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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 to examine disruptions in structural and functional brain connectivity. The longitudinal follow up studies showed group differences of mGluR5 expression, learning, memory, and general motor performance behaviors in the mice as a function of gender and diagnostic groups, paving a way for examining therapeutic response on mGluR5 expression and associated behavioral changes during 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 nine control and FXS subjects. In the human data, uptake of [^{18}F]FPEB showed regional correspondences with those seen in mouse data providing an outstanding translational aspect to investigate FXS related bio-behavior and develop therapeutic approaches.

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

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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 to 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 investigate 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 58 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 (mouse studies):

1. After the original delay of 3+ months for processing all the necessary documents to start experimental work, reported in the previous progress reports, we have been well within the timelines proposed in the SOW.
2. As estimated in the previous report the proposed follow up studies of behavior and mGluR5 expression were supposed to be completed by end of July at the age of 8 months. When we analyzed the data at that point we realized that it would be beneficial for the project to extend the follow up time to the age of one year.
3. We completed all the behavioral and PET imaging studies in FMR1 Knock Out and control mice by the end of January 2019, giving us 4 time points for behavioral studies and investigating modulation of mGluR5 expression.
4. A manuscript, titled "mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" has been submitted and is under the journal review
5.Immunohistochemical and comparative data analyses are still underway for these studies to compare mGluR5 expression in different brain areas *in vivo* and *ex vivo*. At this point 99% of the experimental work for project 1a is completed.

.**Major tasks for Aim 1: b-d (human studies):**

1. Estimated time for the local IRB approval in the SOW was one month (8/1/2017). However, it was obtained 11/2/2018 creating a 4+ month delay in submitting the protocol to HRPO.
2. HRPO Approval: Estimated timeline in the SOW was 2 months. However, it was obtained 2/13/2018. Overall delay: 7.5 months
3. The delays in the approval process delayed starting recruitment and evaluation of subjects for PET, MRI, DTI, MEG studies and behavioral testing. We have interviewed 73 FXS subjects and screened their suitability and interest on voluntary participation for this project. Presently, sixteen PET, MRI and DTI studies have been completed (8 FXS and 8 control subjects). In addition, we have completed MEG studies in 18 subjects due to better compliance with study procedures (9 FXS and 9 control subjects). Individual as well as group analyses of these data are completed. FXS is a rare disorder: a general prevalence with full mutation is estimated at 1/4000 males and 1/5000-1/8000 females (Nui et al, 2017) and we have been able to find 73 affected subjects and their willingness to participate in studies was about 12% due to a variety of reasons including personal family reasons and difficulties with compliance with the test procedures.
4. A manuscript, titled "*In vivo* Imaging of mGlu5 receptor expression in humans with Fragile X Syndrome towards development of a potential biomarker" has been accepted for publication in the Springer Nature journal "Scientific Reports".

Major tasks for Aim 2 (mouse studies):

1. In the second part of the mouse studies we were able to follow accurately the study timelines proposed in the original SOW to test therapeutic response of mGluR5 agonist and antagonist with saline as a reference.
2. We completed the baseline behavioral and PET imaging studies of mGluR5 expression altogether in 20 FMR1 Knock Out and 20 control mice.
3. The mice were treated for 5 weeks with mGluR5 antagonist, MTEP, mGluR5 agonist CDPPB or saline followed by the behavioral and PET imaging studies which were repeated 3 and 6 months later. Presently extensive data analyses have been completed and manuscript writing is in process. However, analyses of the immunohistochemical studies are still in process as described in the SOW. At this point about 97% of the experimental work is completed.

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

Specific Aim 1a.

We have conducted all the behavioral and PET imaging studies of mGluR5 expression, and the manuscript, titled "mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" has been submitted to Journal of Neuroscience (limited). The final immunohistochemical analyses are underway as well as extensive modeling and analyses of connections between different brain region based on *in vivo* and *ex vivo* mGluR5 expression.

Behavioral imaging studies included studies of learning, performance and memory using the Morris water maze; learning and locomotor activity using rotarod; anxiety using elevated plus maze and aggression; social dominance using tube dominance test and open field test to investigate movement and willingness to explore.

In the Morris Water Maze test the longer average swim latencies and slower swim speed of the FMR1-KO mice compared to WT mice on the hidden and/or visible platform tasks are indicative of their motor and spatial learning skills. These deficits were evident at the older ages but not at in youngest age group. The FMR1-KO and WT mice did not differ in their path lengths, and both groups of mice improved in performance over the course of the learning trials; however, the learning rate was marginally reduced in the FMR1-KO mice compared to the WT mice. Somewhat surprising was the slower swim speed of the FMR1-KO mice in the Visible Platform condition evident in the older ages which may suggest gradually emerging sensorimotor issues in FXS. On the Probe condition, the WT mice showed superior retention of the location of the hidden platform compared to the FMR1-KO mice: male WT mice swam faster compared to the FMR1-KO mice to the target quadrant and spent longer time in it.

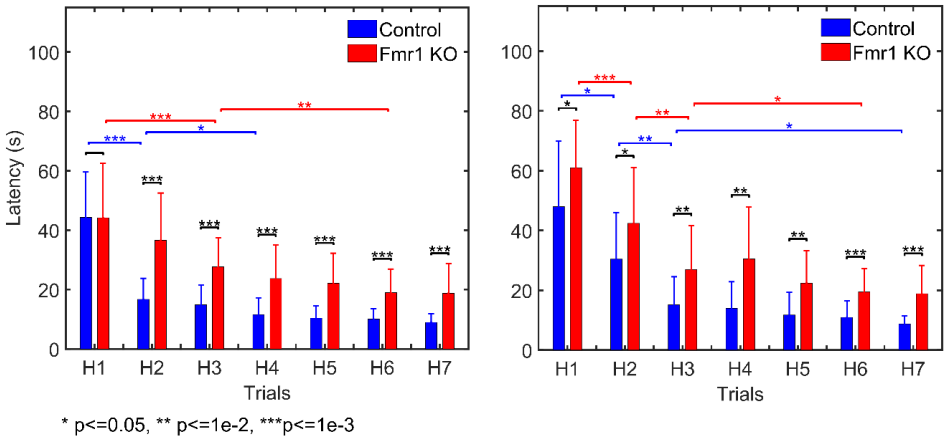


Figure 1. Morris water maze hidden platform latencies of WT and FMR1 KO mice at age groups 2 and 3.

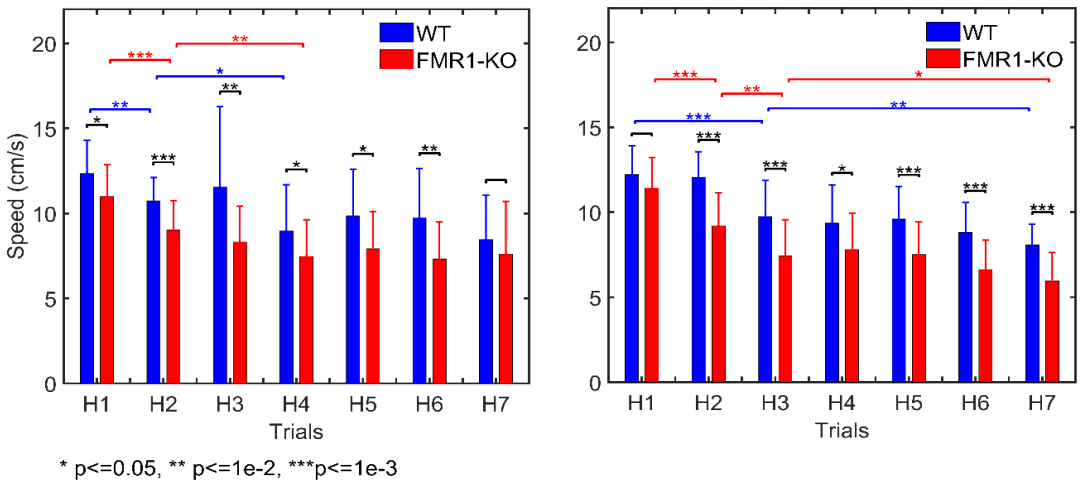


Figure 2. Morris water Maze hidden platform swim speeds for WT and FMR1-KO mice at age groups 2 and 3.

Performance on the Rotarod, Elevated Plus Maze and Open Field Tests showed significant interactions between disorder group and gender on all the tasks, with male FMR1-KO mice doing consistently worse than their female counterparts. On the rotarod task, female FMR1-KO mice held on longer than male FMR1-K mice to the horizontal rod which rotated at increasing speeds, reflecting their superior motor coordination and balance. Clinical features of FXS include motor coordination and human studies show lower scores on motor scales in FXS subjects compared to age-matched peers.

Anxiety is a frequently reported core symptom in FXS. Male FMR1-KO mice were more affected, spending less time than female FMR1-KO mice in the open arm section of the Elevated Plus Maze, a measure of anxiety related behavior. There was no interaction between disease status and gender on the closed arm; however, there was a main effect of disorder: FMR1-KO mice spent less time than WT mice even in the closed arm, regardless of age or gender.

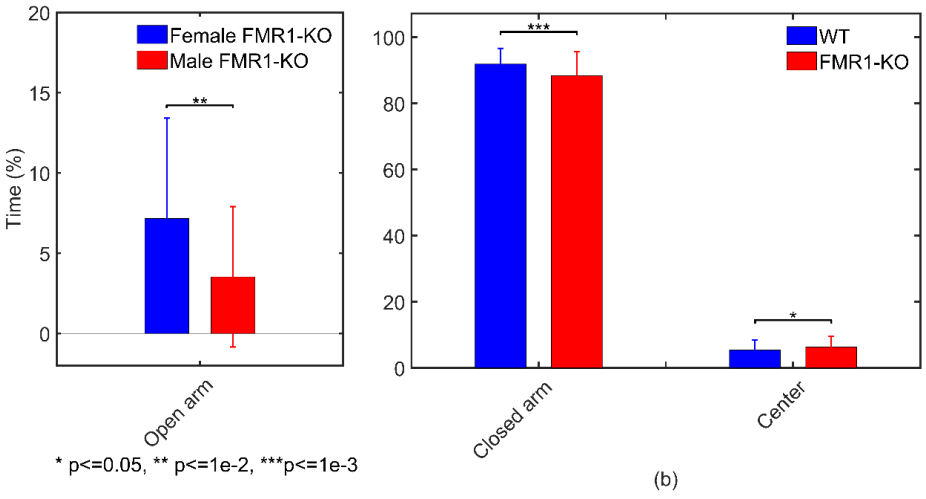
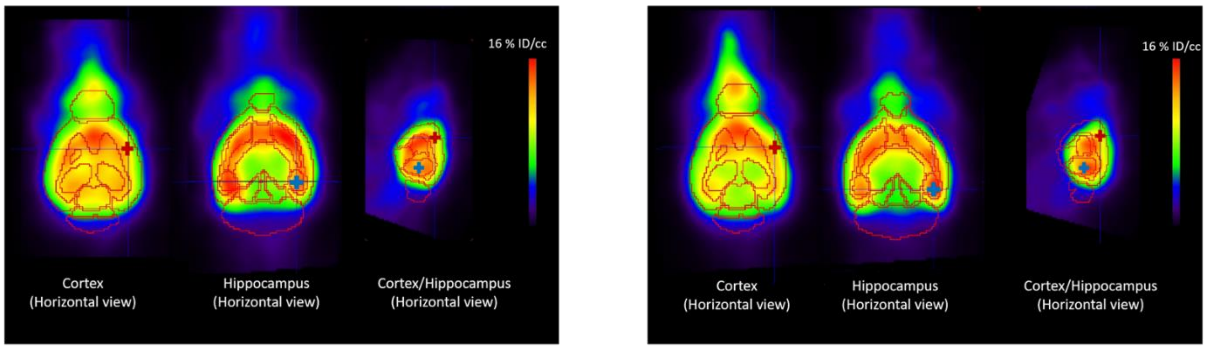


Figure 3. Comparison of the time spent in (a) open arm between female and male FMR1-KO mice, and (b) closed arm between FMR1-KO and WT mice and in center platform between FMR1-KO and WT mice in the youngest age group on the elevated plus maze test.

In the open field test locomotor activity of the mice within a 30 minutes period after being placed in their cage revealed a significant interaction between disorder status and gender (F (1,134) = 23.6, p = 3.3e-6); no other effects were significant. Post-hoc t-tests showed significant differences between FMR1-KO mice and WT mice in both males and females. Whereas male FMR1-KO mice (35.9 m ± 10.8) travelled longer distances than male WT mice (25.4 m ± 20.2) (p = .008), female WT mice (43.8 m ± 15.4) mice travelled longer distances than female FMR1-KO mice (30.3 m ± 7.5) (p = 2.4e-5).

Multivariate analysis of [^{18}F]FPEB binding potential revealed differences between the FMR1-KO and WT mice in multiple brain areas (Fig 5). Below we report significant findings (p<.05), meeting multiple comparison correction criteria.



WT male FMR1-KO male

Figure 4. [^{18}F]FPEB binding potential in a male control mouse and a FMR1-KO male mouse

Binding potentials were significantly higher in WT compared to FMR1-KO mice in cortex (5.013 ± 1.16 vs. 4.508 ± 0.915; p = 3.9 e-6), hippocampus (5.66 ± 1.527 vs. 4.757 ± 1.135; p = 1e-5) and olfactory bulb (4.177 ± 1.327 vs. 3.329 ± .988; p = 1.8e-6) (see Fig. 5) ; no other areas showed significant difference between the two groups The group difference was most evident in cortex and appeared to emerge in age group 2 (F (2,137) = 1.395, p = 0.047; age 2: WT: 6.044 ±1.129; FMR1-KO: 4.592 ± 0.809, p = 0.0002)

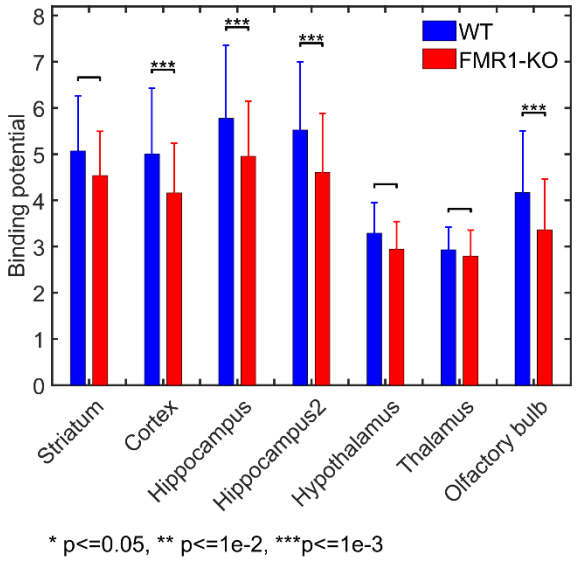


Figure 5. Comparison of [^{18}F]FEB binding potential in WT and FMR1-KO mice in different brain areas.

Accomplishments (cont):
Specific Aim 1b, 1c, 1d: PET, MRI, DTI and MEG Studies in Human Subjects

PET: 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 90 min. Since the patient could not stay still for 90 min the acquisition time was divided into 3 segments of 30 min. To correct for the head motion, reconstruction was done according the following 8 steps. (1) The dynamic reconstruction was done at first without attenuation correction in 30-sec frames; (2) rigid body registration was used to align each reconstructed PET image obtained in step 1 to a selected reference frame. Estimated motion transformations were saved; (3) The acquired attenuation map was registered to the chosen reference frame; (4) The attenuation map obtained in step 3 was transformed using the transformations calculated in step 2. In so doing, we obtained one attenuation map for each frame that is spatially consistent with the measured emission data; (5) A 2nd dynamic reconstruction was done using the frame-dependent attenuation maps calculated in step 4. The scatter correction was also performed using the frame-dependent attenuation map: (6) Each frame was registered (rigid-body registration) to a selected reference frame; (7) The resulting time-averaged image was calculated; (8) Each image was registered to the time-average image obtained in step 7.

Summary of the data reconstruction: steps 1-8 yield a dynamic sequence of motion corrected images for each segment of acquisition. To perform kinetic analyses using data from all 3 segments, all the frames for all segments were registered to a common reference image. Furthermore, the missing time point between the segments were estimated by linear interpolation.

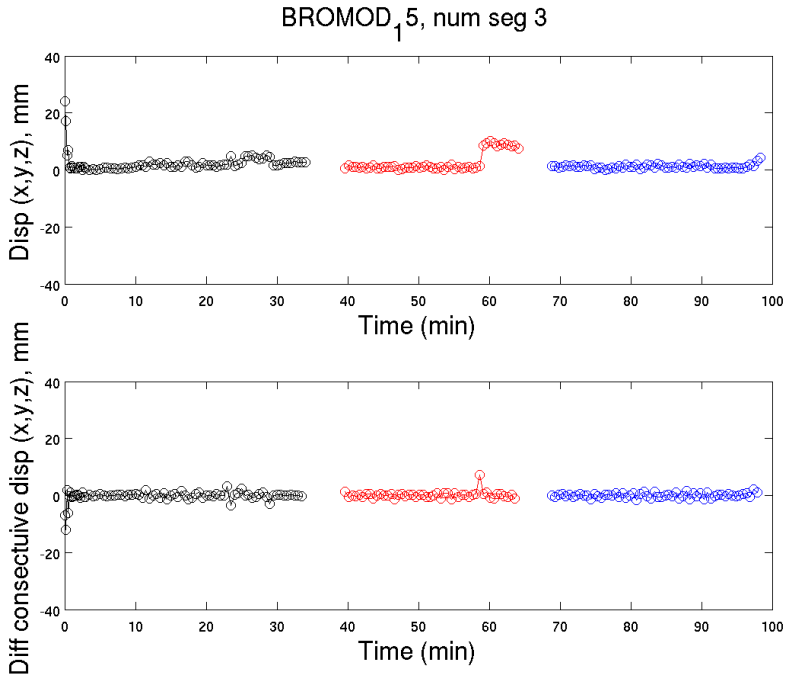


Figure 6. Motion correction as applied to a single subject used for the quantitative analyses.

Accomplishments (cont.)
MRTM2 was then applied to the data to estimate binding potential at the region and voxel level, using the cerebellum white matter as the reference region. To define the ROIs, the masks from the MNI-152 MRI brain atlas were non-rigidly registered to the MPRAGE volume of each subject. The above procedures in the reconstruction will minimize the influence of inter-frame head motion on the kinetic analyses for these studies. However, it does not correct for motion that happens during any of the 30-sec frames.

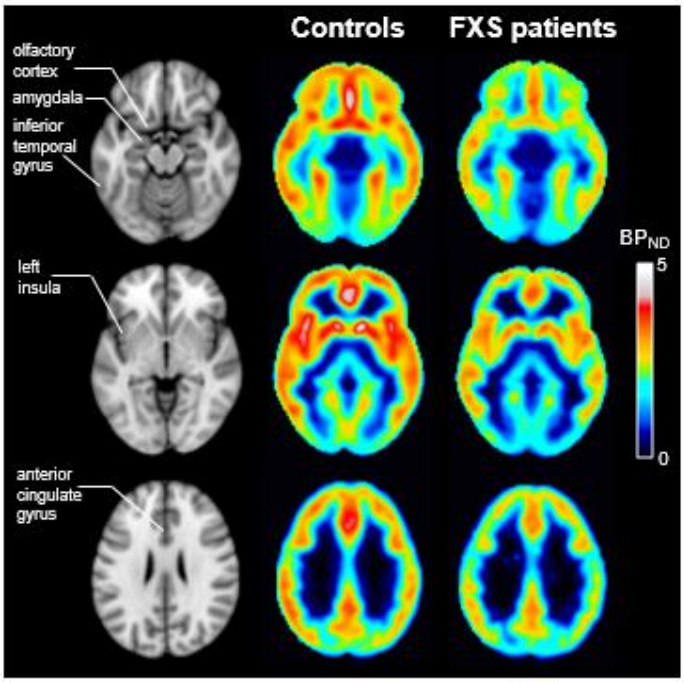


Figure 7. Quantitative distribution of [^{18}F]FPEB accumulation in different brain areas show that the mGluR5-related binding potential is lower in all brain areas in FXS patient compared to the healthy control (HC).

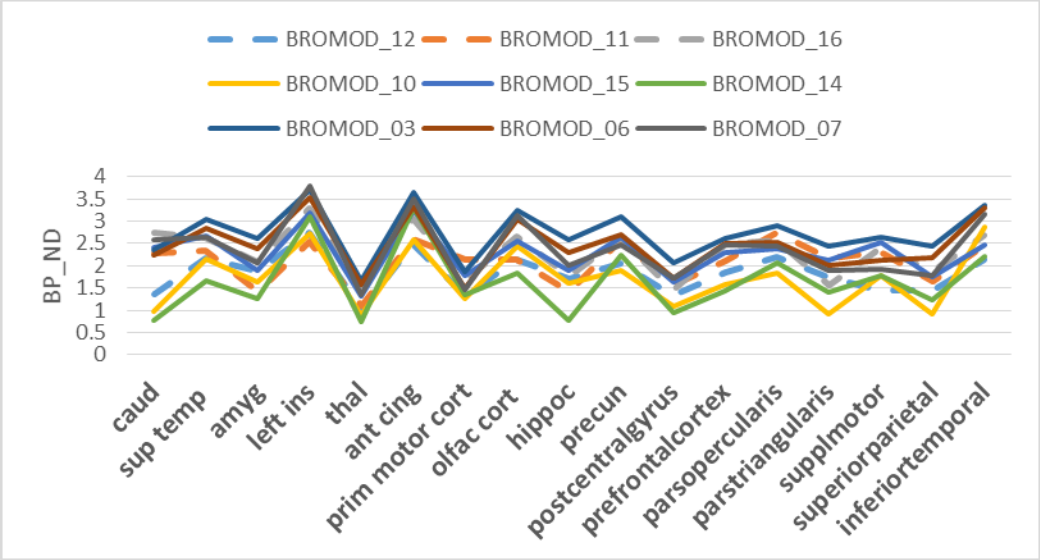


Figure 8. mGluR5-related binding potential in different brain areas.

Accomplishments (cont.)
The FXS patients appear to have lower BP (dashed lines) than healthy controls in all regions. (Fig. 8). There is also large within-group variability in BP. Of the data collected so far, we found a significant group difference ($p<0.05$) in five brain areas: amygdala ($p<0.05$) anterior cingulate gyrus ($p<0.002$), left insula ($p=0.005$), inferior temporal gyrus ($p<0.002$) and olfactory cortex ($p<0.006$) (Fig. 9)

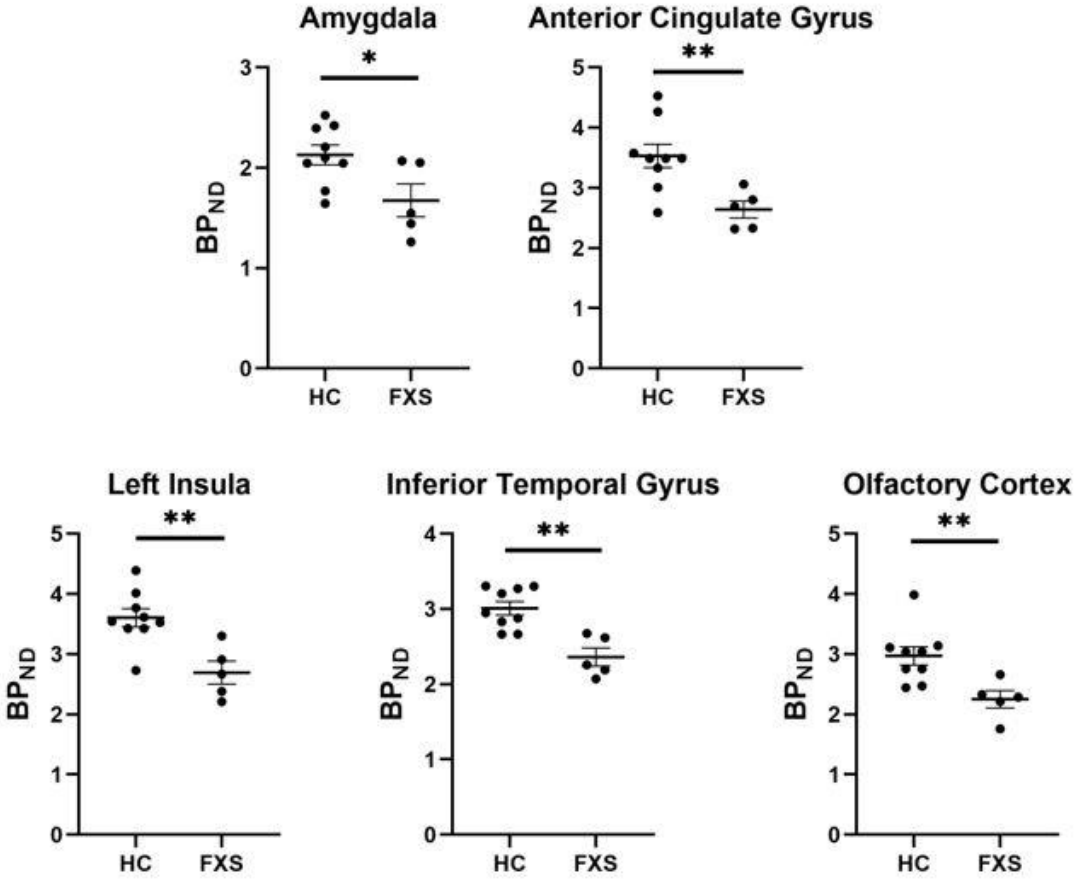


Figure 9. Group differences of the binding potentials in different brain areas in FXS and healthy control (HC) subjects. The results are based on PET imaging of [^{18}F]FPEB binding to mGlu5 receptors.

MRI, DTI and MEG: Disruptions in brain connectivity are considered a hallmark of neuro-developmental disorders like FXS. During this fourth year of the award, we build on our earlier results addressing Specific Aims 1c and 1d: structural and functional connectivity differences between individuals with FXS and age- and gender-matched controls. The findings reveal an apparent convergence of our previously reported differences in brain morphometry (MR) between participants with FXS and matched controls with recent results from white matter tractography (DWI), resting state connectivity (MEG) and mGluR5-related BP (PET-MR), implicating hippocampal and parahippocampal brain areas in the disorder. They lend neurobiological support to a hippocampal-dependent plasticity and learning account of the intellectual disability at the core of
FXS. Below, we report results from MEG (18 participants: nine controls and nine with FXS; one patient had to be excluded due to poor data quality) and DWI (eight FXS and eight controls, the

Accomplishments (cont.)
subset of participants who were able to complete the integrated PET-MRI scan during which diffusion data was collected).

Diffusion Imaging: Data was collected on a 3T integrated PET-MR Siemens scanner (Magnetom Biograph_mMR; 64 gradient directions, b-value: 2000s/mm^2, 66 slices, 2x2x2 mm^3 voxel size, TR=6400ms, TE: 110 ms; phase encoding directions: A>>P, P>>A). Data were analyzed using TRACULA, (Yendiki et al., 2014), an automated global probabilistic tractography tool within the FreeSurfer package, which delineates 18 white matter pathways in the participants' DWI data. For tensor estimation, a ball-and-stick model of diffusion was used to extract fractional anisotropy (FA) and diffusivity measures (radial diffusivity (RD), axial diffusivity (AD) and mean diffusivity (MD). Head motion was quantified in each participant and eddy-current image distortions were removed (Yendiki et al, 2014; Jung, Mody et al, 2019).

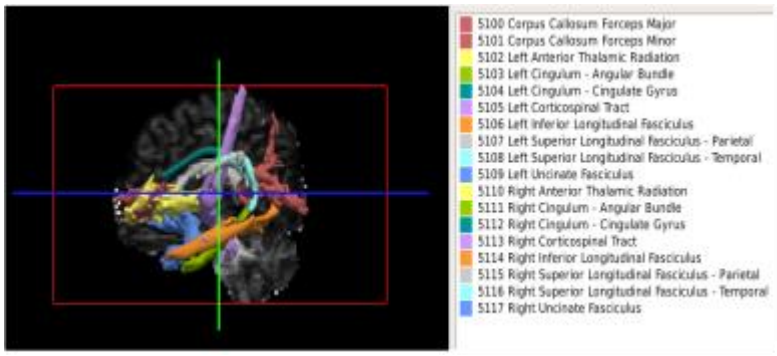


Figure 10: Probability distribution of 18 major white matter pathways generated by TRACULA and displayed in a single control subject

We found significant differences between the groups in (a) FA in left ventral cingulam angular bundle (p=0.05) and right corticospinal tract (p=0.04) (b) MD in right Thalamic Radiation (p=0.04) and (c) AD in Corpus Callosum-right forceps major (p=0.03).
Insofar as the ventral portion of the cingulam angular bundle corresponds to its parahippocampal subdivision, an area associated with spatial memory and navigation which has been implicated in the water maze task (Warburton et al, 1998; Whishaw, 2004), the reduced FA of this tract in the FXS group compared to the control group agrees with findings in our FMR1-KO mice. More specifically, the FMR1-KO mice and human PET participants manifest significantly lower mGluR5-related binding potential in the same regions, assessed with [^{18}F]FPEB during PET imaging.

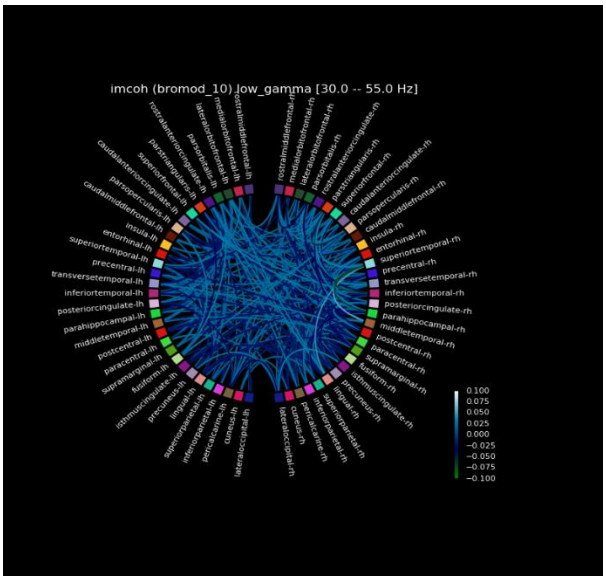
The cingulam bundle has also been implicated in cognitive control and emotions, functions that are known to be impacted in FXS. Brain areas underlying these functions, like the insula and amygdala showed significant group differences (p<.001) in the human PET data (Fig 9). Taken together, these results build on the group difference in cortical thickness in the anterior cingulate (ACC) and parahippocampus that we have reported previously.

Magnetoencephalography (MEG): During this final and fourth year of the award, we focused on data analysis, given the pandemic-related restrictions and concerns with onsite testing. We also looked for changes in MEG activation patterns as part of a longitudinal design, in two of the participants who were able to return a year later for a second session for very preliminary insights into the progression of the disorder.

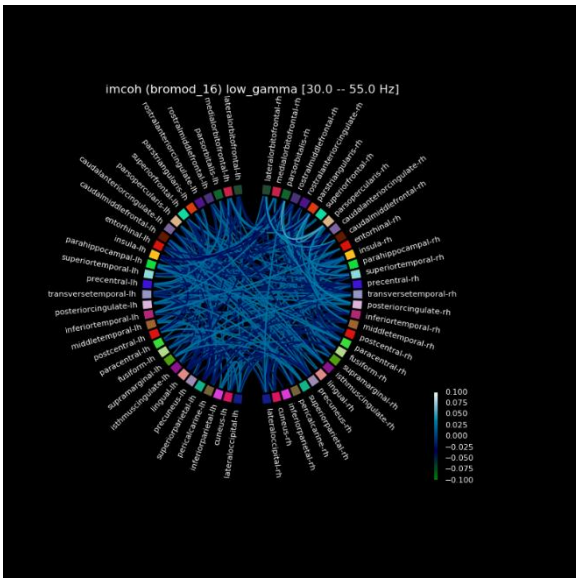
After rigorous curating of the data (8 controls and 10 subjects with FXS) for quality, our resting-state analysis sample (consisting of data from 8 controls and 7 individuals with FXS) were subject to additional functional connectivity analysis to further interrogate our earlier reported findings using imaginary coherence methods (imCOH). Contrary to our earlier findings of stronger gamma band and weaker theta band functional connectivity, specific to the language network in individuals with FXS which was flipped in the age-and gender-matched controls, recent whole brain connectivity analysis suggests a different pattern; viz., a more uniform functional connectivity in the gamma band across both groups but a more contrasting set of correlated and anti-correlated connections in the theta band in the participants with FXS and not in controls. See Figure 11 below for sample subject from each group showing whole brain imCOH connectivity patterns in theta and gamma band.

Low Gamma band (30-55Hz)

Control



FXS



Theta band (4-8Hz)

Control FXS

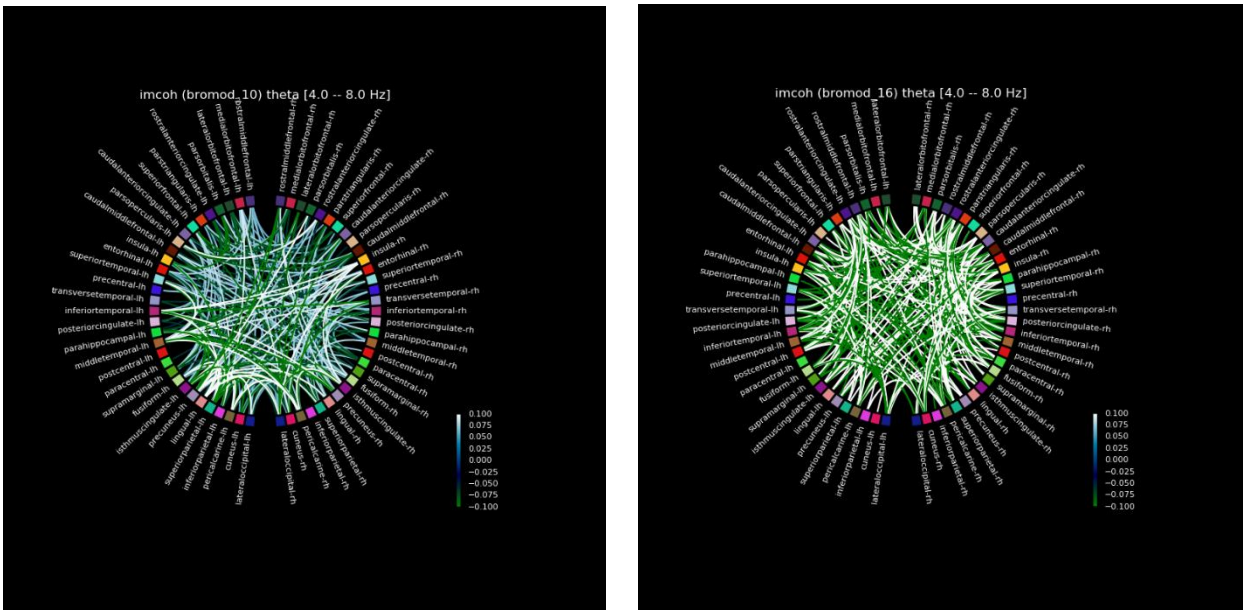


Figure 11. Whole brain functional connectivity in theta and gamma bands in a subject with FXS vs an age- and gender-matched Control

Previous analysis of the role of the motor system in speech and language deficits in FXS pointed to the importance of the pars opercularis as a hub in resting state results (Progress Report, 2020). Additional analysis of this data also points to the supplementary motor area (SMA) in gamma band and its connection to the pars opercularis (imCOH values : Controls: 0.005, FXS:0.016, p=0.029). Taken together, these results help to narrow down potential intervention targets to the pars opercularis and the SMA connectivity in the theta band.

Longitudinal analysis of the source activity in the motor areas (M1, S1, SMA, SP-left and SP-right) in sample Control subject and FXS subject shows a decrease in amplitude across all time windows (tw) relative to the button press response (tw0: -40 to 0 ms; tw1: 20-60 ms; tw2: 60-100 ms) in all 5 motor ROIs except the SMA over the one year between consecutive MEG recording session in the subject with FXS. This change, however, is less consistent in Controls. A complete longitudinal study, with a large number of subjects, would be necessary to see if this pattern holds up with progression of the disease.

Table 1. Change in MNE amplitudes in three time windows relative to button press response in a Control subject and subject with FXS over the course of one year (Y1-Y2)

Control

	Time window 0 (-40 to 0 ms)		Time Window 1 (20 to 60 ms)		Time Window 2 (60-100 ms)	
	Y1	**Y2**	**Y1**	**Y2**	**Y1**	**Y2**
SP-L	4.02	2.39	4.31	3.46	1.29	2.8
M1	4.33	2.06	5.89	3.06	1.88	2.75
SMA	2.0	1.88	2.26	2.79	0.71	2.15
S1	4.2	1.44	5.65	2.98	1.92	3.37
SP-R	1.51	1.69	1.15	0.66	0.44	1.53

FXS

	Time window 0 (-40 to 0 ms)		Time Window 1 (20 to 60 ms)		Time Window 2 (60-100 ms)	
	Y1	**Y2**	**Y1**	**Y2**	**Y1**	**Y2**
SP-L	1.72	1.65	2.4	2.4	4.19	1.45
M1	1.64	0.32	5.24	2.37	5.19	0.42
SMA	2.01	0.61	5.74	4.92	0.69	3.44
S1	1.29	0.45	6.07	3.38	7.8	2.57
SP-R	0.1	0.28	0.3	0.68	0.44	0.19

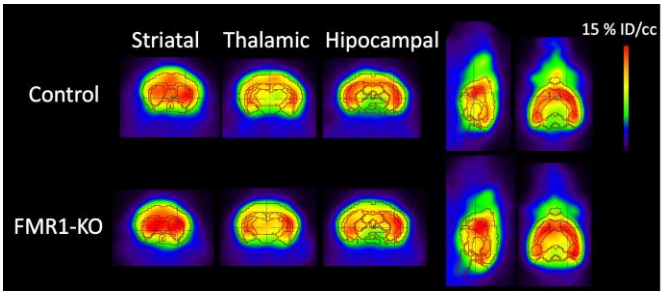
Finally, ongoing resting state analyses aimed at understanding visuospatial deficits in FXS, also appear to implicate the theta band, providing further support for a role for theta band in the pathophysiology of FXS. We observe within the visuospatial network, the inferior parietal cortex shows reduced connectivity in the FXS compared to controls in theta and high gamma band. (Controls: 0.016; FXS: 0.042; p= 0.05).

Results from this fourth year provide insights about the role of the oscillatory mechanisms, specifically, theta and gamma, underlying cognitive dysfunction in FXS

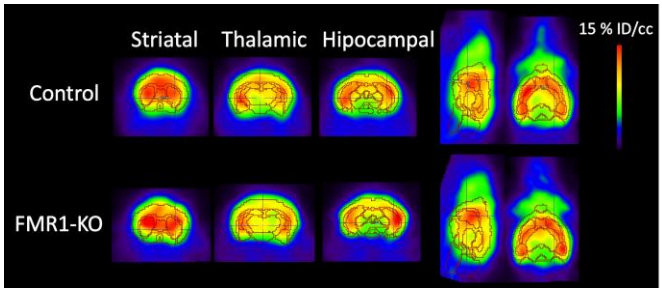
Specific aim 2. We have conducted behavioral studies using the Morris water maze, rotarod, elevated plus maze and open field test as well as PET imaging studies of mGluR5 expression using [^{18}]FPEB in male FXS and control mice at the age of 8 weeks. These baseline studies were followed by drug treatment using mGluR5 agonist, CDPPB, mGluR5 antagonist, MTEP and saline as a control drug. Daily administration of the drugs continued for 5 weeks. Follow up

studies of the drug response on behavior and mGluR5 expression were investigated immediately after the treatment and repeated 3 and 6 months later. These experiments were proceeding according to the original plan and time frame until we had to close the facility because of Covid 19. The mice were euthanized and immunohistochemical experiments were done while the final data analyses of these studies are in process. Manuscript writing of the treatment effect on behavioral and PET imaging data of mGluR5 expression is in process.

(a) Saline treatment



(b) mGluR5 agonist (CDPPB) treatment



(c) mGluR5 antagonist (MTEP) treatment

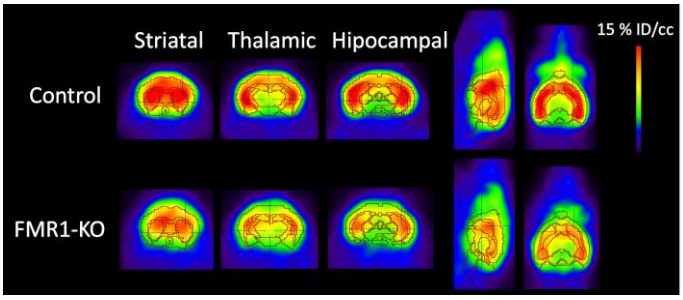


Figure 12. Distribution of [^{18}F]FPEB binding in three coronal (striatal, thalamic and hippocampal brain areas), sagittal and axial levels in male FMR1-KO and control mice 3 months after (a) the saline injections as a control treatment; (b) using mGluR5 agonist (CDPPB) treatment and (c) using mGluR5 antagonist (MTEP) treatment.

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.

This project has provided extensive learning and training opportunities for several team members.

Sepideh Afshar, PhD has been trained to use different mouse brain atlases to localize different brain areas and combine this information for experimental imaging studies. As well she has learned how to quantitate PET imaging studies, to learn brain connections with different behavioral studies as well as to use extensive statistical methods to analyze correlations between animals, sexes, and relation to behavior and mGluR5 expression in different brain areas and their relation to disease progression.

Sevda Lule, PhD, who has extensive experience with behavioral studies learned how behavior can reflect changes on mGluR5 expression in different brain areas and how the combined data predict disease progression.

Yoann Petibon, PhD, championed the motion correction aspects of human PET data acquired in this difficult-to-test population. As well he learned to explore specific brain areas affected in FXS as compared to the normal control subjects to determine disease progression.

Overall, the translational aspect of this project has been an excellent learning opportunity for the whole team to investigate disease progression

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.

We have completed data collection with a subset of patients and matched controls to allow for statistical group comparisons of structural and functional brain differences toward identification of biomarkers that may be used to test drugs targeting mGluR5 for controlling glutamate signaling. Longitudinal analysis of mouse data has guided our predictions and analysis of the human data. During the one-year extension we finalized all the experimental outcomes of the project and the final analyses of the results is in proves. The results will be published in several high-profile journals.

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.

During the remaining time of three months of the funding period we focus publishing of the results.

4. **IMPACT:** D*escribe 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).

This project might have a significant impact on drug development for FXS. Both experimental mouse studies as well as studies in human subjects with FXS show decreased availability of mGlu5 receptors; current mGluR5 focused therapeutic approaches aim, however, to decrease mGluR5 expression based on *ex vivo* studies which show an opposite effect, an excessive mGluR5 expression.

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.

Based on *in vivo* imaging studies of mGluR5 expression we have found that mGluR5 expression is significantly decreased in cortical and hippocampal brain areas both in mouse model and human subjects. This finding could provide new insight for related drug development which has aimed to decrease mGluR5 function based on *ex vivo* data that shows enhanced mGluR5 expression. We will return to this topic when we get all the immunohistochemical studies completed. The big question is "How to mine contrasting *ex vivo* and *in vivo* data for improved intervention".

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.*

After the completion of the immunohistochemical studies this project will probably reveal a new approach for developing therapeutic pharmacological approaches for FXS.

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.*

The interaction with the families of the patients has been mutually rewarding for the study staff and the caregivers. The families appreciate the strategies we use to assure comprehension of the procedures by the patients/their children, and the importance of practice, patience and respect when exposing the patient to a medical procedure to facilitate compliance.

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:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

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.

Because of Covid 19 we had to close activities on March 9, 2020 and to euthanize all the mice. The brains were waiting for immunohistochemical studies about 5 months before we were able to complete them. Now, the experimental work has been completed and data analyses of the immunohistochemical studies is in process while manuscript writing of the statistical data analyses of longitudinal behavioral measures and PET imaging studies of mGluR5 expression is ongoing for Specific Aim 2.

Concerning human studies we had a 7.5 month delay before being able to start recruitment and evaluation of the subjects. FXS is a rare disorder: a general prevalence with full mutation is estimated at 1/4000 males and 1/5000-1/8000 females (Nui et al, 2017) and we have been able to find 73 affected subjects and their willingness to participate in studies was about 12% due to a variety of reasons including personal family reasons and difficulties with compliance with the test procedures. We have extensively worked with different patient groups, the PCPs/clinical providers to expedite enrollment subjects for the project. Also, we were not able to do research patient studies since Covid 19 since March 9, 2020. Under the restricted reopening guidelines by the state effective on August 1, we were able to restart patient studies

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.

An unexpected delay for completion of the project was caused by Covid 19 pandemic and closing of the research facilities starting from March and gradually opening after August 1, 2020.

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

No changes in the protocols but the research activities were closed/restricted during March 9-August 1, 2020.

Significant changes in use or care of vertebrate animals

No changes in the protocols but the research activities were closed/restricted during March 9-August 1, 2020

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).*

Afshar, S., Lule, S., Yuan, G., Qu, X., Pan, C., Whalen, M., Brownell, A-L., Mody. M. "mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" Submitted to Journal of Neuroscience. Federal support is acknowledged in the paper.

Mody, M., Petibon, Y., Han, P., Kuruppu, D., Ma, C., Yokell, D., Neelamegam, R., Normandin, M.D., El Fakhri, G., Brownell, A-L. *In vivo* Imaging of mGlu5 receptor expression in humans with Fragile X Syndrome towards development of a potential biomarker. Submitted to Science Report. Federal support is acknowledged in the paper.

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.

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.*

Mody, M., Ahlfors, S.A, Wreh, C, Yang, J-C, & Brownell, A-L.
"Resting State Connectivity in Fragile X Syndrome". Presented at the International Society for Advancement of Clinical MEG (ISACM) conference in Toronto, Canada in Sept 2019.

Afshar, S., Qu, X., Yuan, G., Lule, S., Pan, C., Whalen, M., Brownell, A-L, Mody. M.Assessment of Glutamatergic Neurosystem in Fragile X Knock Out Mouse Model for Targeted Therapy. Annual Meeting of Division of Clinical Research at MGH. Oct 3, 2019.

Afshar, S., Qu, X., Yuan, G., Lule, S., Whalen, M., Brownell, A-L., Mody, M. Longitudinal assessment of Glutamatergic Neurosystem in Fragile X Knock Out Mouse Model. Annual meeting of SNMMI 2021, June 11-15, 2021. Oral presentation and abstract included to the meeting highlights.

- **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.

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

Nothing to Report.

- **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.

- **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:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report.

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".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	Anna-Liisa Brownell “no change”
Project Role:	PI
Research Identifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Maria Mody “no change”
Project Role:	Co-PI
Research Identifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Sepideh Afshar “no change”
Project Role:	
Research Itendifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Yoann Petibon “no change”
Project Role:	
Research Idendifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Darshini Kuruppu “no change”
Project Role:	
Research Idendifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Paul Han “no change”
Project Role:	
Research Idendifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

Name:	Debra Horng “no change”
Project Role:	
Research Idendifier:	
Nearest person month worked:	
Contribution to the Project:	
Funding Support:	

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.

Nothing to Report.

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://ebrap.org/eBRAP/public/index.htm*** *for each unique award.*

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

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Manuscript

Mody, M., Petibon, Y., Han, P., Kuruppu, D., Ma, C., Yokell, D., Neelamegam, R., Normandin, M.D., El Fakhri, G., Brownell, A-L. "*In vivo* Imaging of mGlu5 receptor expression in humans with Fragile X Syndrome towards development of a potential biomarker". Accepted to Science Reports, 2021. (Federal support is acknowledged in the paper.)

Abstract

Sepideh Afshar, Xiying Qu, Gengyang Yuan, Sevda Lule, Michael Whalen, Anna-Liisa Brownell, Maria Mody. Longitudinal assessment of Glutamatergic Neurosystem in Fragile X Knock Out Mouse Model. SNMMI Annual meeting, June 2021. (oral presentation)

In vivo Imaging of mGlu5 receptor expression in humans with Fragile X Syndrome towards development of a potential biomarker

Maria Mody[1+]*, Yoann Petibon[2+], Paul Han[2], Darshini Kuruppu[3], Chao Ma[2], Daniel Yokell[2], Ramesh Neelamegam[4], Marc D. Normandin[2], Georges El Fakhri[2] & Anna-Liisa Brownell[2]

[1]Athinoula A Martinos Center for Biomedical Imaging, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA. [2]Gordon Center for Medical Imaging, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA. [3]Department of Surgery, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02129, USA. [4]Department of Radiology, University of Texas Health Science at San Antonio, San Antonio, TX 78229, USA.

+These authors contributed equally to this work
*Corresponding author

Abstract

Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by silencing of the Fragile X Mental Retardation (*FMR1*) gene. The resulting loss of Fragile X Mental Retardation Protein (FMRP) leads to excessive glutamate signaling via metabotropic glutamate subtype 5 receptors (mGluR5) which has been implicated in the pathogenesis of the disorder. In the present study we used the radioligand 3-[18F]fluoro-5-(2-pyridinylethynyl)benzonitrile ([^{18}F]FPEB) in simultaneous PET-MR imaging of males with FXS and age- and gender-matched controls to assess the availability of mGlu5 receptors in relevant brain areas. Patients with FXS showed lower [^{18}F]FPEB binding potential ($p<.01$), reflecting reduced mGluR5 availability, than the healthy controls throughout the brain, with significant group differences in insula, anterior cingulate, parahippocampal, inferior temporal and olfactory cortices, regions associated with deficits in inhibition, memory, and visuospatial processes characteristic of the disorder. The results are among the first to provide *in vivo* evidence of decreased availability of mGluR5 in the brain in individuals with FXS than in healthy controls. The consistent results across the subjects, despite the tremendous challenges with neuroimaging this population, highlight the robustness of the protocol and support for its use in drug occupancy studies; extending our radiotracer development and application efforts from mice to humans.

Introduction

Fragile X syndrome (FXS) is a leading cause of inherited intellectual disability[1] and the most common, known single gene cause of autism spectrum disorder [2,3]. Successful treatment of FXS has the potential to positively impact the larger field of neurodevelopmental disabilities. A number of studies have implicated metabotropic glutamate subtype 5 receptors (mGluR5) in FXS[4]; the use of mGluR5 antagonists in Fmr1 Knockout (KO) mouse models has demonstrated a variety of benefits

including reduced seizures, anxiety, and behavioral issues [5,6]. The Fmr1 KO mouse model has shown that the absence of FMRP leads to increased protein synthesis at the synapse and excessive mGluR5 glutamatergic signaling[7] , building on the work of Weiler and colleagues who first identified the connection between FMRP and mGluR5 pathways[8]. However, despite these promising preclinical findings, clinical trials of mGluR5-targeted drugs in FXS have met with limited success. It is unclear as to whether these trials were conducted with optimal outcome endpoints or in the most appropriate age group [9,10,11].

The severity of FXS phenotype is known to be correlated with the magnitude of the FMRP deficit[12]. The *FMR1* gene responsible for FXS is located on the X chromosome, thereby affecting males with FXS more severely than their female counterpart who have one unaffected X chromosome to fall back on[13]. We focused on measuring the availability and distribution of mGluR5 in males with FXS and age- and gender-matched healthy controls to better understand the role of mGluR5 expression in the pathophysiology of FXS in humans. Given the heterogeneity found in neurodevelopmental disorders like FXS, stringent methodological standards were used to obtain reliable delineation and quantification of mGluR5 occupancy differences between healthy and disorder groups, critical for biomarker research.

In the present study, we examined PET data from five adult males with FXS (>200 CGG repeats, full mutation, 26-48 years of age; mean: 34.8 years ± 7.8) and seven age-matched males with normal development (22-43 years of age; mean 31.4 years ± 7.5) from whom PET and MRI data was successfully obtained (*see* **Methods** for details). Participants with FXS were recruited from the hospital database, local clinics and fragile X organizations; typical controls (TC) were drawn from the local community. All subjects completed a high resolution MRI and 90-minute dynamic PET

examination on an integrated whole-body PET-MR (Siemens Biograph mMR) following an intravenous bolus injection of 5 mCi of [^{18}F]FPEB, a safe and reliable radioligand for quantifying regional brain concentrations of mGluR5[14]. Based on *Fmr1* mouse studies of the mGluR theory and the established affinity and specificity of [^{18}F]FPEB to mGluR5, we hypothesized that there would be a significant difference in regional brain concentrations of mGluR5 between the two groups of subjects, validating the use of [^{18}F]FPEB in clinical trials as a tool to confirm target engagement of novel drug agents for mGluR5s in FXS and to monitor dose response.

In a recent study, Brašić and colleagues[17] used the same [^{18}F]FPEB tracer in males with FXS, slightly younger than those in the present study, though incorporating different acquisition protocols and data analysis methods for PET across collaborating sites; they found mGluR5 density was significantly reduced in multiple brain regions including the cingulate, cortex, thalamus and striatum, compared to age-matched males with typical development, thereby supporting the use of [^{18}F]FPEB to measure drug occupancy in clinical trials for mGLuR5 in FXS**.**

Results

Regional and voxel-wise estimates of [^{18}F]FPEB non-displaceable binding potential (BP_{ND}), a measure of mGluR5 availability, were obtained for each subject by multi-linear reference tissue analysis of dynamic PET data [15,16]. We found regional brain uptake was consistent with known mGlu5 receptors based on previous studies [1,17]. A global reduction of [^{18}F]FPEB BP_{ND} was observed in males with FXS compared to control subjects (**Figure 1**).

Region of interest analyses confirmed BP_{ND} differences between FXS and control groups (**Figure 2**). [^{18}F]FPEB BP_{ND} were significantly lower in subjects with FXS compared to controls in the anterior cingulate gyrus (2.67±0.38 vs. 3.56±0.41; $p = 0.004$), insula (2.79±0.47 vs. 3.67±0.36; $p = 0.009$),

inferior temporal gyrus (2.41±0.34 vs. 3.19±0.47; p = 0.008), parahippocampal gyrus (2.10±0.32 vs. 2.65±0.32; p = 0.018) and olfactory cortex (2.27±0.30 vs. 2.98±0.34; p = 0.004) (two-tailed unpaired t-tests, corrected for multiple comparisons using FDR [18] (q=0.05). BP_{ND} was also significantly lower for subjects with FXS subjects in the amygdala (1.70±0.41 vs. 2.25±0.34; p = 0.04), although it did not survive correction for multiple brain region testing. The findings correspond to those in the neuroimaging literature on FXS[19,20].

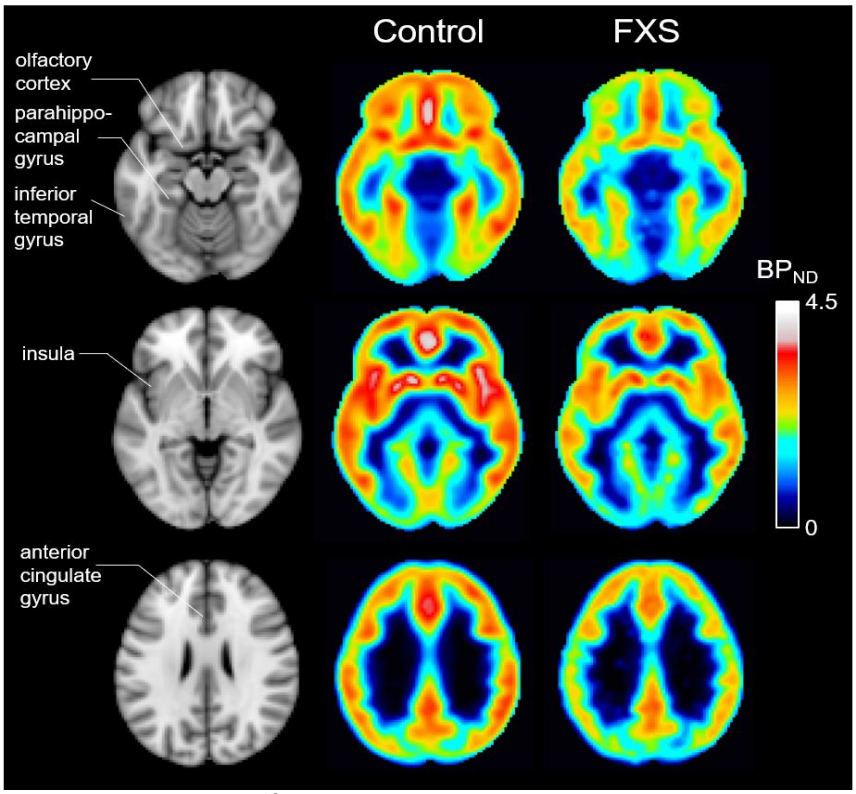


Figure 1. Group-average images of [^{18}F]FPEB BP_{ND} in control subjects and subjects with FXS, and corresponding MR template images. Regions exhibiting significant differences in BP_{ND} between control subjects and subjects with FXS (olfactory cortex, parahippocampal gyrus, inferior temporal gyrus, insula, and anterior cingulate gyrus) are indicated in the MR images.

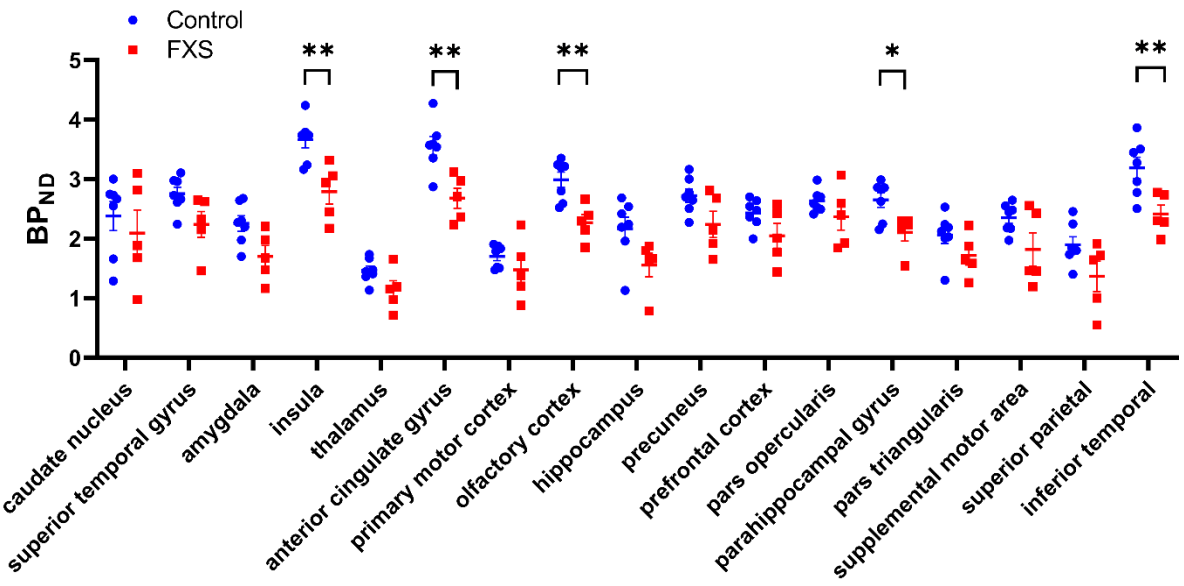


Figure 2. Regional [^{18}F]FPEB BP_{ND} in control subjects (●) and subjects with FXS (■). Error bars indicate standard error of the means. *p< 0.05, **p< 0.01 (corrected for multiple comparisons)

Discussion

Among the core symptoms of FXS is a deficit in visuospatial learning and memory mediated by hippocampus and parahippocampal and inferior temporal regions[21,22]. Our findings of significantly lower [^{18}F]FPEB binding potential (BP_{ND}) in these areas provide support for the role of mGluR5 in the neurobiology of FXS. Anxiety is frequently reported in this population, which may explain the significant group differences we observed in the insula and olfactory cortex [19,23]. Evidence in support of these findings also comes from preclinical studies of the Fmr1 KO mouse using the Morris water maze task and the mirrored chamber test[24,25,26]. Whereas only one of our participants with FXS was on prescription medication for anxiety, a high rate of anxiety in FXS, especially social anxiety, is reported in the literature regardless of gender [12, 27,28]. The amygdala group differences we observed did not hold up after multiple comparison, but would likely have survived correction in a larger sample. Thus, the finding clearly suggests a need for larger studies, perhaps a

narrower age range, and outcome measures to fully capture the profile of the disorder in males and females. Executive functions have also been consistently documented as impaired in FXS [29], implicating the prefrontal cortex. While this area did not show a signal in the present study, it deserves to be examined, especially the gyrus rectus which has been shown to have lower levels of FMRP in individuals with FXS than in controls[13]. Taken together, these approaches along with the genetic etiology of FXS will help advance our understanding of gene-brain-behavior relationships in FXS [30]and related neurodevelopmental conditions like autism spectrum disorder [31]
The role of mGluR5 has been extensively studied in mouse models of FXS. However, findings related to mGluR5 expression in animals and autopsies of humans with FXS have been inconsistent. And clinical trials targeting mGluR5 in FXS have met with repeated failure, pointing to a need for better designed studies and reliable biomarkers in individuals with FXS [32,33].

FXS is currently treated symptomatically using behavioral, educational and psychopharmacological strategies[34] often with unsatisfying results [35,36]. A targeted treatment is lacking.The results of the present study are important as they pave the way for successful human trials by providing a potential biomarker of the impaired mGluR5 mechanism for use in developing targeted drug therapies in FXS. Specifically, using the radioligand [^{18}F]FPEB , which has been shown to reliably bind to mGlu5 receptors, we found the binding potential to mGlu5 receptors to be lower throughout the brain in participants with FXS compared with typical controls. The reduced BP appears to reflect reduced density of unoccupied mGlu5 receptors, potentially a downstream consequence of excessive mGluR5 signaling caused by the lack of FMRP protein in individuals with FXS. However, given the lack of consistency of mGlu5 receptor expression findings across human and mouse models, more studies are needed to understand the functional relationship between these variables. Importantly, this difference in BP_{ND} between the subjects with FXS and control group

was significant in areas consistent with known distribution of mGluR5, and which have been functionally implicated in FXS symptomatology. Our results of overall reduced mGlu5 receptor expression in relevant regions of the brain in individuals with FXS replicate recent findings [17] and advance ongoing efforts for a much-needed measure of mGluR5 target engagement for drugs and treatment of symptoms in clinical trials of FXS and related disorders [37,38].

The participants with FXS in the study had the full Fmr1 mutation with CGG repeats greater than 200. However, some individuals with FXS may present with mosaicism (size or methylation mosaics), which can affect FMRP levels and hence the severity of the condition [39]. There has been significant progress in understanding the physiological role of mGluR5, but treatment with selective mGluR5 antagonists (e.g., MPEP, CTEP, fenobam) have met with limited success in FXS [6, 40, 41] despite evidence for use of this approach[42]. Factors like age are known to influence treatment efficacy as was seen when MPEP was administered to 2-week- vs. 6-week-old Fmr1 KO mice; the immature morphological phenotype of pyramidal neurons in the somatosensory cortex were rescued in the younger and less so in the older KO mice.

Insofar as the precise modulation of synaptic transmission either through the use of selective mGluR5 inhibitors to reduce synaptic excitability or through activation of presynaptic $GABA_B$ receptors is critical for drug efficacy, [^{18}F]FPEB binding potential, an index of mGluR5 availability, may prove to be an invaluable biomarker for optimizing the outcomes of drug trials and improving the lives of individuals with FXS[43]. None of the participants with FXS in the study had an autism diagnosis. However, given the high co-morbidity of FXS and ASD[44], the use of [^{18}F]FPEB to examine differences in mGluR5 binding potential between these disorder groups could significantly contribute to our understanding of the differences in underlying pathophysiology of the disorders; towards development of mechanism-based novel therapeutics in neurodevelopmental disorders. In

one such study, Brašić and colleagues[38], using [^{18}F]FPEB, replicated their earlier findings with FXS; but found that compared to subjects with typical development (TD), individuals with autism spectrum disorder (ASD) had higher mGluR5 expression in cortical areas and no difference in subcortical regions. The participants with ASD were younger than the controls. The PET data were acquired and analyzed at collaborating sites using different scanners, scan protocols and analysis methods, as in the original study. Despite potential methodological confounds, these preliminary findings hint at exciting new opportunities for the development of drugs targeting the observed differences in the underlying pathophysiology of the two disorders.

The failure of a clinical trial may not necessarily reflect a failure to understand the underlying mechanism but rather may reveal treatment parameters to be optimized beyond target identification. Future studies could draw on the protocol and findings from the current study to systematically interrogate larger samples with FXS to address questions of disorder severity, age and cognitive abilities as they relate to mGluR5 availability and its regional distribution in the brain. Zhao and colleagues[45] showed that by taking Nutlin-3, an experimental cancer drug which serves as an inhibitor, mice with FXS lacking the FMRP protein regained their ability to remember what they had seen or smelled in their first study session; this apparent reversal of a memory impairment appeared to specifically relate to reactivation of FMRP affecting neural stem cells and new neurons that they form in the hippocampus. In a recent study, Berry-Kravis and colleagues[46] found that a phosphodiesterase-4D (PRE4D) allosteric modulator helped improve cognitive functions and behavioral outcomes in patients with FXS. Taken together with the results from the present study, the field of FXS appears poised for major breakthroughs in treatment using inhibitors targeting increased mGluR5 signaling or cyclic AMP (cAMP) production; offering promising new directions

for future pharmacological research, as well as approaches to the challenges associated with scanning this population.

In conclusion, the current study supports a role for reduced mGluR5 expression in the underlying pathophysiology of FXS in humans; it holds promise as a potential biomarker for use in clinical trials of FXS that could prove critical in improving pharmacological interventions. Importantly, it builds on our research over the years, from the early development of [^{18}F]FPEB as a PET tracer[47], to its application in the mouse model [48], and in humans [49] and most recently, to humans with FXS evident in the present study.

Methods

Study subjects

The study was approved by the Institutional Review Board at Massachusetts General Hospital; and performed in keeping with the Human Subjects Research Committee guidelines, and in accordance with the Declaration of Helsinki. We enrolled eight males with FXS and eight age- and gender-matched Controls. Of the eight participants recruited in each group, three with FXS were unable to complete the PET-MR protocol and had to be excluded, as also the data from one Control participant due to technical issues. Subjects with FXS were recruited through the hospital's patient database, local clinics, and Fragile X organizations. Those with confirmed FMR1 full mutation (>200 CGG repeats, based on medical records) at screening were eligible to participate. They ranged in age from 26-48 years (mean: 34.8 years ± 7.8), had no comorbid diagnosis and were not on any antipsychotic medications. Two of the participants with FXS were on medication (viz., Losartan, Famotidine) for blood pressure and indigestion, and a third on Ritalin (belonging to the stimulant class of drugs) for ADHD symptoms, and Clonazepam (belonging to benzodiazepines class of

drugs), as needed, when anxious. While the caregivers reported a tendency for participants to be anxious in new settings and with strangers, only one of the participants with FXS was on prescription medication for anxiety on an as-needed basis. Control participants were recruited from the local community. They had no history of neurological or psychological problems and were not on any prescription medication; they had completed at least two years of college, and were between 22-43 years (mean: 31.4 years ± 7.5). The two groups did not differ in age (p= .416). All participants provided informed consent and/or assent prior to the experiment and were compensated for their involvement in the study. Additionally, participants with FXS underwent acclimatization training to familiarize them with the actual scanner and environment prior to their PET-MR session to help alleviate their anxiety.

Data acquisition

Study subjects were scanned for up to 90-min on an integrated whole-body PET-MR (Siemens Biograph mMR) at the MGH Martinos Center for Biomedical Imaging, following an intravenous bolus injection of 5 mCi of 3-[18F]fluoro-5-(2-pyridinylethynyl)benzonitrile ([18F]FPEB) (injected dose: mean 5.15 mCi, SD 0.28; average specific activity at time of injection: 2046 mCi /μmol). Using the published method[49], the radioligand was locally synthesized at the Gordon Center for Medical Imaging in Massachusetts General Hospital under the guidelines of the Radioactive Drug Research Committee and the approval from the FDA.

Each dynamic study was divided into three scanning sessions of up to 35-min, with breaks between sessions. A structural MRI scan was acquired for each participant at the beginning of each session using a 3-D T1 weighted multi-echo MPRAGE sequence[50] with the following parameters: TR = 2530 ms, TEs = 1.69, 3.55, 5.41, 7.27 ms, inversion time = 1100 ms, matrix size = 256×256×176

and voxel size = 1×1×1 mm^3. An attenuation map for PET was generated based on the structural MRI data using a hybrid segmentation and atlas-based technique[51].

Data processing and analysis

Dynamic list-mode PET data for each scanning session were binned into temporal frames of up to 30 sec and were reconstructed and corrected for motion using the following multi-step procedure. First, an initial dynamic reconstruction was performed without correction of attenuation, followed by application of spatial Gaussian smoothing (6-mm full width at half maximum -FWHM) to each reconstructed frame and rigid-body alignment of the activity volume for each frame to a selected reference frame. The attenuation map was then registered to the resulting time-averaged volume and transformed using the previously calculated registration parameters, yielding an attenuation map for each frame. Second, another dynamic reconstruction was performed, incorporating the frame-dependent attenuation map obtained in the first step as well as standard corrections for dead-times, random and scattered coincidences. Note that the attenuation map used during reconstruction also accounted for "static" attenuating medium, such as the scanner's table and MR head coil. Third, activity volumes in each scanning session were smoothed with a 4-mm FWHM Gaussian filter and rigidly registered to a selected reference frame, followed by another registration to the resulting time-averaged volume. Lastly, all the frames in each session were aligned to a common reference frame, taken as the first session's time-average image volume. All PET reconstructions were performed using OP-OSEM 3D with 3 iterations and 21 subsets on a 344×344×127 array with voxel size 2.08×2.08×2.03 mm^3. Image registrations were performed using FSL's flirt method with normalized mutual information as the data consistency criterion and 6 degrees of freedom[52]. To further mitigate motion effects, realignment transformations estimated during the third step were inspected to discard frames associated with substantial frame-to-frame shifts (> 2.5 mm) from the

analysis. Linear interpolation was used to compute time points corresponding to discarded frames as well as missing time points between scanning sessions.

Afterwards, the structural MRI scan for each subject was rigidly aligned to PET space (FSL, flirt), followed by non-rigid registration (FSL, fnirt) of the Montreal Neurological Institute (MNI) template to MRI space for definition of regions of interest (ROIs). In keeping with the range of behavioral symptoms associated with FXS, we explored 18 brain regions that have been implicated across neuroimaging studies of FXS[53, 54]. [^{18}F]FPEB concentration histories were extracted in the following bilateral regions: caudate nucleus, superior temporal gyrus, amygdala, insula, thalamus, anterior cingulate gyrus, primary motor cortex, olfactory cortex, hippocampus, precuneus, prefrontal cortex, pars opercularis, parahippocampal gyrus, pars triangularis, supplementary motor area, superior parietal gyrus, inferior temporal gyrus and cerebellar white matter. Regional time-activity curves (TACs) were fitted using MRTM2[55] to estimate [^{18}F]FPEB binding potential (BP_{ND}) with the cerebellum white matter as reference[16]. The t* value was fixed at $t^* = 30$ min[10] and the k_2' value was estimated by first-pass MRTM analysis of the average TACs [9]. In addition, for visualization purposes, images of [^{18}F]FPEB BP_{ND} were generated for each subject by application of MRTM2 analysis at the voxel level. BP_{ND} images were then transformed to MNI space, followed by spatial Gaussian smoothing (5-mm FWHM) and averaging over subjects in each group.

Note that no region completely devoid of mGlu5 receptors exists in the human brain[56]. As such, kinetic approaches based on reference region modeling could be biased by individual variations in specific radiotracer uptake. A close examination revealed no substantial differences in reference region TACs which appeared similar for the two groups (**Figure 3**).

Statistical analyses

Unpaired t-tests (2-tailed) were applied to test the null hypothesis of no difference in [^{18}F]FPEB BP_{ND} between healthy controls and subjects with FXS for all surveyed regions. The *p*-values were corrected for multiple comparisons by applying the Benjamini, Krieger and Yekutieli false discovery rate method[18] with $q = 0.05$

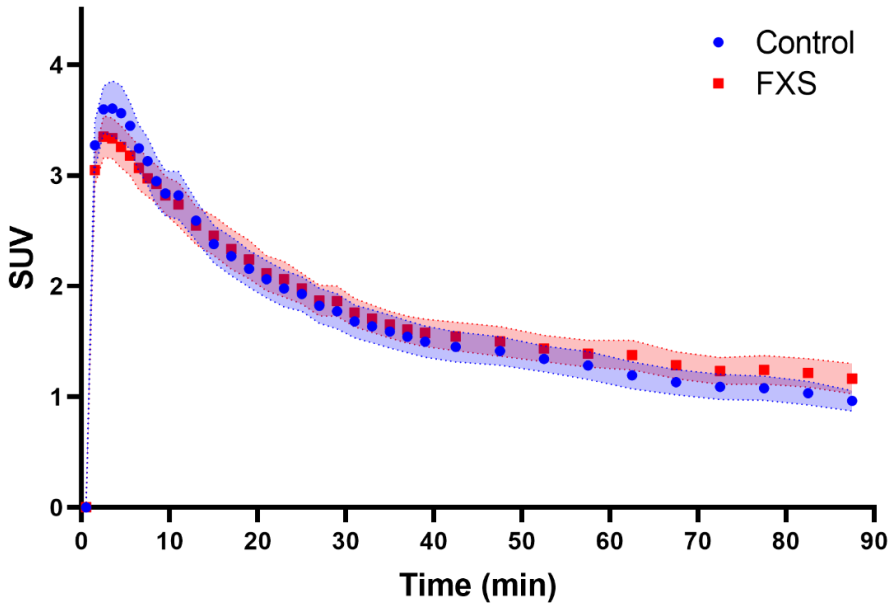


Figure 3. Mean time activity curves in the cerebellum white matter (reference region) for the control subjects (●) and subjects with FXS (■). Envelope represents standard error of the mean. SUV, standardized uptake value.

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Captions

Figure 1. Group-average images of [^{18}F]FPEB BP_{ND} in control subjects and subjects with FXS, and corresponding MR template images. Regions exhibiting significant differences in BP_{ND} between control subjects and subjects with FXS (olfactory cortex, parahippocampal gyrus, inferior temporal gyrus, insula, and anterior cingulate gyrus) are indicated in the MR images.

Figure 2. Regional [^{18}F]FPEB BP_{ND} in control subjects (●) and subjects with FXS (■). Error bars indicate standard error of the means. *p< 0.05, **p< 0.01 (corrected for multiple comparisons)

Figure 3. Mean time activity curves in the cerebellum white matter (reference region) for the control subjects (●) and subjects with FXS (■). Envelope represents standard error of the mean. SUV, standardized uptake value.

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Competing interests: none to declare

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Data availability and Code availability: All datasets generated and/or analyzed during the current study are available from the corresponding authors on reasonable request

Longitudinal assessment of Glutamatergic Neurosystem in Fragile X Knock Out Mouse Model

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Introduction: Fragile X syndrome (FXS) is a monogenic developmental disorder caused by mutations of the Fragile X Mental Retardation 1 (FMR1) gene. Patients with FXS are characterized by learning disabilities and cognitive impairments. In the face of failures of clinical trials with FXS, we focused on PET imaging of FMR1 knockout mice and their performance on the Morris Water Maze (MWM) task, a measure of spatial learning and memory, towards developing a biobehavioral marker for FXS.

Methods: A cohort of FXS mice and age- and gender-matched healthy control mice underwent longitudinal PET imaging using allosteric modulator compound [18F]FPEB (3-[18F]flouro-5-(2-pyridinylethynyl) to examine mGluR5 expression combined with behavioral performance on the MWM and open field tasks at three time points: when mice were between 34-41 days old (A1), between 156-219 days (A3) and between 325-398 days (A4). An additional PET imaging was conducted at age between 84-100 days (A2). The data were statistically analyzed (t-tests and repeated measure ANOVA) to evaluate the effects of independent variables (age and gender) on the dependent variable (FPEB binding potential, BP in PET analysis) in multiple brain regions.

Results: Repeated measure ANOVA (gender and age) of the PET data at each brain area revealed a significant interaction between age and gender at three brain areas, striatum, cortex, and hippocampus ($p<0.041$) in FXS group as well as main effect of time ($p<3.0e-7$) in both groups. Further analysis showed that the difference between male and female mice was significant at age group A1 and A4 ($p<0.051$) in FXS group. In general, females showed lower binding potential than males, and the binding potential reduced with time except a sudden increase at age group A2 in male mice. Furthermore, we observed a significant increase in linear learning rate ($p<0.020$) from the latency trials on finding the hidden platform in FXS group but no difference in the mean latency. Finally, the ANOVA analysis of distance travelled by the mice in an open field test indicated a significant main effect of age ($p=0.005$) and significant reduced distance in FXS group from age group A3 to A4 in keeping with lower binding potential at older ages.

Conclusion: These findings reflect the critical brain areas known to be impacted by the progression of the FXS syndrome namely, striatum, cortex, and hippocampus. In addition, a biobehavioral vulnerability was predicted on the basis of disease progression and hold exciting potential as targets for pharmacological interventions.

Supporting data:

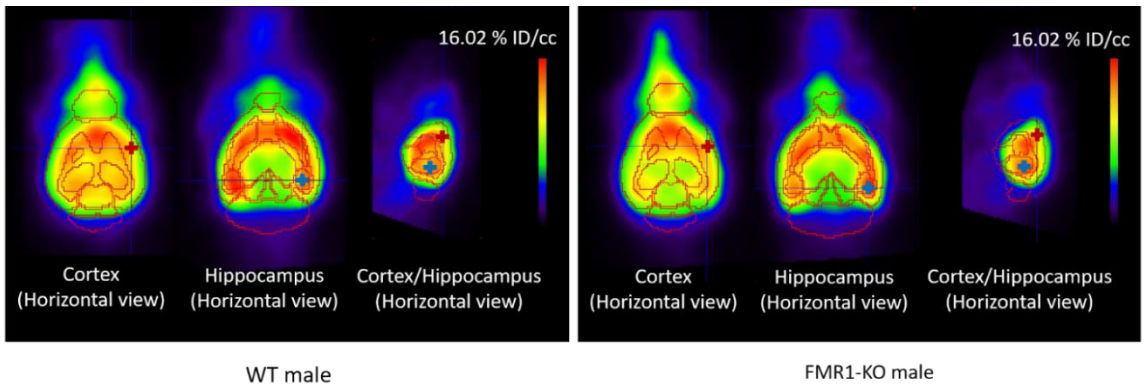

Figure1: [18F]FPEB binding potential in a male control mouse and a FMR1-KO male mouse.

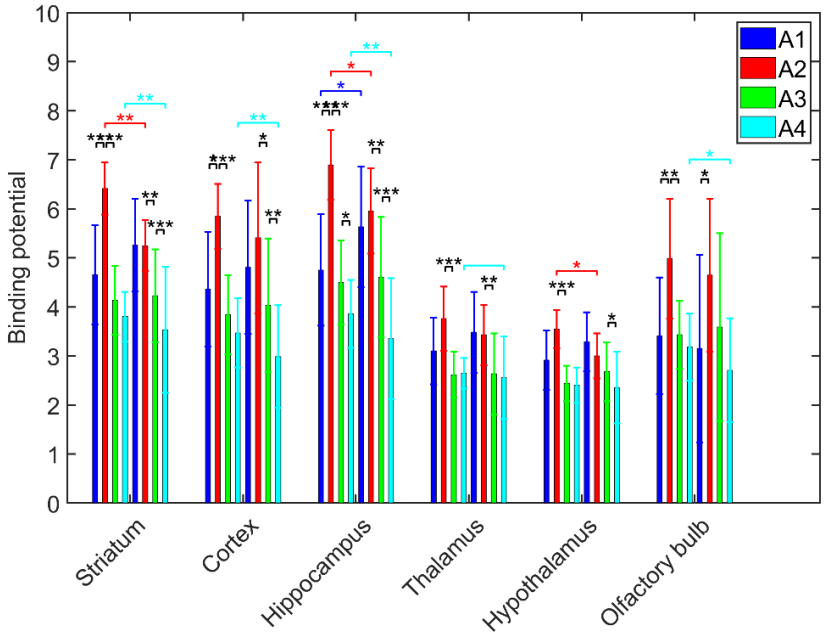

Figure2: Comparison of BP among different age group at different brain areas for both male (first four bars at each area) and female (second four bars at each area) mice.

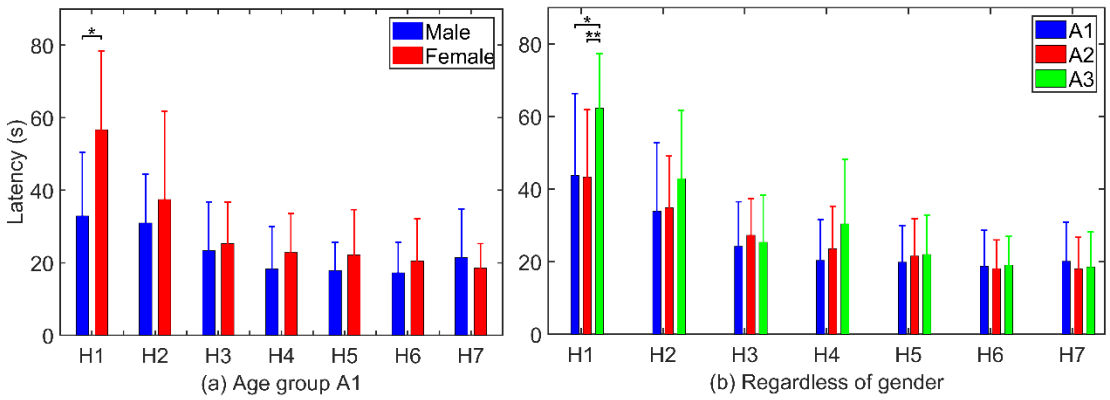

Figure 3: Comparison of Latency between male and female at age group A1 (a) and among different age groups regardless of gender (b).