S.M., Woolrich, M.W., 2015. A symmetric multivariate leakage correction for MEG connectomes. NeuroImage 117, 439–448. http://dx. doi.org/10.1016/j.neuroimage.2015.03.071.

Dale, A.M., Fischl, B., Sereno, M.I., (1999). Cortical Surface-Based Analysis I: Segmentation and Surface Reconstruction, NeuroImage 9(2):179-194

de Lange FP, Jensen O, Bauer M, Toni I. Interactions between posterior gamma and frontal alpha/beta oscillations during imagined actions. Front Hum Neurosci. 2008;2:7. doi: 10.3389/neuro.09.007.2008.

Fischl, B., and Dale, A.M., (2000). Measuring the Thickness of the Human Cerebral Cortex from Magnetic Resonance Images, Proceedings of the National Academy of Sciences, 97:11044-11049.

Fischl, B., A. van der Kouwe, C. Destrieux, E. Halgren, F. Segonne, D. Salat, E. Busa, L. Seidman, J. Goldstein, D. Kennedy, V. Caviness, N. Makris, B. Rosen, and A.M. Dale, (2004). Automatically Parcellating the Human Cerebral Cortex, Cerebral Cortex, 14:11-22.

McFarland DJ, Miner LA, Vaughan TM, Wolpaw JR. Mu and beta rhythm topographies during motor imagery and actual movements. Brain Topogr. 2000;12:177–186. doi: 10.1023/A:1023437823106.

Sanes JN, Donoghue JP. Oscillations in local field potentials of the primate motor cortex during voluntary movement. Proc Natl Acad Sci U S A. 1993;90:4470–4474. doi: 10.1073/pnas.90.10.4470.

Wang J-Q, Tueckmantel W, Zhu A, Pellegrino D, Brownell A-L. Synthesis and preliminary biological evaluation of 3-[^{18}F]Fluoro-5-(2-pyridinylethynyl)benzonitrile ([^{18}F]FPEB) as a PET radiotracer for imaging metabotropic glutamate receptor subtype 5. Synapse 2007;61(12):951-61.