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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 five FXS subjects while only MEG studies were done in 9 FXS subjects. In the human data, uptake of [^{18}F]FPEB shows regional correspondences with those seen in mouse data providing an outstanding translational aspect to investigate FXS related biobehavior and develop therapeutic approaches.

15. SUBJECT TERMS
FXS, mGluR5, FMR1, PET, MRI, DTI, MEG, humans, moue 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 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 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 report, we have been well within the time lines 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 "Behavior and 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 95% 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 time line 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 + behavioral testing. So far we have interviewed 73 FXS subjects and screened their suitability and interest on voluntary participation for this project. Based on the response we have received, we have interviewed 9 age matched control subjects. Presently PET, MRI and DTI studies have been completed in 14 subjects (5 FXS and 9 control subjects) and studies with two additional FXS subjects are in process. In addition, we have completed MEG studies in 17 subjects due to better compliance with study procedures (9 FXS and 8 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.

Major tasks for Aim 2 (mouse studies):

1. In the second part of the mouse studies we were able to follow accurately the study time lines 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 is in process as well as immunohistochemical analyses of brain as described in the SOW. At this point about 85% of the experimental 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

Accomplishments cont.

Specific Aim 1a.

We have conducted all the behavioral and PET imaging studies of mGluR5 expression, and the manuscript, titled "Behavior and mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" has been submitted to Neuropsychopharmacology (Appendix). 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.

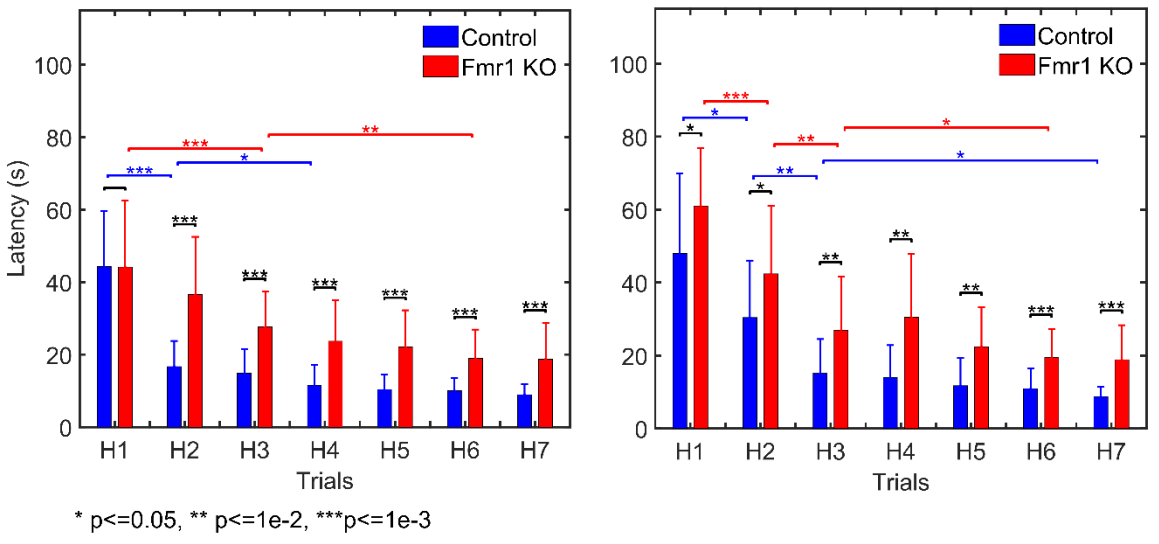


Figure 1. Morris water maze hidden platform latencies of WT and FMR1 KO mice at age groups 2 and 3.

Accomplishments (cont.)

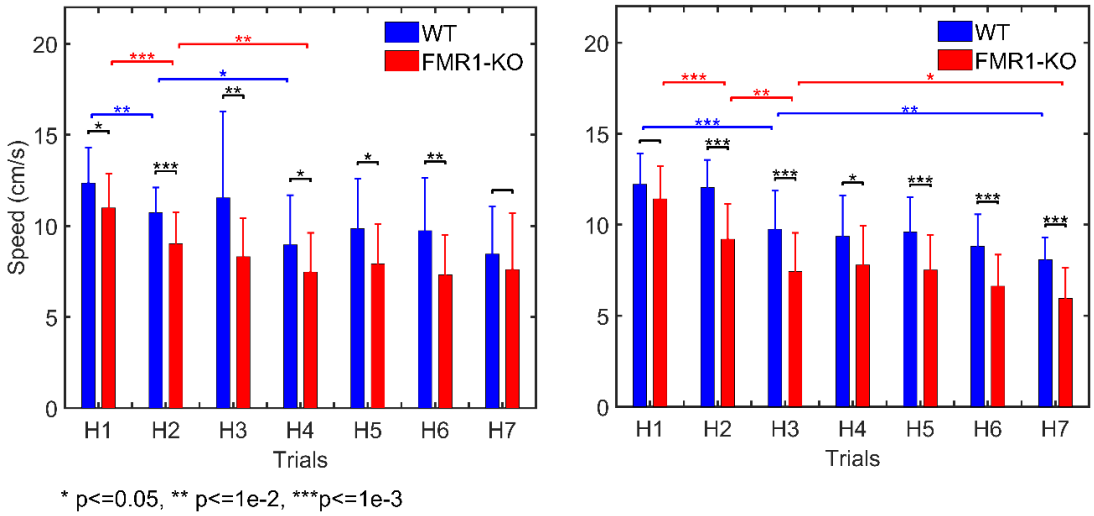


Figure 2. Morris water Maze hidden platform swim speeds for WT and FMR1-KO mice at age groups 2 and 3

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.

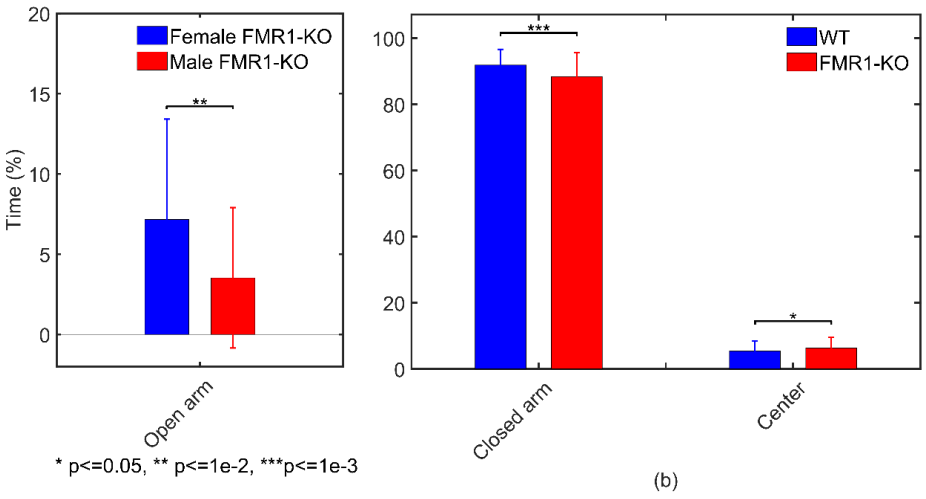
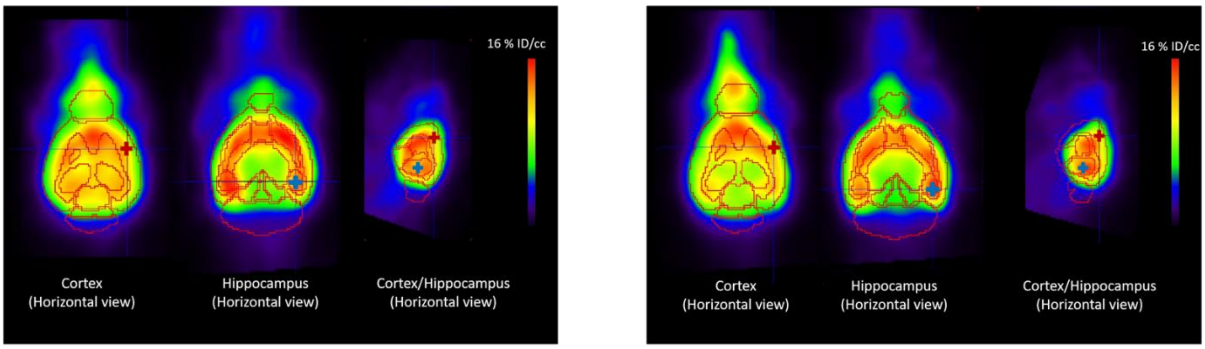


Figure 3. Comparison of the time spent in (a) open arm between female and male FMR1-KO mice, and (b) closed arm between FMR1-KO and WT mice and in center platform between FMR1-KO and WT mice in the youngest age group on the elevated plus maze test.

Accomplishments (cont.)

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 4). Below we report significant findings (p<.05), meeting multiple comparison correction criteria.



WT male FMR1-KO male

Figure 3. [^{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)

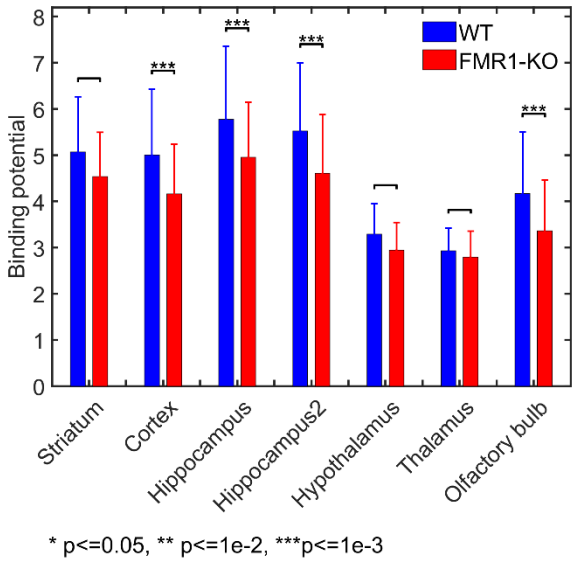


Figure 4. Comparison of [^{18}F]FEB binding potential in WT and FMR1-KO mice in different brain areas.

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.

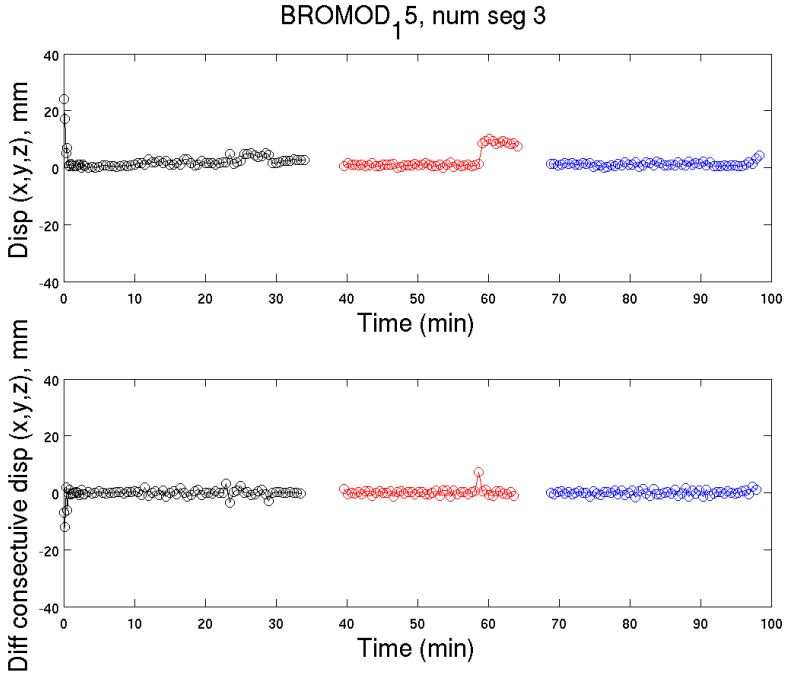


Figure 6. Motion correction as applied to a single subject used for the quantitative analyses.

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.

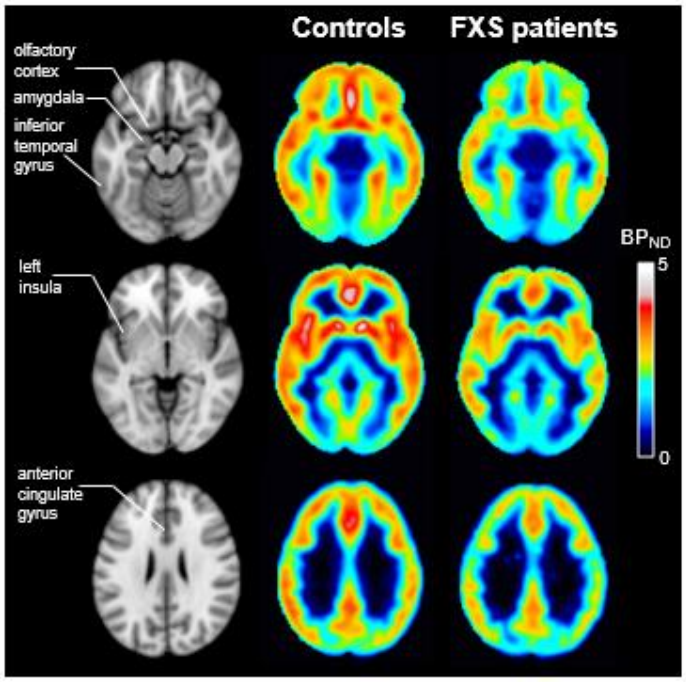


Figure 7. Quantitative distribution of [^{18}F]FPEB accumulation in different brain areas show that the mGluR5-realted binding potential is lower in all brain areas in FXS patient compared to the healthy control (HC).

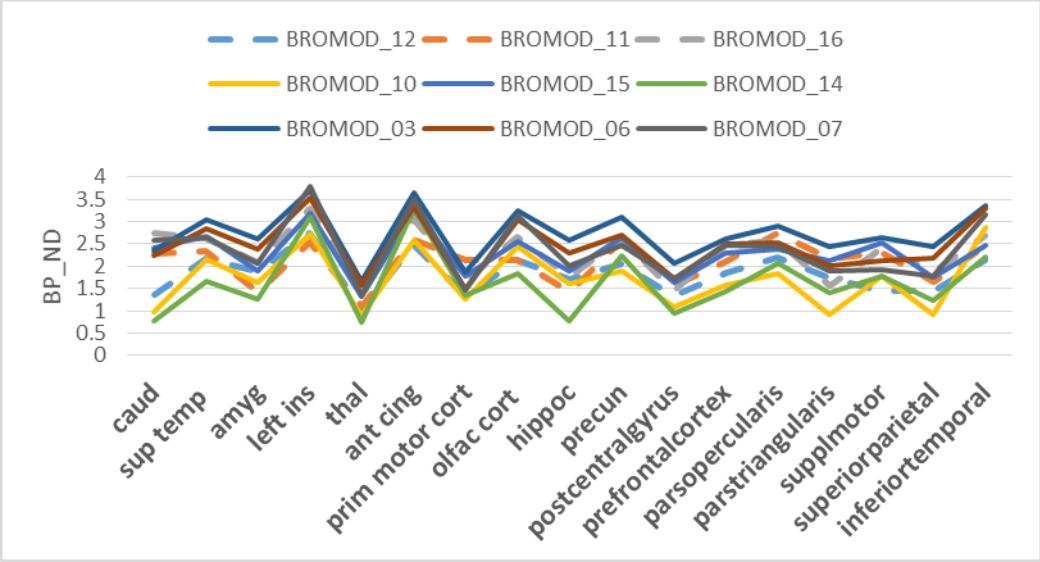


Figure 8. mGluR5-related binding potential in different brain areas.

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)

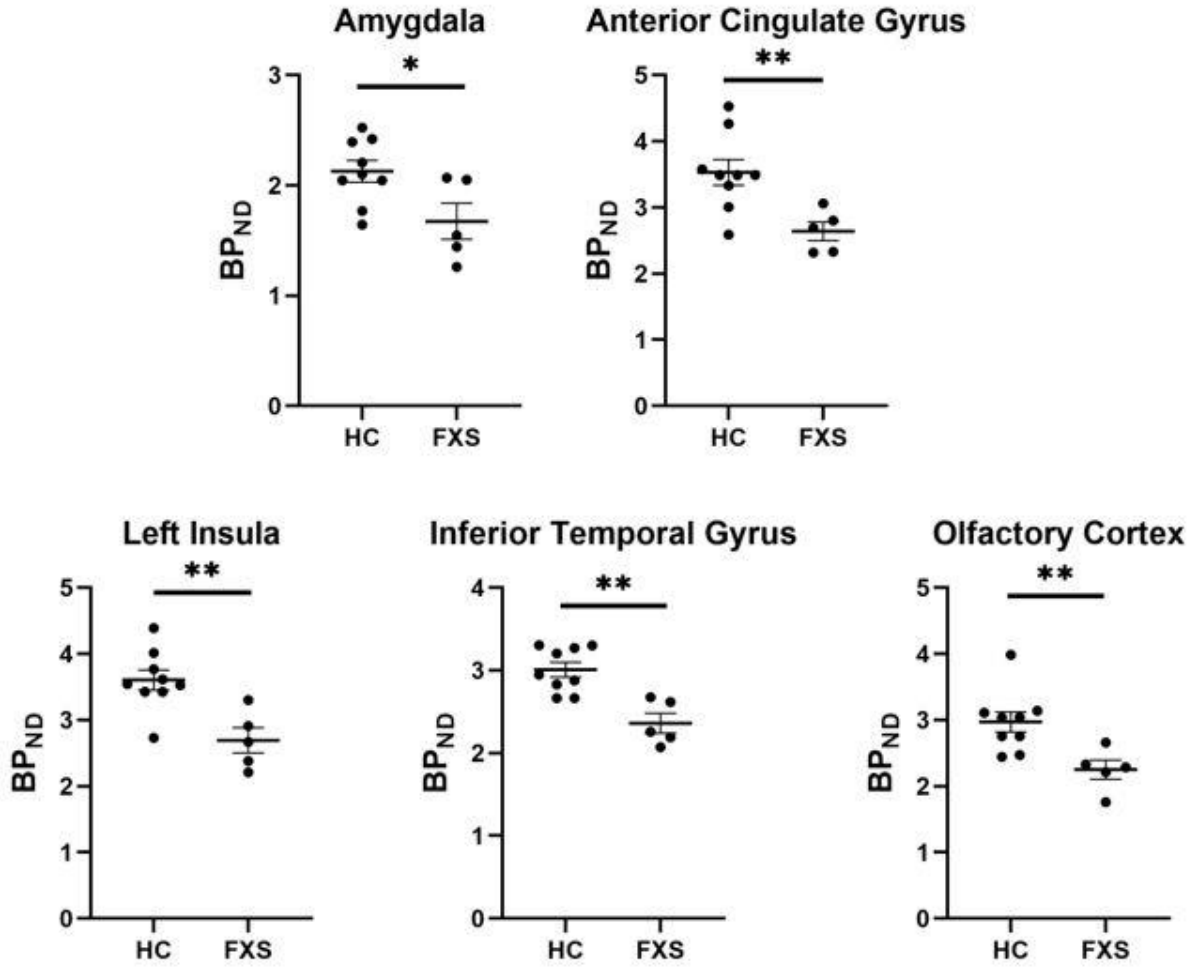


Figure 9. Group differences of the binding potentials in different brain areas in FXS and healthy control (HC) subjects. The results are based on PET imaging of [^{18}F]FPEB binding to mGlu5 receptors.

MRI, DTI and MEG: Disruptions in brain connectivity are considered a hallmark of neurodevelopmental disorders like Fragile X Syndrome (FXS). During this third 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; see above), 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 (17 participants: eight controls and nine with FXS; one patient had to be excluded due to poor data quality) and DWI (five FXS and nine controls, the subset of participants who were able to complete the integrated PET-

Accomplishments (cont.)

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).

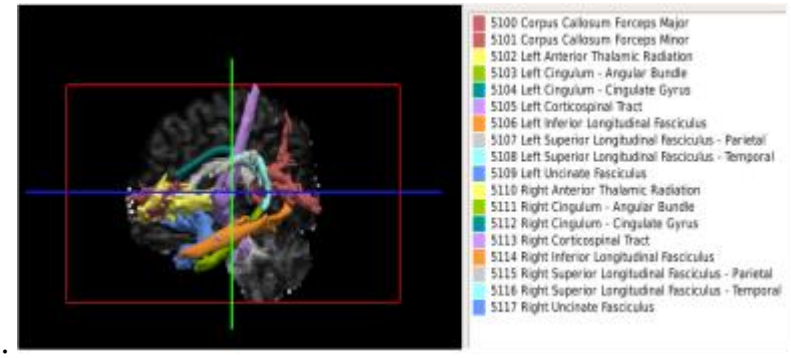


Figure 10: Probability distribution of 18 major white matter pathways generated by TRACULA and displayed in a single control subject

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 miceand human PET participants manifest significantly lower mGluR5-related binding potential in the same regions, assessed with [^{18}F]FPEB during PET imaging (see Fig 4 and Fig 11).

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 8). 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): In our previous report, we focused on MEG Resting State (RS) functional connectivity of the motor system (M1, S1, SMA, SP) to the rest of the brain in the alpha band. We found significant differences between the FXS and Control groups in connections to the pars opercularis, a key structure in Broca's area/inferior frontal gyrus (IFG) responsible for speech production. Stronger connectivity of the FXS group compared to the

Accomplishments (cont.)

Control subjects, to the pars opercularis on the right rather than on the left also raised questions about atypical or weaker lateralization of language in FXS. These results were presented at International Society for the Advancement of Clinical MEG conference in Toronto last year (Mody et al. (2019). Resting State Functional Connectivity in Fragile X Syndrome). Motivated by these findings and known deficits in speech and language in FXS, our current analyses go beyond the alpha band to examine the connectivity of the pars opercularis not just to select motor areas (viz., SP and SMA) but also other receptive and expressive language areas (viz., pars triangularis, superior temporal, transverse temporal/Heschl's gyrus) and in multiple frequency bands (theta, beta and gamma) for a comprehensive understanding of the breakdown in language and potential relations with sensorimotor dysfunction in FXS.

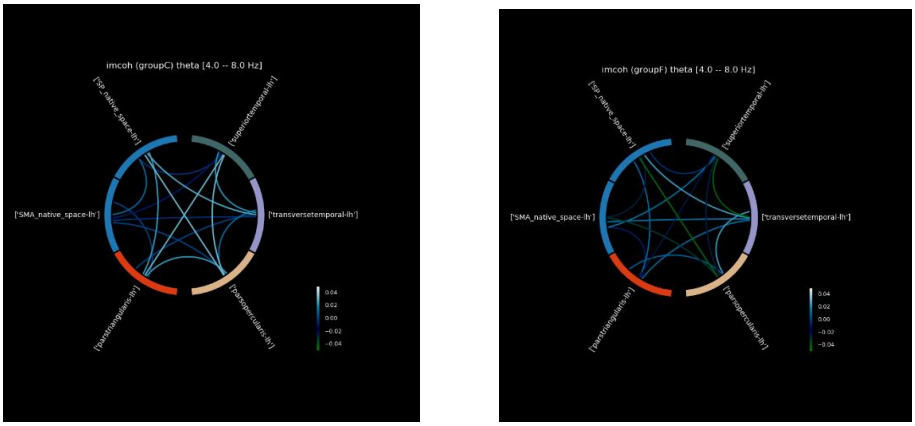
We conducted imaginary coherence (imCOH) analysis of the MEG RS data (5 min, eyes open paradigm) to guard against partial volume current effects, drawing on time-frequency decomposition (Wavelet analysis) across the power spectrum. We found significant differences ($p<.05$) between the groups in select connections and frequency bands. Whereas the FXS participants showed stronger connections than the control participants within this network of motor and language areas, (see Fig. 11), the strength of the connection between the pars opercularis and superior temporal region showed an interesting flip across theta and gamma bands: compared to the control group, the FXS group showed weaker connectivity in theta band ($p=0.019$) but stronger connectivity in the high gamma (60-90 Hz) band ($p=0.051$) for this connection. None of the other connections showed this reversal between the groups across bands.

Theta oscillations play a significant role in memory, providing top-down control from frontal to hippocampal areas of memory encoding and retrieval functions, thought to be mediated by attention. This dovetails well with our findings of stronger fronto-temporal theta band connectivity in control participants, poorer behavioral performance of our FMR1-KO mice on the Morris Water Maze test, and reduced [^{18}F]FPEB binding potential in the hippocampal region in the FXS compared to the control group. Gamma rhythms, on the other hand, may be reflective of local excitation-inhibition interactions in the brain; our finding may serve as a potential signature of excessive mGluR5 expression in keeping with the mGluR5 theory of FXS.

Accomplishments (cont.)

Theta (4-8 Hz): Control > FXS in pars opercaularis- superior temporal connectivity

Controls FXS



High Gamma (65-90 Hz): FXS > Control in pars opercaularis- superior temporal connectivity

Controls FXS

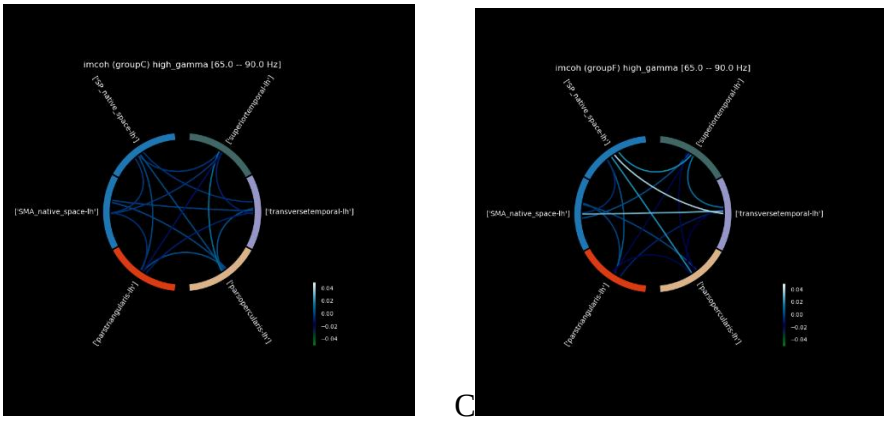


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Figure 11: Resting State MEG: Functional connectivity between select motor and language regions in theta and high gamma frequency bands, using imaginary coherence analysis, in FXS and age-and gender-matched Control groups.

MEG analysis of button press data with alternating left and right hands for 5 min yielded no significant difference between the groups in behavioral performance or amplitude of the brain response to the button press, neither in motor preparatory phase (-40ms-0 ms; $p = 0.09$) nor in the post-button press response period (0-100 ms) ($p= 0.08$).

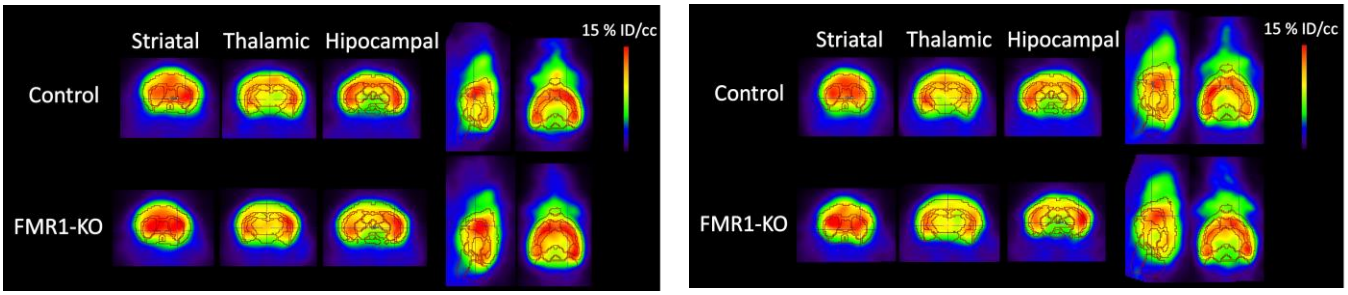
Taken together, results from multimodal imaging (DTI, PET, MEG) appear to converge on a neurobiological model of FXS suggesting a potential role for a fronto-hippocampal theta circuit.

Accomplishments (cont.)

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 are now in process as well as the final data analyses of behavioral and PET imaging data of mGluR5 expression.

(a) Saline treatment

(b) mGluR5 agonist (CDPPB) treatment



(c) mGluR5 antagonist (MTEP) treatment

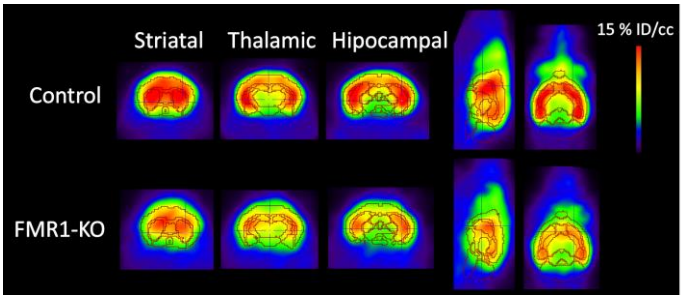


Figure 12. Distribution of [^{18}F]FPEB binding in three coronal (striatal, thalamic and hippocampal brain areas), sagittal and axial levels in FMR1-KO and control mice 3 months after (a) the saline injections as a control treatment; (b) using mGluR5 agonist (CDPPB) treatment and (c) using mGluR5 antagonist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

Accomplishments (cont.)
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.

During our ongoing recruitment process, the study and its potential benefits have been made known to various developmental disability school programs and the staff that work with individuals with FXS, as well as to disability organizations via parents of the participants.

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 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. During the upcoming one-year extension we will finalize all the experimental outcomes of the project and the results will be published in several high profile journals.

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).

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 pharamacological 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.

We were able to complete experimental *in vivo* studies in mice for specific aim 2 before we had to close the facility because of Covid 19. Since we had to close activities on March 9, 2020 we had to euthanize all the mice and the brains are waiting for immunohistochemical studies in order to complete all experimental studies for specific aim 2. Statistical data analyses of longitudinal behavioral measures and PET imaging studies of mGluR5 expression are ongoing.
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 7% due to a variety of reasons including personal family reasons and difficulties with compliance with the test procedures. We have extensively worked with different patients groups, the PCPs/clinical providers to expedite enrollment subjects for the project. Also we have not been able to do research patient studies since Covid 19 since March 9, 2020. Under the new reatricted reopening guidelines by the state effective on August 1, the first patient studies will be done on August 10, 2020.

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.

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

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

Despite the tight state and institutional restrictions, we will continue to offer practice sessions supplemented by virtual feedback session to help with compliance on the day of the actual scan.

Significant changes in use or care of vertebrate animals

Expanded follow up time up to one year of age of behavior and mGluR5 expression in the FXS and control mice as explained above.

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. "Behavior and mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" Submitted to Neuropsychopharmacology, 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 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.*

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.

- **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 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:	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:	
Project Role:	Michael Whalen “no change”
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:	Sevda Lule “no change”
Project Role:	
Research Idendifier:	
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://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) 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

Afshar,S., Lule, S., Yuan, G., Qu, X., Pan, C., Whalen, M., Brownell, A-L., Mody. M. "Behavior and mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model" Submitted to Neuropsychopharmacology. (21 ppages)

Behavior and mGluR5 expression in Fragile X syndrome: a longitudinal study using a FMR1 Knockout mouse model

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Abstract

Fragile X syndrome (FXS) is a monogenic developmental disorder caused by the silenced Fragile X Mental Retardation 1 (FMR1) gene. In the face of failures of clinical trials with FXS, we focus on PET imaging of metabotropic glutamate subtype 5 receptor (mGluR5) function in FMR1 knockout (FMR1-KO) mice and their performance on four different behavioral tests, including Morris Water Maze (MWM) test, rotarod tests, elevated plus maze, and open field test, towards developing a biobehavioral marker for FXS. A cohort of FMR1-KO mice and age- and gender-matched cohort of healthy control mice underwent PET imaging to examine mGluR5 expression, implicated in FXS, in combination with performance on four behavioral tests at three ages. ANOVA of the PET data revealed significant disorder status x brain area interaction effects. These interactions emphasize that the key brain areas wherein a significant difference is observed between FMR1-KO and control mice are cortex, hippocampus, and olfactory bulb with binding potential (BP) decreased in FMR1-KO mice. ANOVA of the MWM data yielded a significant age x disease status interaction effect: at later ages, both male and female FXS mice showed significantly slower MWM latencies than the control mice as well as slower learning rate, in keeping with induced BP in both cortex and hippocampus in FMR1-KO mice. ANOVA of other behavioral tests indicated the effect of gender in behavioral alteration as disorder progresses. These findings reflect biobehavioral vulnerabilities predicted on the basis of disease status and hold exciting potential as targets for pharmacological intervention.

1. Introduction

Fragile X syndrome (FXS) is a monogenic developmental disorder caused by mutations of the Fragile X Mental Retardation 1 (FMR1) gene. The silenced FmR1 gene leads to suppression of the fragile x mental retardation protein (FMRP) [1]. As such, individuals with FXS fail to produce normal levels of the FMRP due to defective FMR1 gene functioning, which give rise to intellectual disability and other neurological symptoms.

Individuals with FXS typically have mild to moderate learning disability and are characterized by multiple deficits including cognitive impairments not limited to visuospatial processing deficits, speech and language delays, and attention problems, [2–4]. The cognitive phenotype also includes deficits in executive function, short-term memory, and impulse control [5]. The behavioral phenotype might include anxiety, hyperactivity and social interaction deficits [6]. There is no single dominant behavioral or cognitive deficit associated with FXS and to date, no specific medication for treatment of the disorder.

Current pharmacological treatments for FXS draw on medications designed for other disorders that share similar symptoms with FXS [2–4]. Understanding the neural mechanism underlying the disorder and alterations in the brain activity with progression of the disorder, are key to identifying the main factors contributing to the imbalanced neurocircuitry implicated in FXS. These factors can help optimize treatment by efficient targeting of the affected neural systems. In other words, finding an effective and targeted treatment requires identification of a robust biomarker to assess the progression of the disease and its response to intervention.

Behavioral tests have been widely used to assess fragile X phenotypes and track response to different treatments, [7–11] . Among the various behavioral tests used in preclinical studies of FXS, the more well-known ones include the Morris Water Maze (MWM) test, which is used to measure hippocampal-dependent learning, including spatial memory and long-term memory [12]; the rotarod test to measure striatal dependent motor learning and balance [13], the elevated plus maze (EPM) test to assess anxiety level and anxiety-related behaviors [14], and open field (OF) test to index general locomotor activity level and willingness to explore [15]. Whereas behavioral tests provide valuable metrics of symptom severity for use in tracking progression of a disorder, neuroimaging methods like positron emission tomography (PET) allow us to probe the underlying neurobiological mechanism for potential use in clinical trials towards improving outcomes. To this end, Bear and colleagues [16] put forth a metabotropic glutamate receptor 5 (mGluR5) theory of FXS.

According to the mGluR5 theory, suppression of the FMRP results in exaggerated mGluR5 signaling, affecting relative glutamate and GABA levels and creating excitatory-inhibitory neurotransmission imbalance. Increased protein synthesis occurs and induces formation of excessive long and thin dendritic spines resembling immature cortical networks [17–20] and deficits in neurogenesis and synaptic maintenance [21].

A member of the G-protein-coupled receptor family, mGluR5 is expressed post-synaptically and involved in modulating synaptic transmission and thus neuronal excitability. Due to its regulatory role in synaptic maintenance, impaired expression of mGluR5 leads to disorders with strong developmental origins, like Fragile X and Autism Spectrum Disorders and hence serves as an intervention target.

Based on these findings, there has been a growing interest in pharmacological methods to modulate mGluR5 expression as a potential approach to slowing the progression of the disorder. Different drug agents targeting mGluR5 receptors have been developed and tested in both pre-clinical [8,9] and clinical studies [22,23].

In order to understand how mGluR5 functioning and related glutamate transmission in the brain is affected in individuals with FXS, it is essential to investigate the receptor's availability in vivo. To this end, PET can be used with mGluR5-targeted radiolabeled ligands to measure receptor density, known as binding potential. PET imaging has been utilized to investigate the role of the mGluR5 in human studies involving a variety of disorders; for example, schizophrenia [24,25], Alzheimer's disease [26,27], and autism [28]; and in mouse and rat models, for example., Parkinson's disease [29,30], amyotrophic lateral sclerosis (ALS) [31]. However, mGluR5 receptor density in human studies of FXS has primarily been examined in postmortem tissue [32] and not in-vivo. Promising findings from preclinical studies using drugs to block mGluR5, have failed to extend to human subjects in clinical trials, raising questions about our understanding of the impaired mechanism and its relationship to the symptoms.

In this study, we use [^{18}F]FPEB (3-fluoro-5-(2-pyridinylethynyl) benzonitrile) [33] a compound with high specificity and binding affinity for mGluR5 to assess the glutamatergic system in FXS using a FMR1-knockout (FMR1-KO) mouse model.

In the first part of this study, we conducted behavioral tests at 1 month, 5 months and 1 year of age in FMR1-KO and gender- and age- matched wild type (WT) mice, targeting the progression of core deficits in spatial learning, memory, hyperactivity, anxiety and attention associated with FXS. The tests included MWM, rotarod, EPM, and OF tests known to tap these functions. Next, the mice underwent PET imaging with [^{18}F]FPEB to measure mGluR5 density. To the best of our knowledge, we are among the first to systematically examine differences in mGlu5 receptor density, *in vivo*, in age and gender matched FMR1-KO and WT mice; in relation to behavioral differences between the mouse groups, associated with symptoms of the FXS, and over the course of the disease using a longitudinal study design. The overarching objective of this study is to develop a biobehavioral marker specific to FXS for use in drug development and testing and improving outcomes of clinical trials.

2. Methods

2.1 Experimental animals

The animal studies were approved and done under the guidelines of the Subcommittee on Research Animals of the Massachusetts General Hospital and Harvard Medical School in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Altogether 56 mice, 28 FMR1-KO mice (B6.129P2-Fmr1tm1Cgr/J) and 28 WT mice (C57BL/6NJ) comprising 14 male and 14 female mice in each group, were purchased from the Jackson Laboratories, Maine, at 4 weeks of age. After acclimation, the first set of behavioral studies were conducted between 34-41 days of age followed by PET imaging at ages 48-55 days. Behavioral and PET imaging studies were repeated two more times within one year to track the progression of the disease. The data were divided into 3 groups corresponding to the age of the mice: less than 55 days (group 1), more than 55 days but less than180 days (group 2) and more than 180 days (group 3). During the one year follow up, about 20% of the mice were lost mainly because of their aggressive behavior and fighting. In the last round of experiments there were 24 FMR1-KO (12 male and 12 female mice) and 20 WT (9 male and 11 female).

The animals were typically housed in groups of 4 mice per cage, of the same gender and genetic background. The housing and other environmental resources were standardized to avoid extraneous influences on behavioral measures. Additionally, behavioral studies were always conducted before the PET imaging studies to avoid the effects of anesthesia and imaging induced stress on behavioral performance.

2.2 Behavioral tests

Behavioral studies including Morris water maze, rotarod, elevated plus maze and open field tests were performed during a one-week period and were followed by PET imaging studies to investigate mGluR5 expression.

The behavioral studies were repeated at three timepoints corresponding to the specific age ranges at testing, and conducted before the imaging studies.

2.2.1 Morris Water Maze test

The MWM test [34] is primarily a test of spatial learning and reference memory [12]. Mice were placed in a pool of water, in one of four random starting positions per trial, and were allowed to locate and rest upon a hidden platform within an allotted time of 90 sec on each trial to escape the water. A probe trial was used to measure retention of the hidden platform's location based on how long the mice spent in the target quadrant which had contained the hidden platform, when returned to the pool 24 hours after completion of the hidden trials. A total of 7 hidden trials were performed, 1-2 trials/day.

A visible platform test was conducted to assess any difference in visual acuity or sensorimotor function between the two groups of mice and its impact on spatial learning latencies of the two groups. Two visible platform trials were performed.

2.2.2 Rotarod test

The rotarod test [35] measures motor coordination and learning [13]. In this test, the mouse is placed on an automated rotarod apparatus (Harvard Apparatus), which accelerates from 4 to 40 rpm over 60 sec. Each mouse was assessed three times per day, with a five minute rest intervals in between trials, for three consecutive days. Maximum trial duration was 300 seconds, or until the mouse fell off the rotating rod.

2.2.3 Elevated Plus Maze (EPM)

The EPM test [36] is widely used with rodents to assess the anxiety responses and mechanism underlying anxiety-related behavior [14]. The apparatus consists of two 130 - 8 cm platforms with an 8 - 8 cm square area at their intersection, elevated at 60 cm above ground. The closed arms of the platform had 10-cm walls, whereas the open arms had none. Each mouse was placed in the central area of the maze and video-recorded for five minutes to estimate the amount of time spent in the different sections of the maze (open arm, closed arm and center platform)

2.2.4 Open Field test

The OF test [37] is a common measure of general locomotor activity and willingness to explore [15]. In the test the movement of the rodent in an open field within a 30-minute period is video recorded and the distance travelled calculated from it using ANY-maze software (Stoelting Co., Wood Dole, IL).

2.3 PET imaging

In preparation for PET imaging, animals were first anesthetized with isoflurane/ oxygen (1.5-2% isoflurane with oxygen flow of 2L/min) and the tail vein was catheterized for injection of the radioligand. The mouse was positioned in the microPET scanner (Triumph II, Trifoil Imaging, Inc) and administered with [^{18}F]FPEB (150-200 uCi i.v.). Dynamic volumetric imaging data were acquired for 45 mins followed by CT scan to obtain data for attenuation correction and anatomical registration of the PET data.

The PET data were processed using maximum-likelihood expectation-maximization (MLEM) algorithm with 30 iterations of dynamic volumetric images (9x20", 7x60" and 7x300"), and corrected for uniformity, scatter, and attenuation. CT data were processed using the modified Feldkamp algorithm with a 512×512×512 matrix volume and pixel size of 170 μm. Co-registration of CT and PET images and analysis of PET images were implemented with PMOD 3.2 software (PMOD Technology, Zurich, Switzerland).

Finally, the six regions of interest (viz., striatum, cortex, hippocampus, thalamus, hypothalamus and olfactory bulb) implicated in FXS [4,38–40], were rendered on all coronal and axial slices by fusing borders of different brain areas from the Allen mouse brain atlas with the fused CT-PET images. Time-activity curves were extracted from different brain areas and the values for the binding potential calculated using the cerebellum as a reference area.

2.4 Statistical analysis

The data were submitted to multivariable analysis and t-tests to evaluate the effects of independent variables (disease status, age, and gender) on behavioral measurements and regional ^{18}F-[FPEB] binding potential. All the statistical analysis was done in MATLAB (2018a).

3. Results

Below we report significant findings on the behavioral tasks as well as with PET imaging, focusing on results involving disease status. A complete list of all significant findings may be found in Table 1. All reported significances ($p < 0.05$) met criteria for multiple comparisons.

3.1. *Behavioral Findings*

Morris Water Maze

The latencies and swim speeds of the mice were recorded under three test conditions: hidden platform, probe and visible platform.

A multivariate analysis (disease status, sex, and age) of the latencies yielded a significant three-way interaction (F (2,130) = 3.44; p = 0.035). The interactions between age and gender (F (2,130) = 9.98, p = 9.2e-5) as well as disorder status and age (F (2,130) = 7.40, p = 0.009) were also significant; however, there was no interaction between gender and disorder status (F (1,141) = 8.0, p = 0.375). Post-hoc t-tests revealed a significant difference between the groups, with FMR1-KO mice taking significantly longer to locate the hidden platform than WT mice at age group 2 (FMR1-KO: 27.4 sec ±11.6 , WT: 16.7 sec ± 6.2*; p* = .00005) and age group 3 (FMR1-KO: 31.6 sec ±13.2, WT: 19.8 sec ± 9.9; p = . 0004) (see Fig. 1). Both groups showed reduced latencies over the course of the seven learning trials. An exponential function fit to the data showed that the rate of learning to locate the hidden platform was marginally reduced in the FMR1-KO mice compared to WT mice at age group 2:(WT: 0.21 ± 0.08 , FMR1-KO: 0.15 ± 0.08; p=0.024) and age group 3 (WT: 0.25 ± 0.11, FMR1-KO: 0.19 ± 0.9; p=0.075) Analysis of performance on the probe test yielded a main effect for disorder group (F (1,141) = 11.3, p = 0.027). The WT mice spent more time than the FMR1-KO mice in the target quadrant (WT: 11.5 sec ± 5.65; FMR1-KO: 9.21sec ± 3.55, p = 0.019). The visible platform condition yielded a significant age x gender interaction (F (2,130) = 4.14, p = 0.018). There were no other main effects or interaction effects.

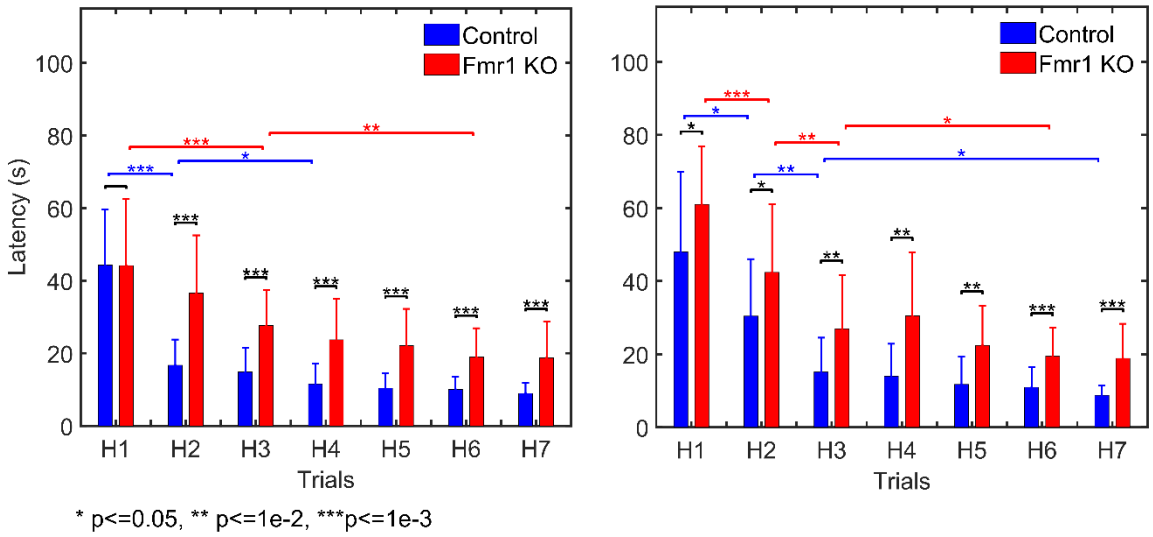


Figure 1: MWM hidden platform latencies of WT and FMR1-KO mice at age groups 2 and 3.

Analysis of swim speed in the hidden platform condition also yielded significant two-way interactions between disorder group and age (F (2,140) = 5.90, p = 0.003) as well as age and gender (F (2,140) = 6.53, p = 0.012) but not between disorder group and gender (F (1,141) = 0.03, p = 0.858). Based on post hoc t-tests, FMR1-KO mice swam more slowly than WT mice at age group 2 (FMR1-KO: 8.37 cm/s ± 2.14, C: 10.23 cm/s ± 2.26; p = 0.007) and at age group 3 (FMR1-KO: 7.98 cm/s ±1.86, WT: 9.97 cm/s ± 1.73; p = 2.3e-5) (see Fig. 2). In the visible platform condition, there was a significant interaction between disorder group and age (F (2,140) = 5.04, p = 0.008) but no other two way interactions. (F (2,140) = 46.8, p = 0.137; F (1,141) = 12.1, p = 0.747). Compared to the WT mice, FMR1-KO mice had slower swim speeds at the older ages (age group 2: FMR1-KO = 7.06 cm/s ± 1.9, WT = 9.23 cm/s ± 1.8; p = 1.3e-5; age level 3: FMR1-KO = 6.48 cm/s ± 1.3, WT = 9.37 cm/s ± 1.5; p = 6.4e-9), but not in age group 1. The FMR1-KO mice also had slower swim speed than WT mice in the probe condition, but only in the male group. There was a significant two way interaction between disease status and gender (F (1,141) = 3.57, p = 0.051). The interaction between disease status and age was also significant (F (2,140) = 6.50 p = 0.002) as well, but not between age and gender (F (2,140) = 1.98, p = 0.142).

The post-hoc t-test showed a significant difference between WT and FMR1-KO (p = 0.0001) in male mice, with WT (15.7 cm/s ± 2.5) swimming faster than FMR1-KO mice (13.1 cm/s ± 2.4). Furthermore, at age group 2 and 3 there was a significant difference in swim speed between WT and FMR1-KO (p=0.008 at age group 2 and p=9.3e-5 at age group 3). In both age groups, WT mice swam faster (15.9 cm/s ± 2.0 at age group 2 and 16.1 cm/s ± 2.3 at age group 3) than

FMR1-KO mice (13.7 cm/s ± 3.3 at age group 2 and 12.6 cm/s ± 3.0 at age group 3) in the probe test.

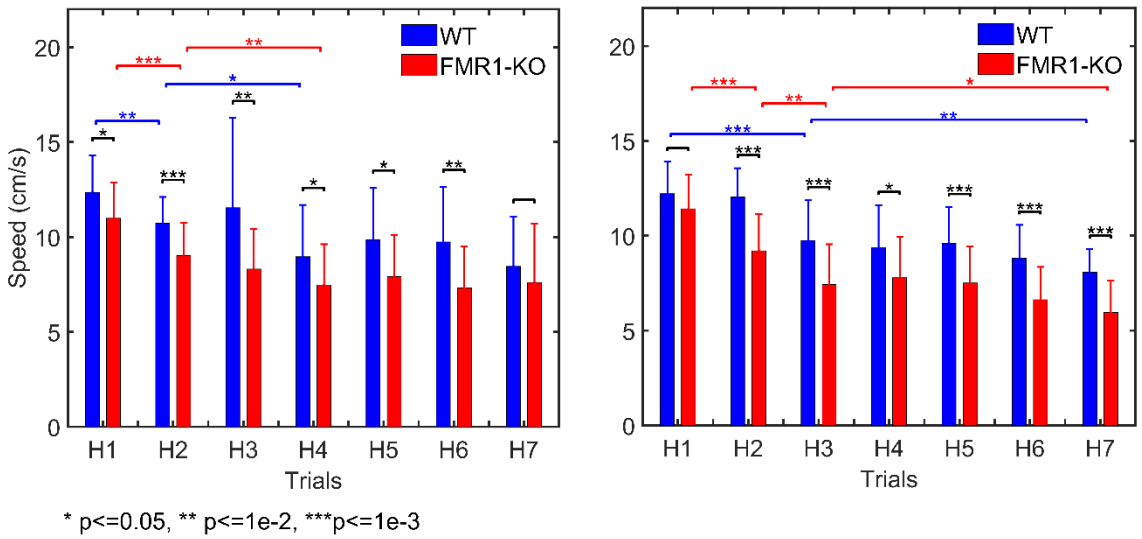


Figure 2: MWM hidden platform swim speeds for WT and FMR1 KO mice at age groups 2 and 3.

Rotarod Test:

Analysis of the different mouse groups' performance revealed a significant interaction between disorder group and gender (F (1,137)=5.75, p=0.017). Female FXS mice had significantly longer latency on the rotating rod than their male FXS counterparts (46.9 sec ±16.8 vs. 33.8 sec ±11.5; p=.003). There was also a main effect for age (F (2,136)=11.31, p=2.8e-5); no other main or interaction effects were significant ($F_{\text{gender} \times \text{age}}$ (2,136)=15.3, p=0.706; $F_{\text{disorder} \times \text{age}}$ (2,136)=22.3, p=0.299.

Elevated Plus Maze:

Behavior of the mice in the open and closed arms as well as the Center platform reflected different response patterns in the two groups of mice. Analysis of the time spent in the open arm of the maze revealed significant interactions between gender and age (F (2,138) = 2.88, p = 0.059) as well as gender and disorder group (F (1,139) = 4.77, p = 0.03). In post-hoc testing, we found male FMR1-KO mice spent less time (3.5% ± 4.3) than female FMR1-KO mice (7.1% ± 6.2) in the open arm of the maze (p = 0.004) (see Fig. 3). There was no such gender difference in the WT group (p = .678). In the closed arm condition, there was a main effect for disorder (F (1,139) =11.78, p = 0.0007). FMR1-KO mice spent less time than WT mice in the closed arm, regardless of age or gender. There was also a significant interaction between age and disorder

group for the Center platform of the maze (F (2, 138) = 4.92, p = .008); the FMR1-KO mice spent more time than WT mice in the Center section of the maze in the youngest age group (p = .020)

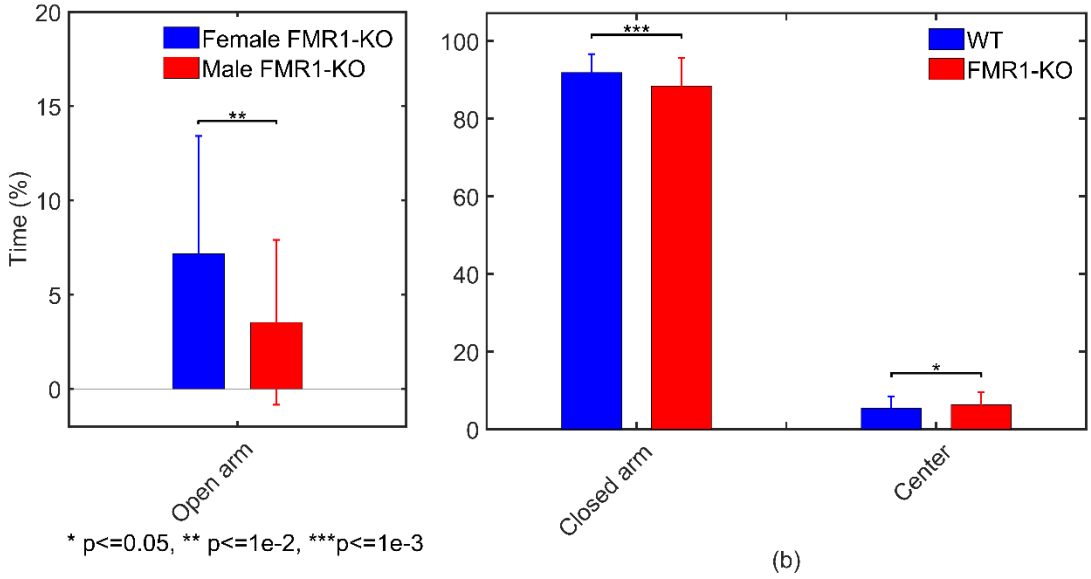


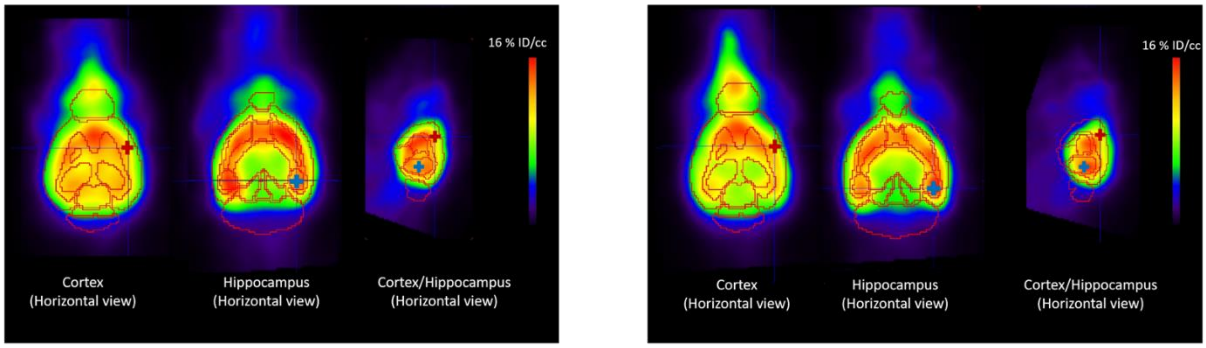
Figure 3: : Comparison of the time spent in (a) open arm between female and male FMR1-KO mice, and (b) closed arm between FMR1-KO and WT mice and in center platform between FMR1-KO and WT mice in the youngest age group on the elevated plus maze test.

Open Field Test

The locomotor activity of the mice within a 30 minutes period after being paced 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)

3.2 PET Results

Multivariate analysis of [^{18}F]FPEB binding potential revealed differences between the FMR1-KO and WT mice in multiple brain areas (Fig 4). Below we report significant findings (p<.05), meeting multiple comparison correction criteria.



WT male FMR1-KO male

Figure 4: [18F]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)

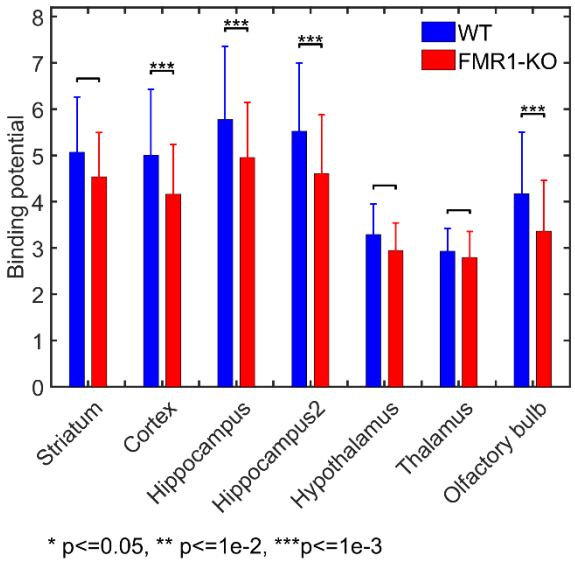


Figure 5: Comparison of [^{18}F]FEB BP in WT and FMR1-KO mice in different brain areas.

4. Discussion

Metabotropic glutamate receptors have been implicated in the pathogenesis of a variety of neuropsychiatric disorders. Of particular interest here is mGluR5, the exaggerated signaling of which appears to account for the sensory and cognitive impairments that characterize Fragile X syndrome. A reduction in mGluR5 signaling by drugs that block these receptors has worked in mice, reducing the number of seizures and improving functional connectivity in sensorimotor networks but has been less effective in social and cognitive domains [41]. The present study examined mGluR5 activity using PET imaging with [^{18}F]FPEB and behavioral performance on a number of tasks in FMR1 knockout mice and age-matched WT mice, incorporating both males and females, followed longitudinally, to better understand brain-behavior relationships at the molecular level in FXS and changes with progression of the disorder. We found that FMR1-KO mice typically did worse than the WT mice on all the tests; they also had lower mGluR5-related binding potentials in the brain. Below we explore these findings in greater detail.

Behavioral Findings: Interpretation and implications

The Morris Water Maze test is widely used in preclinical studies to evaluate spatial learning and memory under different conditions. The longer average swim latencies and slower swim speed of the FMR1-KO mice compared to WT mice on the hidden platform task imply that motor dysfunction might confound interpretation of the hidden platform latency in terms of cognitive dysfunction. 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 also observed in other studies [42,43]. 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. Taken together, performance of the mice on the various components of the MWM test are in keeping with learning deficits observed in FXS [44,45] .

Performance on the Rotarod, Elevated Plus Maze (EPM) 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. This was in keeping with expectations in that male FMR1-KO mice are more severely affected than females with the disorder; given the absence of an unaffected second X chromosome to make up for the affected one as found in females. On the rotarod task, female FMR1-KO mice held on longer than male Fmr1 mice to the horizontal rod which rotated at increasing speeds, reflecting their superior motor coordination and balance. Clinical features of FXS include motor coordination [46,47],

and human studies show lower scores on motor scales in FXS subjects compared to age-matched peers [48,49]. Similarly,

Anxiety is a frequently reported core symptom in FXS [50,51]. As on the Rotarod test, male FMR1-KO mice were more affected, spending less time than female FMR1-KO mice in the open arm section of the EPM, 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: surprisingly, FMR1-KO mice spent less time than WT mice even in the closed arm, regardless of age or gender. We speculate that the height of the elevated maze made FMR1-KO mice anxious and led to their spending less time than WT mice in both open and closed arms of the EPM; and the increased vulnerability of the male FMR1-KO mice contributed to the disorder x gender interaction in the open arm; in contrast, the FMR1-KO mice spent more time than the WT group in the Center piece of the maze which was evident at the youngest age (in group 1) reflecting their anxiety at the very outset, in initiating movement to either the open or closed arm.

In the Open Field test which measures general locomotor activity level and a willingness to explore, we found a significant difference between FMR1-KO and WT mice in both males and females, regardless of age. Female WT mice travelled longer distances than female FMR1-KO mice whereas the results were reversed in the male group: the FMR1-KO mice were more active, covering longer distances than the WT mice in keeping with hyperactivity symptoms associated with FXS, which, not surprisingly, was more evident in males with the disorder, as found in other studies [11,52–56]. The findings, however, have not been consistent in the literature and warrant further study.

PET Findings: Behavior and the mGluR5 theory

Our findings revealed overall lower mGluR5 binding by [^{18}F]FPEB, a radioligand selected for its high specificity and affinity to mGluR5, in the FMR1-KO mice compared to the WT mice. Specifically, we found significant differences in BP between the two groups of mice in cortex, hippocampus and olfactory bulb, emerging in age group 2 at around 5 months of age.

The brain areas implicated in our study dovetail well with known deficits in in individuals with FXS. Importantly, the FMR1-KO and WT mice in our study showed significant differences on a number of behavioral tests, of spatial memory, learning, anxiety, neuromuscular coordination, and motor activity reflecting the mGluR5-implicated areas in PET imaging with these mice. That these differences in behavioral performance and corresponding [^{18}F]FPEB binding potential were modulated by age and/or gender effects shed light on the nature of the disease and its progression, while providing potential mechanistic insights for use in treatment design and pharmacological intervention.

FXS has been associated with alterations in sensory development and function [48,49]. As such, BP differences in the olfactory bulb related to mGluR5 dysfunction in FXS may contribute to the sensory impairments in the disorder, including decreased sensitivity to odors [40]. This finding

may also be associated with the anxiety of the FMR1-KO mice on the Elevated Plus Maze test, associated with the amygdala, which is known to be connected to the olfactory bulb. Interestingly, the amygdala, olfactory bulb, and hippocampus jointly play a role in emotion, memory and learning; as such, our results suggest a circuit-specific impact and point to a use of brain networks as potential targets in treatment [57,58]. The findings here also provide an opportunity for insights into other neurodevelopmental disorders like autism spectrum disorder which shares several deficits with FXS in language, learning and sensorimotor domains [59] while providing interesting contrasts in the visuospatial domain [60]

Conclusion and Future Directions

In conclusion, the use of a longitudinal study design, including both male and female mice with FXS, and their performance on a comprehensive set of behavioral tasks accompanied by PET imaging, has yielded some important insights about this disorder. Whereas the impact of FXS on sensorimotor systems appears to affect males more severely than females with the disorder, the impact on cognitive skills like memory and learning appears to be affected by age; emerging at around 5 months of age, providing a potential time window for treatment efficacy based on the results in the present study. Future studies of humans and mice affected by FXS would also benefit from connectivity analyses that explore subcortical circuitry involving the hippocampus and amygdala as potential outcomes of pharmacological treatment.

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Author contributions: ALB, MW and MM planned experiments and drafted this work. SA, SL, GY, XQ, and CP conducted experimental studies.SA, SL, ALB and MM analyzed the data. SA and MM did statistical analyses. SA and SL composed the manuscript under supervision of MM and ALB. All authors contributed to critically revising this work and approved the final version.

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Table 1: Main findings of PET imaging

Comparison of interest	*p*-value	Significant effect (Binding potential)
Age×Brain area	6.3e-10	
In cortex, WT versus FMR1-KO mice	3.9e-6	FMR1-KO <WT 4.508±0.915<5.013±1.166
In hippocampus, WT versus FMR1-KO mice	1e-5	FMR1-KO <WT 4.757±1.135<5.666±1.527
In olfactory bulb, WT versus FMR1-KO mice	1.8e-6	FMR1-KO <WT 3.329±0.988<4.177±1.327
In cortex, Disorder x Age	0.047	FMR1-KO<WT at age 2 4.592±0.809 < 6.044±1.219

Table 2: Main findings of behavioral tests

Comparison of interest	P-value	Significant effect	
1.1. MWM test latency (hidden platform)		**Average latency (s)**	**Learning rate (exponential rate)**
Disorder×Gender×Age	0.035		
Gender×Age	9.2e-5		
Disorder×Age	0.0009		
At age group 2, WT versus FMR1-KO mice	0.00005	FMR1-KO >WT 27.4±11.6>16.7±6.2	FMR1-KO <WT 0.15 ± 0.08<0.21 ± 0.08
At age group 3, WT versus FMR1-KO mice	0.0004	FMR1-KO >WT 31.6±13.2 >19.9±9.9	FMR1-KO <WT 0.19 ± 0.9<0.25 ± 0.11
At age group 1, female versus male	5.3e-5	Female>Male 36.8±20.2 >20.0±10.8	Difference is not significant
1.2. MWM test latency (probe)		**Average latency (s)**	
Disorder WT versus FMR1-KO mice	0.019	WT> FMR1-KO 11.51±5.65>9.21±3.50	
1.3. MWM test latency (visible platform)		**Average latency (s)**	
Gender×Age	0.018		
At age group 1, Male versus female mice	0.013	Female>male 15.10±7.50>11.50±3.70	
1.4. MWM test speed (hidden platform)		**Average velocity (cm/s)**	
Gender×Age	0.012		
Disorder×Age	0.003		
At age group 2, WT versus FMR1-KO mice	0.007	FMR1-KO <WT 8.37±2.14<10.23±2.65	
At age group 3, WT versus FMR1-KO mice	2.3e-5	FMR1-KO <WT 7.98±1.86<9.97±1.73	
At age group 2, female versus male	0.024	Female>male 9.53±2.48>8.87±2.51	
1.5. MWM test speed (visible platform)		**Average velocity (cm/s)**	
Disorder×Gender×Age	0.015		

Disorder×Age	0.008	
At age group 2, WT versus FMR1-KO mice	1.3e-5	FMR1-KO <WT 7.06±1.9<9.3±1.8
At age group 3, WT versus FMR1-KO mice	6.4e-9	FMR1-KO <WT 6.48±1.3<9.4±1.5
1.6. MWM test speed (probe)		
Disorder×Age	0.002	
Disorder×Gender	0.051	
In male mice, WT versus FMR1-KO mice	0.0001	FMR1-KO <WT 13.1±2.5<15.7±2.6
At age group 2, WT versus FMR1-KO mice	0.008	FMR1-KO <WT 13.7±3.2<15.9±2.0
At age group 3, WT versus FMR1-KO mice	9.3e-5	FMR1-KO <WT 12.6±3.0<16.1±2.3
2. Rotarod test		**Staying time (s)**
Disorder×Gender	0.017	
Age	2.8e-5	Age group1>age group3>age group2 46.79±13.89>35.29±14.14> 31.51±8.55
In FMR1-KO mice, female versus male	0.003	Female>male 46.9±16.8>33.8±11.5
3.1. Elevated plus maze (open arm)		**Spent time (%)**
Disorder×Gender	0.030	
Gender×Age	0.059	
In female mice, WT versus FMR1-KO mice	0.0008	FMR1-KO >WT 7.1±6.2>2.9±3.8
In FMR1-KO mice, male vs. female	0.004	male< female 3.5±4.3 < 7.1±6.2
At age group 2, female versus male	0.003	Female>male 6.4±4.9>2.6±3.1
3.2. Elevated plus maze (closed arm)		**Spent time (%)**
Disorder WT versus FMR1-KO mice	0.0007	WT> FMR1-KO 91.87±4.78>88.40±7.31
Gender Male versus female mice	0.056	Male>female 90.99±5.52>89.10±7.16
4. Open field test		**Distance traveled (m)**
Disorder×Gender	3.3e-6	
In FMR1-KO mice, males versus females	0.013	males>females 35.9±10.8>30.3±7.5
In WT mice, males versus females	0.0001	Female>male 43.8±15.4>25.4±20.2
In male mice, WT versus FMR1-KO mice	0.008	FMR1-KO >WT 35.9±10.8 >25.4±20.2
In female mice, WT versus FMR1-KO mice	2.4e-5	FMR1-KO <WT 30.3±7.5 <43.8±15.4