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Developmental Acoustic Exposure as a Novel Approach to Treat Fragile X Syndrome

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: The main goal of the proposed studies was to test the hypothesis that a combination of sound exposure and minocycline treatment provided to the mouse model of Fragile X Syndrome (FXS), will cause a long lasting benefit in terms of sensory hypersensitivity and behaviors as adults. The current project period has been highly productive, despite the slow recovery from the pandemic-related limits on in-person training activities. The collaborative group of this project (Razak/Ethell/Binder labs) has published four papers and two reviews on FXS in 2021-22. In Rais et al (2022), we showed that post-natal deletion of FMRP leads to cortical EEG deficits in Fmr1 KO mice, and more importantly, post-natal re-expression of the protein was sufficient to reverse EEG, cellular and behavioral phenotypes. The main implication is that optimal treatment windows can be developed at post-natal ages in FXS. This was one of the major aims of the project. In Pirbhoy et al (2021), we showed that manipulation of the endocannabinoid system by increasing 2-AG reduces cortical hyperexcitability and behavioral measures of anxiety in Fmr1 KO mice. Likewise, in Jonak et al., (In Press), our group showed that GABAb receptor agonist, baclofen, reduced EEG abnormalities in Fmr1 KO mice. These studies serve to identify both EEG outcome measures and novel treatments to reduce symptoms in FXS. In Razak et al., (2021), we review the literature on how EEG measures in rodents and humans can provide translation relevant biomarkers for both circuit exploration and treatment development. In Ethell et al (2021) we review the role of interneurons in autism circuit abnormalities, highlighting the PV dysfunctions in FXS we have discovered. We highlight many of the mechanisms and therapeutic pathways we have identified in the project periods as a paradigm for translational neurodevelopmental disorders work. These papers provide the foundation for the current grant in testing the same phenotypes following sound exposure and minocycline. We have a paper that shows peripheral cochlear function is not different between WT and KO mice that is currently being submitted. This study indicates central auditory processing changes underlie hyperexcitability abnormalities. We showed in previous periods that sound exposure or MMP9 inhibition given separately reduces phenotypes when given during development. We have obtained substantial amount of data on one of the main goals of comparing different acoustic exposure rates on FXS phenotypes. In the next project year (no cost extension period), we will test the combination approach and identify longevity of development vs. adult treatments.

15. SUBJECT TERMS
Fragile X Syndrome, Sound Exposure, Development, Cortical Hyperexcitability

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*
The proposed studies will test the hypothesis that a combination of sound exposure and minocycline treatment during early development will have long-lasting beneficial effects on symptoms of sensory hypersensitivity in a pre-clinical mouse model of Fragile X Syndrome (FXS), a leading known genetic cause of autism. These studies will identify for the first time the long-term efficacy of treatment during specific developmental windows in FXS. These data will have broad implications in the design of clinical trials for neurodevelopmental disorders by specifying optimal developmental treatment windows. The specific impact will be on the following areas of encouragement as identified in the call for proposals:
    i. Identification and testing of novel therapies
    ii. Research to establish early treatment
    iii. Research to understand pathophysiology of FXS.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*
Fragile X Syndrome, Sound Exposure, Development, Cortical Hyperexcitability

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.

The 3 major aims of the project are:

1. To identify optimal repetition rates of sound exposure to alleviate phenotypes of auditory hypersensitivity.
2. To test the hypothesis that combined sound exposure and minocycline treatment from P5 to P21 will reverse phenotypes of hyperexcitability in *Fmr1* KO mice.
3. To test the long-term impact of the combination treatment on auditory hypersensitivity.

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.

Major activities
During the reporting year:
1. We have analyzed PV protein level with western blotting in auditory cortex (AC) of P21 male mice raised in a regular vivarium which we term as 'normal exposure' (NE) (WT, n=4, Fmr1 KO, n=4), mice that were raised in a sound attenuated chamber and unexposed to external sounds, which we term 'attenuated exposure (AE)(WT, n=4, Fmr1 KO, n=4), and mice that were sound exposed (SE) during the developmental period to 14 kHz sounds with a repetition rate 5 Hz (5 Hz WT, n=4; 5 Hz Fmr1 KO, n=4) or 1 Hz (1 Hz WT, n=4; 1 Hz Fmr1 KO, n=4). This will allow us to identify optimal acoustic exposure rates during development. We found that PV protein level significantly increased in AC of both WT and Fmr1 KO mice exposed to 14 kHz sounds with a 5 Hz repetition rate, but not 1 Hz (Fig. 1). This supports our hypothesis that the specific sound parameters used are important in activating specific

cortical mechanisms, and the beneficial effects are not due to non-specific effects of sensory environment.

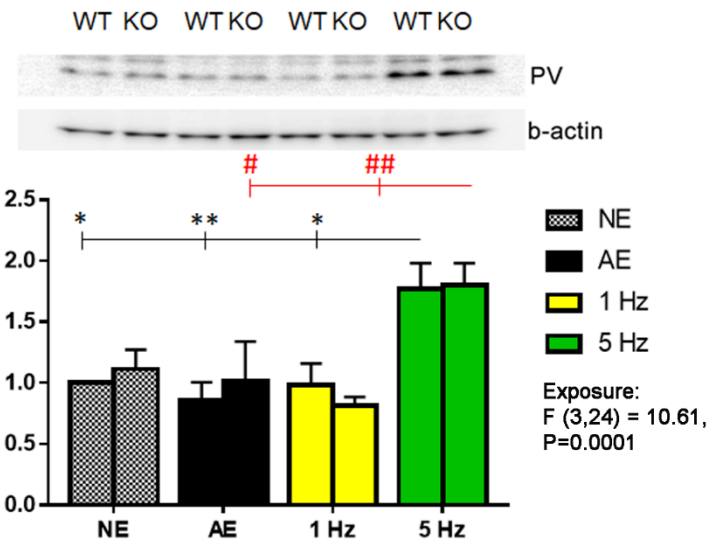


Fig. 1. PV protein levels increase in auditory cortex of both WT and KO P21 male mice after exposure to 14 kHz sounds with 5 Hz, but not 1 Hz repetition rate.
PV protein levels were normalized to β-actin in P21 auditory cortex of WT and *Fmr1* KO mice in NE, AE, 1 Hz SE and 5 Hz SE groups. In P21 auditory cortex of WT and *Fmr1* KO exposed to 14 kHz sounds with 5 Hz repetition rate (5 Hz), there was an increase in PV level compared to NE, AE and 1 Hz SE mice. Statistical analysis was done using two-way ANOVA with Bonferroni's post-hoc test (AE WT n=4; AE *Fmr1* KO n=4; 5 Hz WT n=4; 5 Hz *Fmr1* KO n=4; 1 Hz WT n=4; 1 Hz *Fmr1* KO n=4); * depicts sound exposure effect in WT mice, # shows sound exposure effect in *Fmr1* KO mice; */# $p < 0.05$; **/##, $p < 0.01$). There were no genotype differences.

2. We have also analyzed PV, PNN and cFos immunoreactivity with immunohistochemistry in AC of P21 male WT and *Fmr1* KO mice in NE, AE, 1 Hz SE and 5 Hz SE groups. We confirmed that similar to mice on FVB background (Kulinich et al., 2020), there was a decrease in the density of PV-positive cells in layers (L)4 and L5-6 AC of AE *Fmr1* KO mice compared to their WT counterparts on C57bl6 background (Fig. 2A). While PV cell density was increased in L4 AC of KO mice exposed to both 5Hz and 1 Hz repetition rate sounds, increase in PV intensity was observed only in AC of mice exposed to 5 Hz, but not 1 Hz (Fig. 2A-D), which corroborated western blot analysis results (fig. 1).

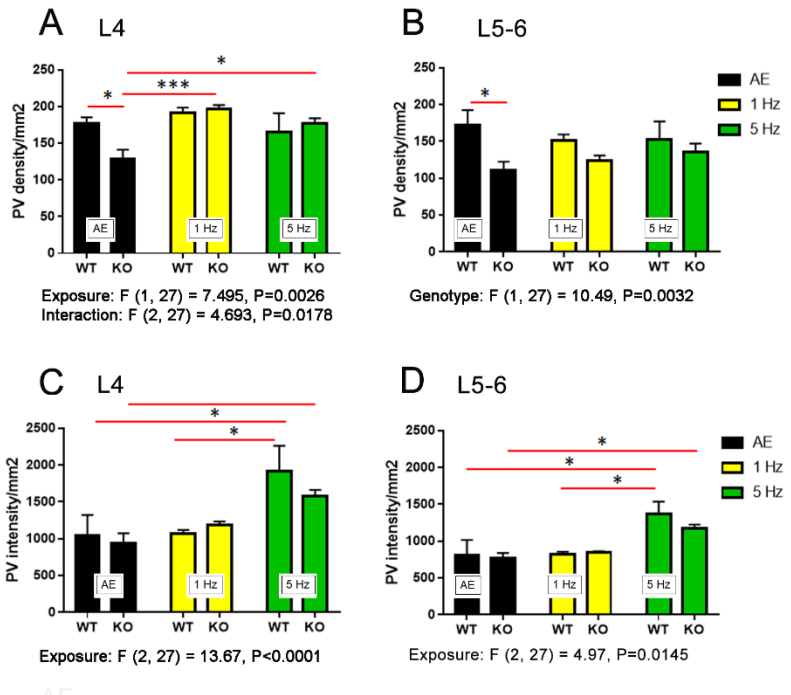


Fig. 2. Increase in PV intensity was observed only in AC of mice exposed to 14 kHz sounds with 5 Hz, but not 1 Hz repetition rate.
Analysis of PV cell density and intensity in L4 and L5-6 auditory cortex (AC) of WT and *Fmr1* KO mice in AE, 1 Hz SE and 5 Hz SE groups. Statistical analysis was done using two-way ANOVA with Bonferroni's post-hoc test (AE WT n=5; AE *Fmr1* KO n=6; 5 Hz WT n=7; 5 Hz *Fmr1* KO n=5; 1 Hz WT n=3; 1 Hz *Fmr1* KO n=7; *$p < 0.05$; *** $p < 0.001$).

3. There was a decrease in overall cFos cell density in L4 and L5-6 AC of Fmr1 KO mice exposed to 14 kHz sounds with 5 Hz repetition rate, but not 1 Hz (Fig. 3). Yet again, the influence of sound repetition

rates on outcomes suggests specific cortical mechanisms being activated. Importantly the sound exposure during development results in less number of cells being excited (as measured with cFos).

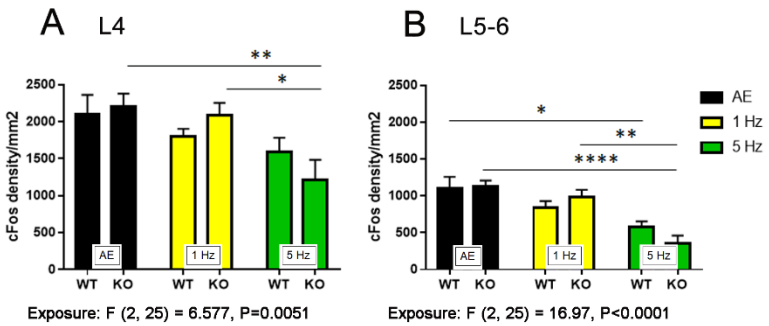


Fig. 3. Decrease in overall cFos cell density was observed in AC of KO mice exposed to 14 kHz sounds with 5Hz but not 1 Hz repetition rate. Analysis of cFos+ cell density in L4 (A) and L5-6 (B) auditory cortex of WT and *Fmr1* KO mice in AE, 1 Hz SE and 5 Hz SE groups. Statistical analysis was done using two-way ANOVA with Bonferroni's post-hoc test (n=5-7 mice; *p < 0.05; **p < 0.01 **** p < 0.0001).

4. There was no difference in density of PNN-positive cells observed in L4 and L5-6 auditory cortex of AE WT and AE KO and mice exposed to sounds with 5 Hz and 1 Hz repetition rate (Fig. 4A-C). Interestingly, there was a decrease in PNN cell intensity in AC of KO exposed to sounds with both 5 Hz and 1 Hz repetition rate (Fig. 4D).

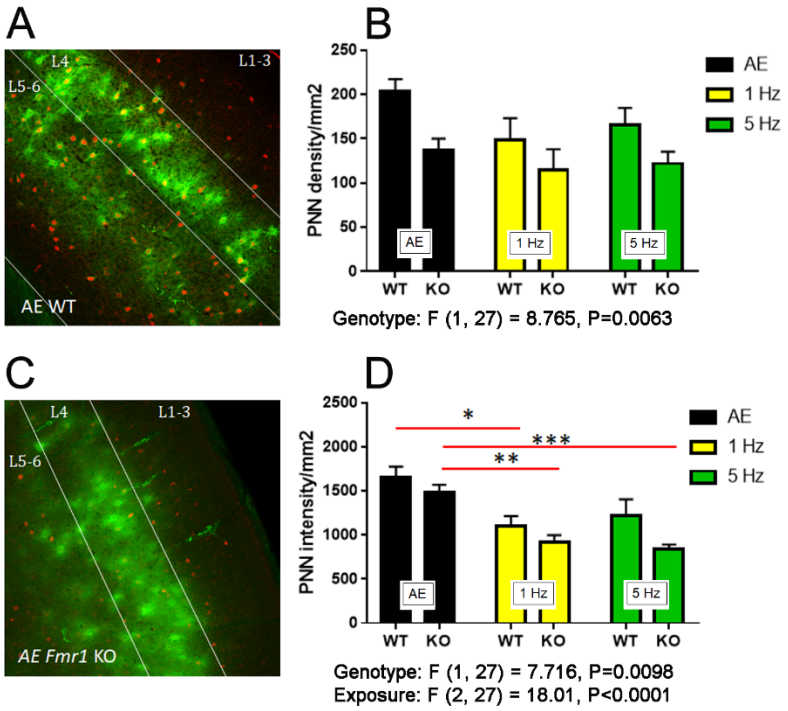


Fig. 4. Decrease in PNN intensity was observed in AC of KO mice exposed to 14 kHz sounds with both 5 Hz and 1 Hz repetition rate
Representative images of PNN positive cells (green) and PV (red) in AC of AE WT (A) and AE KO (C) and analysis of PNN cell density (B) and intensity (D) in L4 auditory cortex of WT and *Fmr1* KO mice in AE, 1 Hz SE and 5 Hz SE groups. Statistical analysis was done using two-way ANOVA with Bonferroni's post-hoc test (n=5-7 mice; *p < 0.05; ** p < 0.01, **** p < 0.0001).

5. To further investigate the effect of sounds with different repetition rates we have completed EEG recordings in mice raised in AE and SE environments. We have already published the data for P25-P26 NE mice (Pirbhoy et al., 2020). An EEG 2-channel electrode implant surgery was performed at P21 and after the 4-5 days recovery period (P25-P26) EEG recordings were performed. We have recorded EEG from littermates raised in sound attenuation box (AE WT, n=11, AE *Fmr1* KO, n=16); and those exposed to 14 kHz sound with 1 Hz repetition rate (SE 1Hz WT, n=14, SE 1Hz *Fmr1* KO, n=16) and 5 Hz repetition rate (SE 5Hz WT, n=14, SE 5Hz *Fmr1* KO, n=14). Similar to NE mice (Pirbhoy et al., 2020), we found a significant decrease in **synchronization to chirp-modulated sound** at 20-40Hz beta/gamma range in AC of AE KO and SE 1 Hz KO mice but not SE 5 Hz KO mice compared to their WT counterparts (Fig. 5). In frontal cortex, synchronization to chirp-modulated sound was reduced in KO mice in gamma range in all groups (not shown).

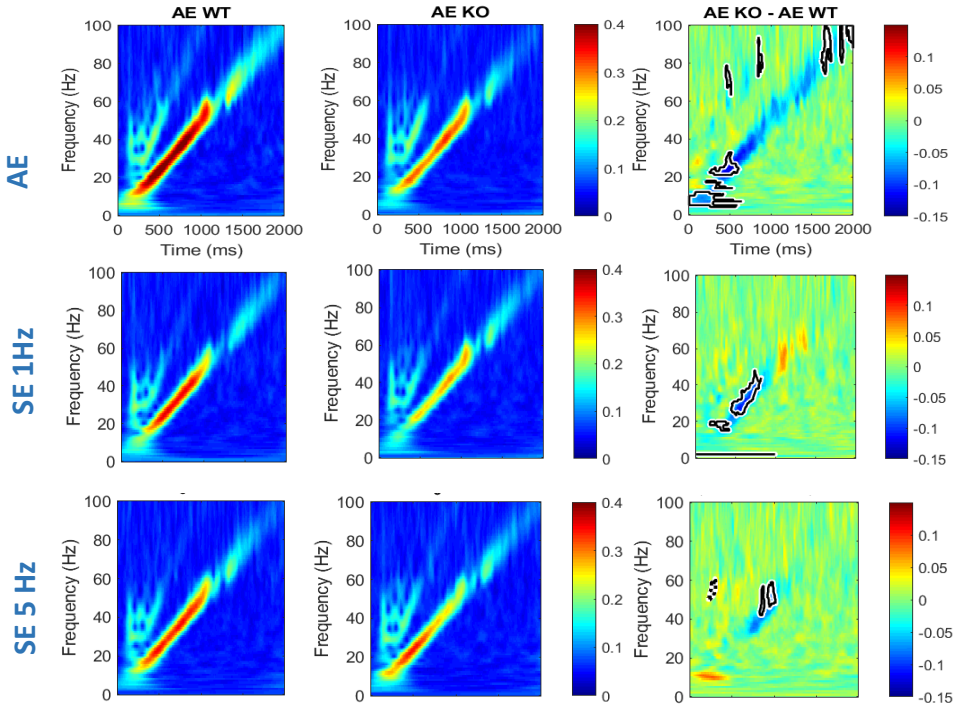


Fig. 5. Synchronization to chirp is impaired in auditory cortex (AC) of AE KO and SE 1 Hz KO mice but not SE 5 Hz KO mice compared to their WT counterparts
Grand average matrices were calculated for each genotype (WT, left and KO, middle panels), and then average ITPC WT value was subtracted from KO value (right panels) for AC. Statistical cluster analysis reveals time x frequency bands that are significantly different between groups highlighted with bolded black borders.

6. We also compared chirp responses in mice raised under the 5 Hz repetition rate sound exposure (SE 5Hz) to Naïve mice (NE). Similar to our previous findings, naïve KO mice showed a reduced synchronization to sound in gamma frequency compared to naïve WT mice (raised in the regular vivarium, **Fig.6**). However, we observed a significant improvement in phase-locking to Chirp in gamma range in the auditory but not frontal cortex of *Fmr1* KO mice exposed to sound trains with 5 Hz habituating repetition rate compared to naïve KO mice and similar to naïve WT (**Fig.6**). Sound exposure with 1Hz repletion rate did not improve responses to chirp in neither auditory nor frontal cortex (data not shown). This finding indicates improvement in ability of cortex to produce synchronous stimulus-induced oscillations in mice after the exposure to sound trains with 5 Hz repetition rate during P9-P21 period.

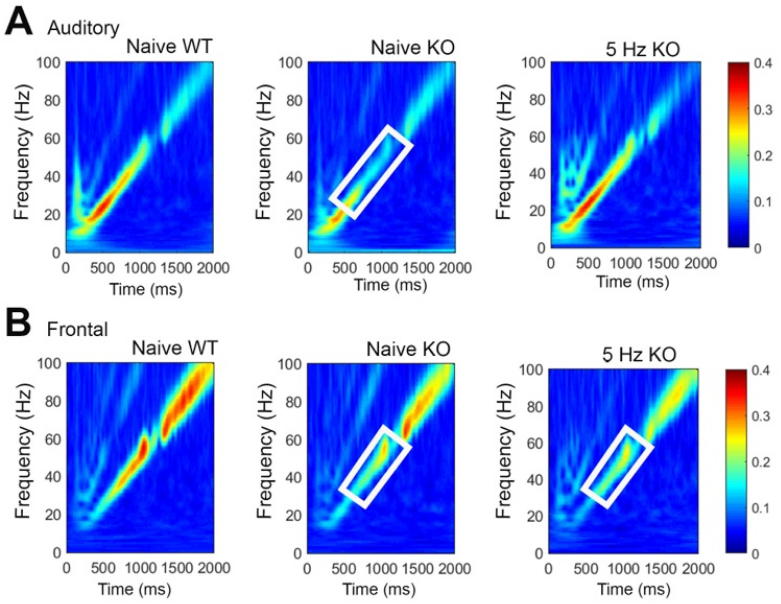


Fig.6. Phase locking to frequency-modulated sound "chirp" in auditory and frontal cortices of P26-P28 mice raised in a regular vivarium (naive) and KO mice exposed to 14 kHz sound trains with 5 Hz repetition rate during developmental period P9-P21. Top raw, auditory cortex, bottom raw, frontal cortex. White boxes show significant differences compared to naive WT mice.

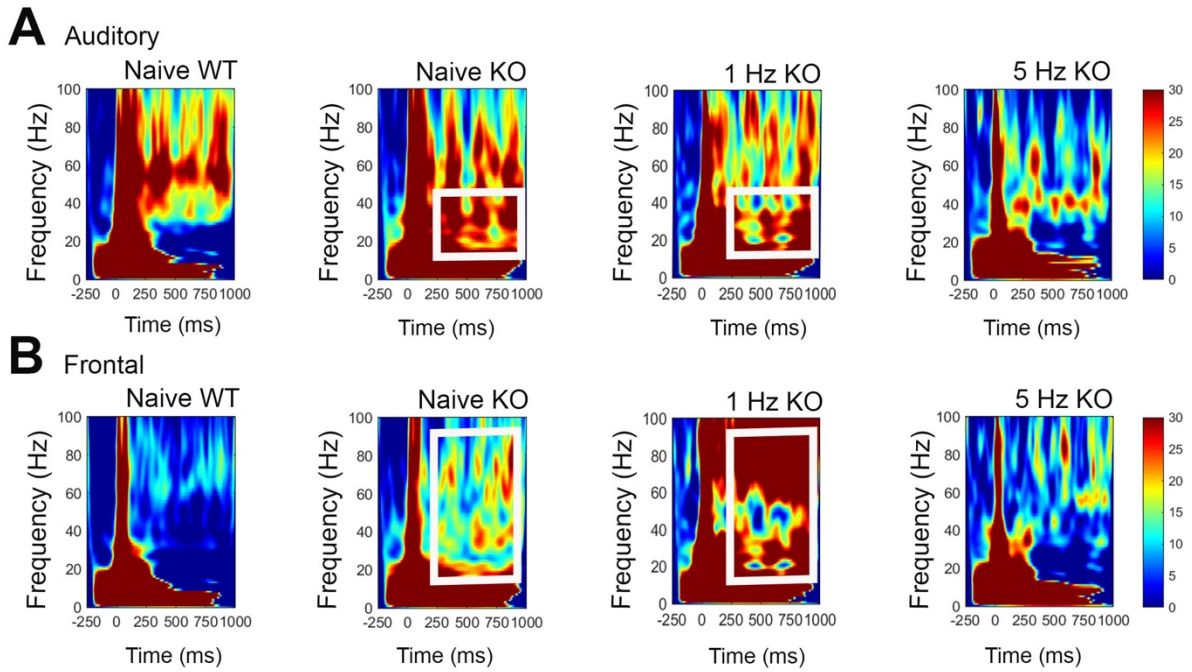
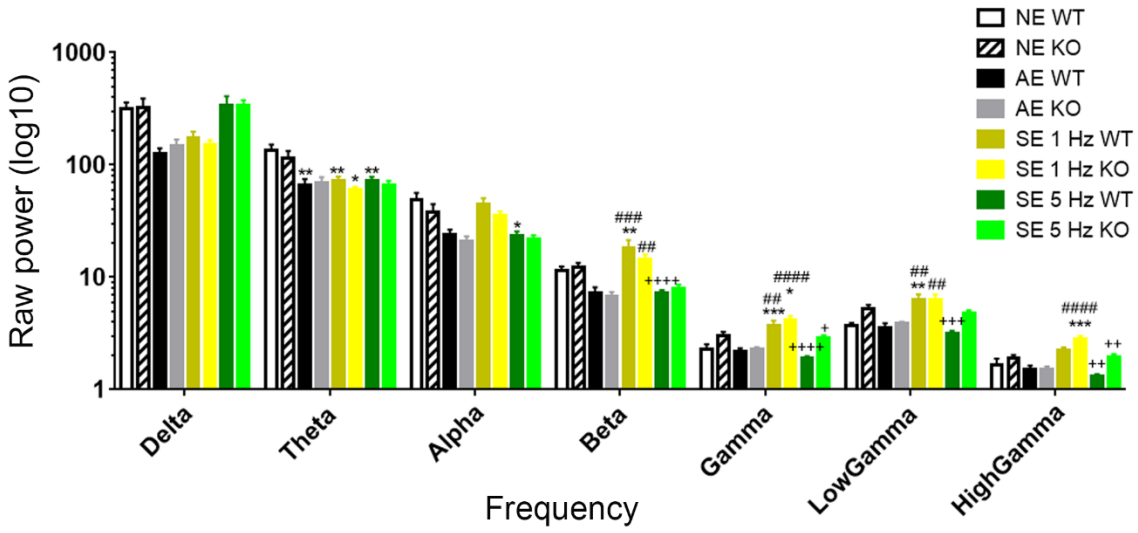


Fig. 7. Sound-induced power in auditory (A) and frontal (B) cortices of P26-28 WT and *Fmr1* KO mice raised in a regular vivarium (naïve) and exposed during development to 14 kHz sound trains with 1 and 5 Hz repetition rate during P7-P21 period. Trains of 100 ms duration broad band noise with 4 Hz sound presentation were used to generate event related potentials (200 repetitions) for time × frequency analyses. After correcting for baseline single trial power was averaged over all trials. White boxes show a significant difference compared to naïve WT (un-published data).

7. **Analysis of event-related potentials (ERPs)** in response to broadband sound showed significantly increased ongoing responses and decreased habituation to sound in AC and FC of naive *Fmr1* KO mice compared to naïve WT mice (see boxes in **Fig.7**), similar to our published findings (Pirbhoy et al., 2020). However, *Fmr1* KO mice exposed to a sound trains with 5 Hz repetition rate normalized ongoing responses and habituation in both AC and FC to WT levels. Whereas KO mice exposed to sound trains with 1 Hz repetition rate still showed increased on-going responses to sound compared to naïve WT mice.

8. **Analysis of resting EEG power** in auditory cortex (AC) of WT and *Fmr* KO mice raised in regular vivarium (naïve), sound attenuated environment and exposed to 14 kHz tone with a repetition rate 1 Hz and 5 Hz showed exposure effect for all frequency bands (delta, $P<0.0001$; theta, $P<0.0001$; alpha, $P<0.0001$; beta, $P<0.0001$; gamma, $P<0.0001$) and genotype effects for gamma frequency ($P=0.0025$, including low gamma, $P=0.0075$ and high gamma, $P=0.0025$). In AC, we found a trend showing decreased power for all frequencies in mice raised in attenuated sound environment (AE) compared to naïve mice (NE), with the significant difference in theta power and no genotype differences. Developmental exposure to sound with 1 Hz repetition rate (SE 1 Hz) resulted in lower theta power, but higher beta and gamma power, including low gamma and high gamma, in both WT and *Fmr1* KO compared to naïve mice. Moreover, increased beta and gamma power was observed in AC of mice exposed to 1 Hz sound compared to sound attenuated mice, both WT and *Fmr1* KO. Exposure to sound with 5 Hz repetition rate (SE 5 Hz) resulted in lower theta and alpha power compared to naïve mice with a trend for beta power. Although gamma power was not different between SE 5Hz and NE or AE mice, SE 5 Hz showed significantly lower beta and gamma power (including low and high gamma) than SE 1 Hz (**Fig. 8**).

Fig.8. Resting EEG in auditory cortex of P26-P28 WT and Fmr1 KO mice raised in vivarium (naïve, normal exposure, NE; WT, n=22; KO, n=20), sound attenuated environment, AE (WT, n=11; KO, n=17), exposed to 14 kHz with repetition 1 Hz (WT, n=14; KO, n=16) and 5 Hz (WT, n=14; KO, n=14). Stars depict comparison of WT or KO to corresponding NE WT or NE KO; pound signs depict comparison to AE WT or AE KO and plus signs depict difference between mice exposed to 1 Hz and 5 Hz. Statistical analysis is done using 2-way ANOVA with Tukey's post-hoc test. * p<0.05, **/##/++ p<0.01, ***/+++ p<0.001, ####/++++ p<0.0001.



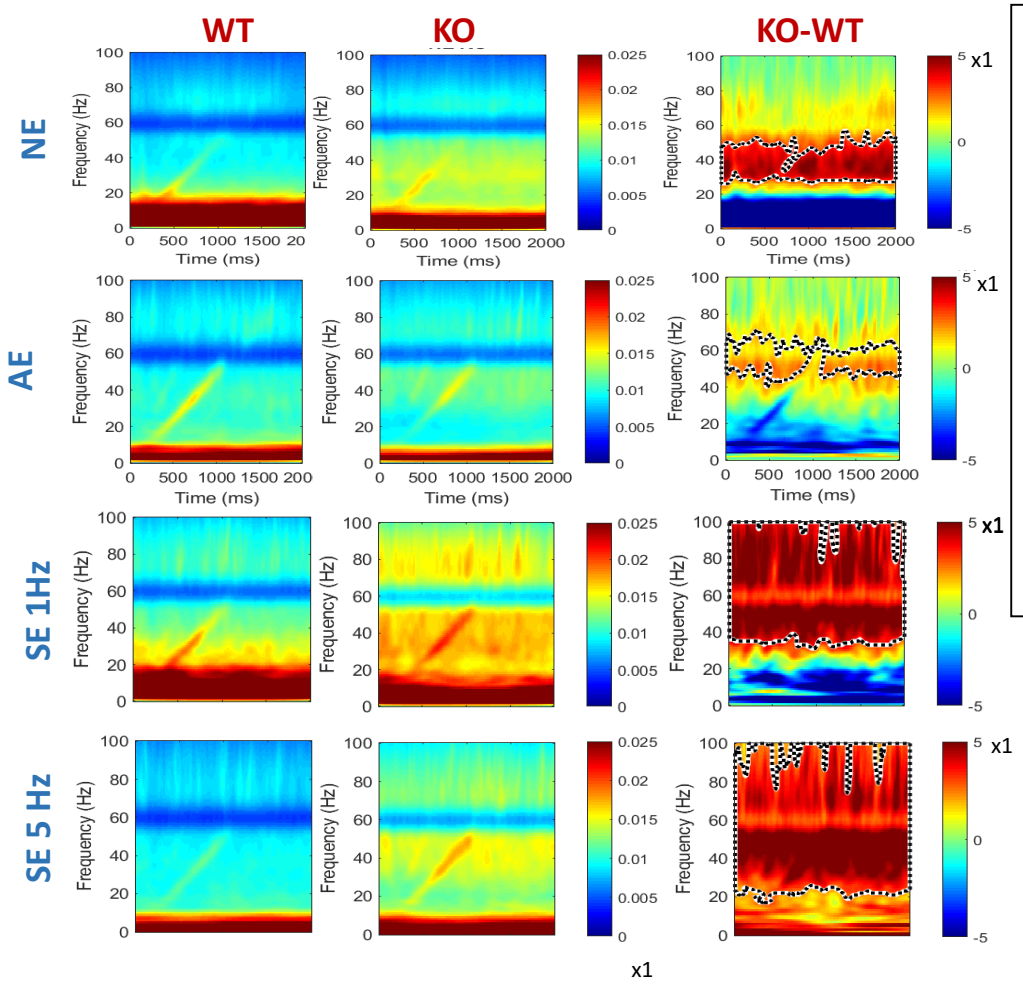


Fig. 9. Gamma background Single Trial Power (STP) during chirp stimulation is reduced in auditory cortex of AE KO mice, but increased in SE 1Hz and SE 5Hz KO mice. Grand average matrices were calculated for each genotype (WT, left and KO, middle panels), and then average STP WT (n=11-14) value was subtracted from KO (n=14-16) value (right panels). Statistical cluster analysis reveals time x frequency bands that are significantly different between groups highlighted with bolded black borders

9. Analysis of **background non-phase locked single trial power (STP)** in beta and gamma range during the chirp presentation in AC and FC showed a significant increase in both SE 1Hz and SE 5Hz KO compared to NE and AE mice, but a decrease in AE mice compared to NE mice (**Fig. 9**). This is similar to our observations of increased baseline beta and gamma power in the SE 1 Hz mice (**Fig. 8**).

10. The MEA EEG experiments have been completed from AE adult mice (10 WT and 10 *Fmr1* KO). We found that adult KO resting low gamma power was significantly increased in the right temporal brain region compared to the WT controls, while there was no difference in all the other regions. Additionally, the chirp ITPC in adult KO was significantly increased in the right frontal and left temporal brain regions compared to the WT controls, while there was no difference in all the other regions. Lastly, the ERP power was significantly increased in the left and right frontal, right medial and right temporal brain regions of adult KO, while there was no difference in the left medial and left temporal brain regions. We will compare these data with SE mice with the different repetition rates using MEA EEG in next project period. The NE mice data are already published (Jonak et al., 2020).

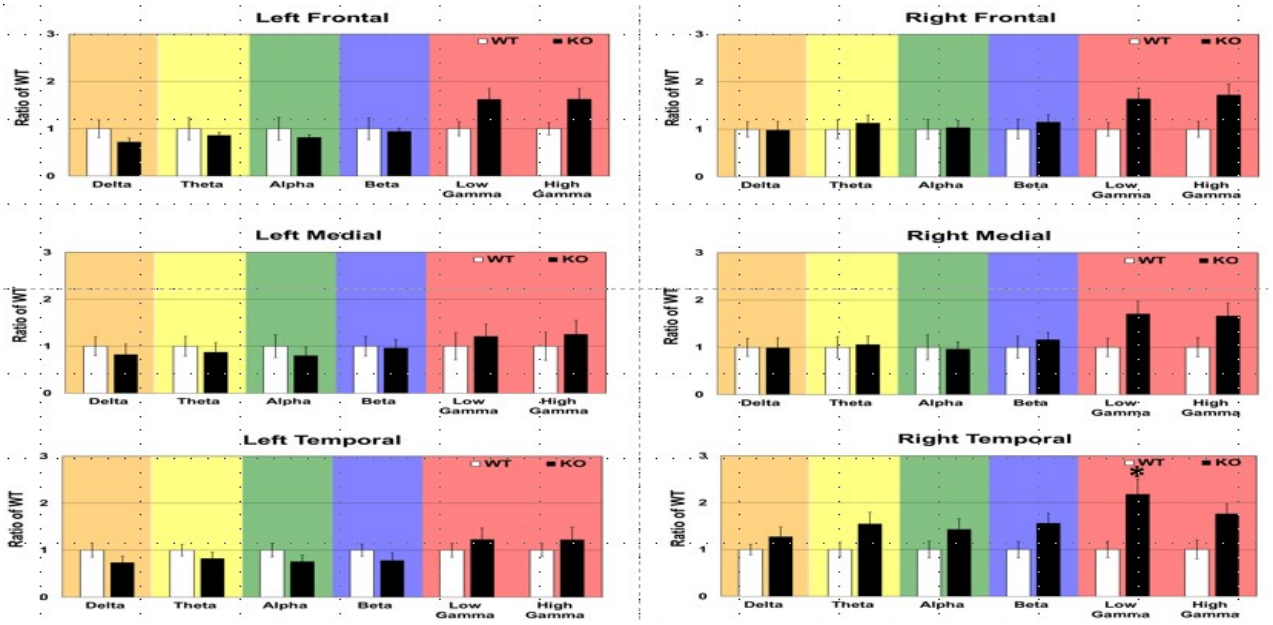


Fig.10. Quantification of ratio of power across frequency bands in sound attenuated *Fmr1* KO to WT MEA EEG for distinct brain regions. Values above 1 indicate higher EEG power in *Fmr1* KO compared with WT mice. Gamma band power was elevated in the mice raised in sound attenuated conditions, similar to normally raised Fmr1 KO mice. *p<0.05. n=10 *Fmr1* KO, n=10 WT mice.

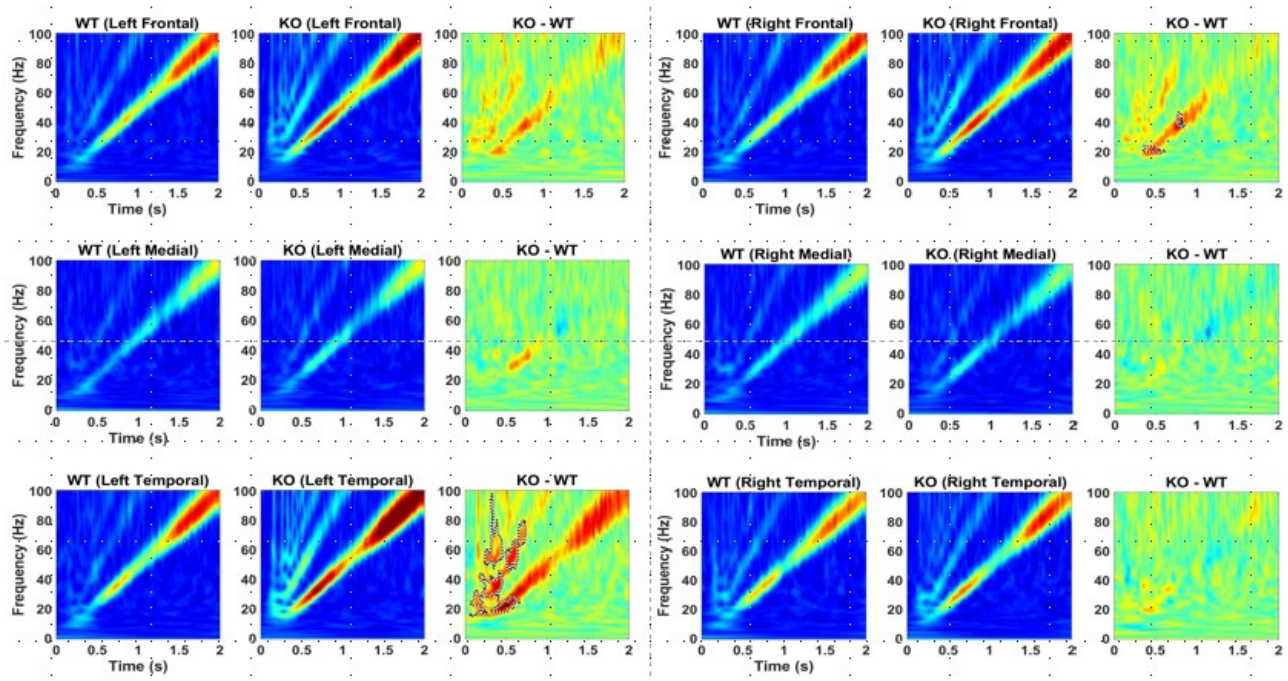


Fig.11. Multielectrode array analysis of auditory chirp stimulation in sound attenuated WT *vs. Fmr1* KO mice. For each brain region, the left panel shows the averaged WT intertrial phase coherence (ITPC or phase-locking factor), the middle panel shows the averaged *Fmr1* KO ITPC and the right panel shows KO-WT. Scales at the bottom show ITPC and ITPC difference in μV^2/Hz. Significant increases in ITPC in *Fmr1* KO compared to WT mice are outlined in black dashed outlined areas. Red areas in the right panels (KO-WT) are positive ITPC differences since ITPC values in KO are greater than WT mice.

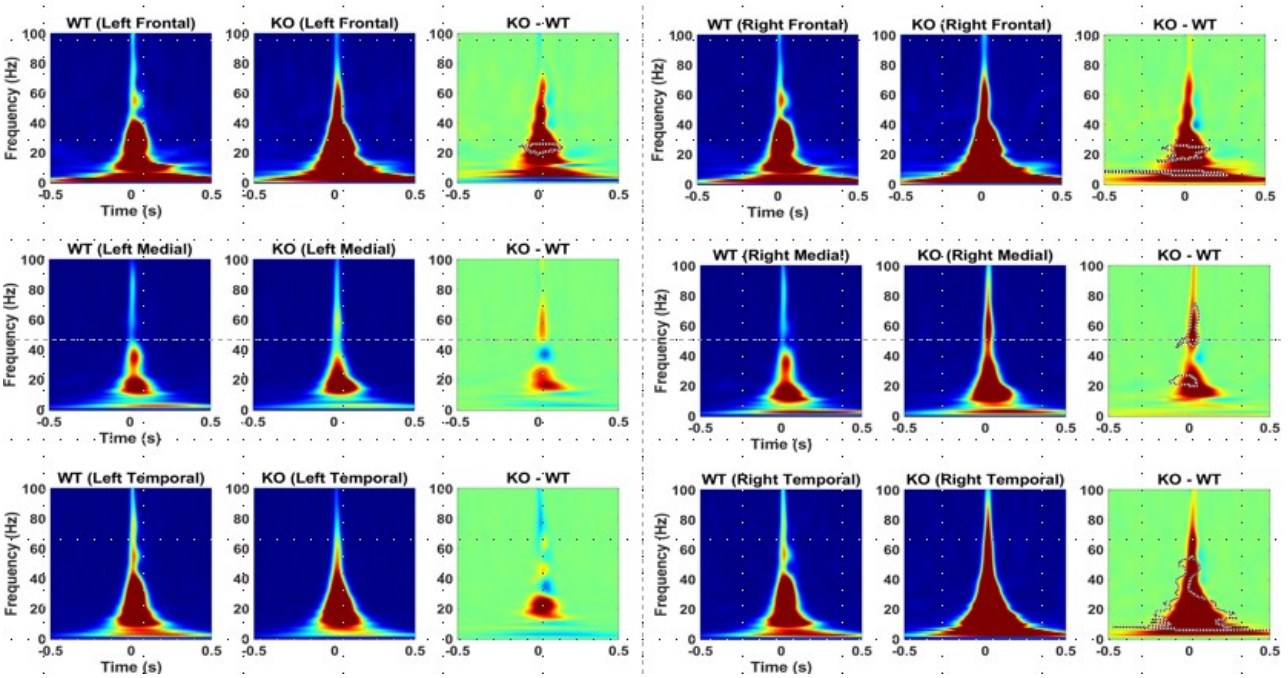


Fig.12. Single-trial event-related power following auditory stimulation in sound attenuated WT *vs. Fmr1* KO mice. For each brain region, the left panel shows the averaged WT event-related power, the middle panel shows the averaged *Fmr1* KO event-related power and the right panel shows KO-WT (subtraction). Scales at the bottom show power and power difference in μV^2/Hz. Significant increases in event-related power in *Fmr1* KO compared to WT mice are outlined in black dashed areas.

11. We also tested the sensitivity of MEA EEG measurements to treatments using a GABAb agonist (baclofen) and found phenotype and dose dependent effects and published these results in Jonak et al. (In Press). These data set the stage in terms of group comparisons to test minocycline and sound exposure effects in the next year of the project using MEA.
12. In Rais et al. (2022), to achieve *Fragile X mental retardation gene* (*Fmr1)* deletion and re-expression in excitatory neurons during the postnatal day (P)14-P21 period, we generated $Cre^{CaMKIIa}/Fmr1^{Flox/y}$ (cOFF) and $Cre^{CaMKIIa}/Fmr1^{FloxNeo/y}$ (cON) mice, respectively. We showed that cortical and behavioral phenotypes can be reversed with post-natal expression of FMRP in forebrain excitatory neurons alone, opening up this window for potential therapeutic approaches in FXS.

13. Undergraduate student Nadia Farooq was trained to setup and perform behavioral experiments on mice including open field, elevated plus maze, sociability and social novelty tests (Rais et al., 2022). Graduate student Xin Tao was trained to develop and perform a probabilistic reversal learning and social conditioned place preference test in mice. These behavioral tests will be used for the project to determine efficacy of minocycline and sound exposure. Graduate student Katilynne Croom was trained to perform EEG recordings from developing and adult mice, and will perform the combination treatment experiment in the next reporting period.

When examined in relation to the approved SOW, we accomplished the following:
Major task 1
Sub-task 1 – accomplished. We have recruited and trained a team of post-docs, graduate students, undergraduate students and technicians to perform all experiments proposed.
Sub-task 2 –We have recorded EEGs and ERPs from sound exposed (SE) mice. The ERP data is already published (Kulinich et al., 2020). The EEG time x frequency analysis work has been completed in NE mice and published (Pirbhoy et al., 2020). The AE and the SE data are completed and manuscript is currently under preparation. We have completed the AE MEA EEG data for electrophysiological analysis. The SE MEA EEG data will be completed by the end of 2022.
Sub-tasks 3 – We have completed the 5 Hz and 1 Hz exposure rate data collection and histological analysis across groups.
Sub-task 4 – We have trained an undergraduate student to design and conduct behavioral experiments and this will be completed by the end of this year.

Major tasks 2 and 3 – We have already published (Lovelace et al., 2020) on the EEG responses following minocycline treatment in the Fmr1 KO mice (NE). Because minocycline can have other effects in the brain besides MMP-9 inhibition, we also tested and published the data on a specific MMP-9 inhibitor (Pirbhoy et al., 2020). Currently we have trained a graduate student (Katilynne Croom) to record EEGs and another graduate student (Xin Tao) to conduct behavioral results which will be obtained from mice treated with the combination of sound and minocycline in the next reporting period. This will cover both long term and acute effects of treatment combination.

Key Outcomes:
Publications

1. Jonak CR, Pedapati EV, Schmitt LM, Assad SA, Sandhu MS, DeStefano L, Ethridge L, Razak KA, Sweeney JA, Binder DK, Erickson CA. Baclofen-associated neurophysiologic target engagement across species in fragile X syndrome. *Journal of Neurodevelopmental Disorders. In Press,* 2022.
2. Holley, AJ, Shedd A, Boggs A, Lovelace, J, Erickson C, Gross C, Jankovic M, Razak KA, Huber K and Gibson JR. A sound-driven cortical phase-locking change in the *Fmr1* KO mouse requires *Fmr1* deletion in a subpopulation of brainstem neurons. *Neurobiology of Disease*, https://doi.org/10.1016/j.nbd.2022.105767, *In Press*. 2022.

3. Rais M, Lovelace JW, Shuai XS, Woodard W, Bishay S, Estrada L, Sharma AR, Nguyen A, Pirbhoy PS, Palacios AR, Nelson DL, Razak KA and Ethell IM. Functional consequences of postnatal interventions in a mouse model of Fragile X Syndrome. *Neurobiology of Disease*, 162:105577, doi: 10.1016/j.nbd.2021.105577, 2022.
4. Pirbhoy PS, Syed R, Jonak CR, Perez PA, Wiley M, Hessamian K, Lovelace JW, Razak KA, DiPatrizio NV, Ethell IM, Binder DK. Increased 2-arachidonoyl-*sn*-glycerol levels normalize cortical responses to sound and improve behaviors in *Fmr1* KO mice. *J. Neurodevelopmental Disorders.* 13(1):47, doi: 10.1186/s11689-021-09394-x, 2021.

Reviews:
1. Razak KA, Binder, DK and Ethell IM. Neural correlates of auditory hypersensitivity in Fragile X Syndrome. *Frontiers in Psychiatry*, 12:720752. doi: 10.3389/fpsyt.2021.720752, 2021.
2. Contractor, A., Ethell, I.M. and Portera-Cailliau, C., 2021. Cortical interneurons in autism. *Nature Neuroscience*, *24*(12), pp.1648-1659.

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.

We have trained 2 post-docs, 4 graduate students, 1 undergraduate student, and 2 research technicians on the project aims.
Post-docs, Patricia Pirbhoy and Anna Kulinich have been trained in EEG, MEA analyses, pharmacological treatment approaches, immunohistochemistry, histological analyses and behavioral analyses. They were each trained on analyzing complex EEG data and writing papers. Dr. Pirbhoy wrote and received her own grant funding from FRAXA, providing an important skillset for future success. They also first-authored papers during the reporting year as listed above. Graduate student Maham Rais was trained in EEG, histological and undergraduate student Nadia Farooq on behavioral analysis. Maham Rais also successfully renewed an NIH F31 training grant for year 2. This prestigious and competitive fellowship will be a major aspect of professional development for Maham. She has now graduated and has started a post-doc position at Seattle Children's Hospital. Three other graduate students, Mawaheb Kassir, Xin Tao and Katilynne Croom have been trained on these aims, particularly to develop behavioral analysis and EEG analysis tools. Mawaheb also received an NIH F31 training grant in the reporting period, making it two in two years of students co-trained in this grant. Research technician, Carrie Jonak, is trained to conduct MEA 30 channel EEG recordings. Research technician, Stephen Brookshire, has been trained in genotyping, mouse colony maintenance, histology and IHC analysis. He contributes by managing the resources so all trainees are optimally productive and the progress is maintained.

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 published 4 papers and 2 review articles during the reporting period (see above).
Trainees and PIs attended and presented at several conferences and seminars:

2022	Symposium talk "Astrocyte-mediated mechanisms of abnormal Inhibition in FXS", 18th International Fragile X Conference of National Fragile X Foundation, San Diego, CA 07/2022
2022	Poster Presentation "Astrocyte-mediated mechanisms of abnormal inhibition in Fragile X Syndrome" Cell Symposia: The Biology of Neuropsychiatric Disorders 2022, Sitges, Spain, 05/2022
2022	Psychiatry and Neuroscience Grand Rounds Talk "Cortical Hyperexcitability in Fragile X Syndrome: from molecule to circuit and behavior", 03/16/2022 (virtual)
2022	NRSC Seminar "Astrocyte-mediated mechanisms of inhibition and discovery of novel interventions for treating neurodevelopmental disorders", UCR, 02/22/2022
2022	Symposium talk "Excessive MMP-9 Activity Contributes to the Development of Sensory Hypersensitivity in Fragile X Syndrome by Regulating Perineuronal Nets", Winter Conference on Brain Research, 02/2022 (virtual)
2022	NIH/NIAAA seminar "Astrocyte-mediated mechanisms of inhibition and discovery of novel interventions for treating neurodevelopmental disorders", 01/13/2022 (virtual)
2022	Croom, K, Rumschlag, JA, Kokash, J, Erickson, M, Binder, DK, Huber, K and **Razak KA**. Auditory Temporal Processing Across Development in Two Different Mouse Models of Autism. Gordon Research Conference on Fragile X and Autism-related Disorders, Barga, Italy.
2022	Tao X, Brookshire SW, Zhou AQ, Li YR, Newman-Tancredi A, Varney M, **Razak KA**. Acute and Chronic Administration of a Selective and Biased Serotonin 1A Receptor Agonist Reduces Audiogenic Seizures in Developing Fmr1 Knockout Mice. Gordon Research Conference on FXS and other ASD

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.

The main objective is to complete the combined sound exposure + minocycline treatment during early development to identify benefits in terms of sensory, anxiety, social phenotypes. The mice are available and the students have been trained to accomplish this goal.

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).

Our project is the first to test the idea that modifying sensory environment during development impacts autism specific behaviors and electrophysiological correlates. We have published 14 papers and 5 reviews during the funding period that have generated a novel understanding of pathophysiological mechanisms of FXS sensory hypersensitivity, novel therapeutic approaches and novel measurement and analysis methods. The unpublished data generated in this reporting period continue to point to differential regulation of PV/PNN inhibitory system in the cortex, mainly during the early developmental period, as a neural correlate of hypersensitivity. The 5Hz vs 1Hz repetition rate exposure produced different effects on PV/PNN, cFos expression, and responses to sound, indicating specific effects on cortical mechanisms. These data suggest novel ways to think about therapeutics in FXS. The finding that MEA EEG responses are sensitive to pharmacological approaches (e.g., baclofen) has generated considerable interest in both academic and pharma companies who are developing other novel approaches.

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.

Our data that sound exposure during early development is beneficial has broad impact on sensory hypersensitivity in multiple disorders with sensory dysfunction. The PV/PNN system is implicated in a number of neurodevelopmental disorders including Rett Syndrome and Schizophrenia. Our finding that these cell types and their activities can be modulated by sound exposure with specificity for different repetition rates has major implications for treating multiple disorders.

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.*

Our development of the 30 channel MEA EEG skull recordings from awake and freely moving mice has generated much interest in pharmaceutical companies to test developing drugs. The system is being used to test novel drugs (e.g., PDE inhibitor, Jonak et al., 2021 and baclofen, Jonak et al., In Press). It was important to show sensitivity of the recordings to treatment effects, and that is indeed what was shown in

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

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

- *improving public knowledge, attitudes, skills, and abilities;*

- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report in this year annual progress report.

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

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.

None

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.

There was a slow recovery due to Covid19 in 2021 in terms of in person training. We are delayed by ~6 months in where we expected to be in terms of data collection and trainee mentorship. We expect to make that up during the no cost extension period, and are on track to complete the projects as proposed.

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.

None to report

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

None

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents

None

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).*

Publications

1. Jonak CR, Pedapati EV, Schmitt LM, Assad SA, Sandhu MS, DeStefano L, Ethridge L, Razak KA, Sweeney JA, Binder DK, Erickson CA. Baclofen-associated neurophysiologic target engagement across species in fragile X syndrome. *Journal of Neurodevelopmental Disorders. In Press,* 2022.
2. Holley, AJ, Shedd A, Boggs A, Lovelace, J, Erickson C, Gross C, Jankovic M, Razak KA, Huber K and Gibson JR. A sound-driven cortical phase-locking change in the *Fmr1* KO mouse requires *Fmr1* deletion in a subpopulation of brainstem neurons. *Neurobiology of Disease*, https://doi.org/10.1016/j.nbd.2022.105767, *In Press*. 2022.
3. Rais M, Lovelace JW, Shuai XS, Woodard W, Bishay S, Estrada L, Sharma AR, Nguyen A, Pirbhoy PS, Palacios AR, Nelson DL, Razak KA and Ethell IM. Functional consequences of postnatal interventions in a mouse model of Fragile X Syndrome. *Neurobiology of Disease*, 162:105577, doi: 10.1016/j.nbd.2021.105577, 2022.
4. Pirbhoy PS, Syed R, Jonak CR, Perez PA, Wiley M, Hessamian K, Lovelace JW, Razak KA, DiPatrizio NV, Ethell IM, Binder DK. Increased 2-arachidonoyl-*sn*-glycerol levels normalize cortical responses to sound and improve behaviors in *Fmr1* KO mice. *J. Neurodevelopmental Disorders.* 13(1):47, doi: 10.1186/s11689-021-09394-x, 2021.

Reviews:

1. Razak KA, Binder, DK and Ethell IM. Neural correlates of auditory hypersensitivity in Fragile X Syndrome. *Frontiers in Psychiatry*, 12:720752. doi: 10.3389/fpsyt.2021.720752, 2021.
2. Contractor, A., Ethell, I.M. and Portera-Cailliau, C., 2021. Cortical interneurons in autism. *Nature neuroscience*, *24*(12), pp.1648-1659.

Books or other non-periodical, one-time publications.

None

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.*

Conference presentations:

1. Ethell IM, Symposium talk "Astrocyte-mediated mechanisms of abnormal Inhibition in FXS", 18th International Fragile X Conference of National Fragile X Foundation, San Diego, CA 07/2022
2. Ethell IM, Poster Presentation "Astrocyte-mediated mechanisms of abnormal inhibition in Fragile X Syndrome" Cell Symposia: The Biology of Neuropsychiatric Disorders 2022, Sitges, Spain, 05/2022
3. Ethell IM, Symposium talk "Excessive MMP-9 Activity Contributes to the Development of Sensory Hypersensitivity in Fragile X Syndrome by Regulating Perineuronal Nets", Winter Conference on Brain Research, 02/2022 (virtual)
4. Croom, K, Rumschlag, JA, Kokash, J, Erickson, M, Binder, DK, Huber, K and Razak KA. Auditory Temporal Processing Across Development in Two Different Mouse Models of Autism. Gordon Research Conference on Fragile X and Autism-related Disorders, Barga, Italy, 2022.
5. Tao X, Brookshire SW, Zhou AQ, Li YR, Newman-Tancredi A, Varney M, Razak KA. Acute and Chronic Administration of a Selective and Biased Serotonin 1A Receptor Agonist Reduces Audiogenic Seizures in Developing Fmr1 Knockout Mice. Gordon Research Conference on FXS and other ASD, 2022.

Seminar Presentations:

1. Ethell IM Psychiatry and Neuroscience Grand Rounds Talk "Cortical Hyperexcitability in Fragile X Syndrome: from molecule to circuit and behavior", 03/16/2022
2. Ethell IM NRSC Seminar "Astrocyte-mediated mechanisms of inhibition and discovery of novel interventions for treating neurodevelopmental disorders", UCR, 02/22/2022
3. Ethell IM NIH/NIAAA seminar "Astrocyte-mediated mechanisms of inhibition and discovery of novel interventions for treating neurodevelopmental disorders", 01/13/2022
4. Razak KA. 'Mechanisms and biomarkers of auditory hypersensitivity in Fragile X Syndrome'. Brain Awareness Day Invited Speaker, Department of Psychiatry and Behavioral Neurosciences, McMaster University, Hamilton, CA.
5. Razak KA. 'Mechanisms, biomarkers and treatment of auditory hypersensitivity in Fragile X Syndrome'. Sensory Biology Seminar Series, University of Wyoming, Laramie, WY.

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.

None

Technologies or techniques

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

None

- **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.

None

- **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.*

None

3. **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:	*Khaleel A. Razak*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Razak, along with co-PI and collaborator, designed experiments and trained post-docs and graduate students to collect and analyze EEG data and write publications.*
Funding Support:	*DOD*

Name:	*Iryna M. Ethell*
Project Role:	*PI*

Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1.2*

Contribution to Project: *Dr. Ethell, along with co-PI and collaborator, designed experiments and trained post-docs and graduate students to collect and analyze the biochemistry, immunohistochemistry and behavioral data and write publications.*
Funding Support: *DOD*

Name: *Devin K. Binder*
Project Role: *Collaborator*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *1*

Contribution to Project: *Dr. Binder, along with PIs, designed experiments and trained post-docs and research technicians to collect and analyze the MEA EEG data and write publications.*
Funding Support: *DOD*

Name: *Stephen Brookshire*
Project Role: *Lab Technician and Manager*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*

Contribution to Project: *Mr. Brookshire is involved with genotyping and colony maintenance and obtaining histology data.*
Funding Support: *DOD*

Name: *Carrie Jonak*
Project Role: *Research Associate and Manager*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Ms. Jonak is involved with genotyping and colony maintenance and obtaining all the MEA EEG data.*
Funding Support: *DOD*

Name: *Dr. Anna Kulinich*
Project Role: *Post-doc*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *6*

Contribution to Project: *Dr. Kulinich is involved with mouse breeding and genotyping, designing the sound exposure chamber and controlling exposure of mice in this project and analyzing PV/PNN/cFos/MMP9 protein levels by immunohistochemistry and western blotting and EEG recordings .*
Funding Support: *DOD and internal funds*

Name:	*Xin Tao*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Ms. Tao is a doctoral student being trained to develop behavioral analysis in all aims*
Funding Support:	*DOD*

Name:	*Mawaheb Kassir*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Ms. Kassir is a doctoral student being trained to develop EEG recordings. In particular, she is developing analytical tools to analyze the complex MEA EEG datasets to examine cross regional interactions.*
Funding Support:	*DOD*

Name:	*Katilynne Croom*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*Mrs. Croom is a doctoral student being trained to develop EEG recordings. In particular, she is trained to perform minocycline administration and sound exposure to study effects on EEG responses.*
Funding Support:	*DOD*

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.

Razak KA
Nothing to report

Ethell IM
Nothing to report

Binder DK
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."

Nothing to report

4. **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.*

5. **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.*

Attached following this page.