

AWARD NUMBER: W81XWH-19-1-0521

TITLE: Developmental acoustic exposure as a novel approach to treat Fragile X Syndrome

PRINCIPAL INVESTIGATOR:

Khaleel A. Razak

CONTRACTING ORGANIZATION: Regents, University of California

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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. This project addresses a major gap in the autism literature on when the appropriate treatment window is. The first year of the project period has been highly productive, despite the loss of a significant amount of time and resources to the pandemic-related shut-downs of our university. We have published five papers and two reviews in 2020 from this project. In Kulinich et al (2020), we showed the beneficial effects of early life 5Hz repetition rate sound exposure on hyperexcitability correlates in Fmr1 KO mice. This is one of the major aims of the project. In Lovelace et al (2020), we showed that minocycline treatment in adult mice has an impact on sound evoked EEG responses, but not resting EEG compared to vehicle treatment. In Pirbhoy et al (2020), we showed that a more specific inhibitor (SB-3CT) that works like minocycline to reduce matrix metalloprotease-9 (MMP9) given to developing mice (~P22) works to reduce EEG phenotypes, reverses inhibitory cell phenotypes and improves behaviors even with an acute injection. In Jonak et al (2020), we used a state-of-the-art 30 channel multielectrode array (MEA) system to record EEGs from mouse skull and showed regional differences in EEG phenotypes in adults. In Nguen et al (2020), we showed that auditory hypersensitivity correlates are found in the P21-P34 window. These papers provide the foundation for the current grant in testing the same phenotypes following sound exposure and minocycline. We have also analyzed pilot data in the reporting year to show that in the adults, peripheral cochlear function is not different between WT and KO mice. These indicate central auditory processing changes underlie abnormalities. We have now shown separately that sound exposure or MMP9 inhibition reduces phenotypes when given during development. In the next project year, 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 successfully expanded our colony of *Fmr1* KO mice with C57Bl6/J background to conduct major experiments of the proposal using *Fmr1* KO and WT male littermates. We have collected 12 (5 WT and 7 *Fmr1* KO) auditory cortex tissue samples of male mice raised in silence for protein analysis such as parvalbumin levels, total TrkB, 816 pTrkB, 515 pTrkB and pAkt/Akt; 10 brains for immunohistochemistry (IHC, 4 WT and 6 *Fmr1* KO) and 16 mice for MEA recordings.
2. Published the data collected using 5 Hz repetition rate of sound exposure during early development to reveal highly promising beneficial effects on neural correlates of hyperexcitability in *Fmr1* KO mice (Kulinich et al., 2020). This is one of the main aims of the project.

3. Developed and tested a state-of-the-art MEA EEG system and identified a number of phenotypes in the Fmr1 KO mice related to cortical hypersensitivity (Jonak et al., 2020). This applies to all 3 aims as the MEA will be used to test outcomes following early treatment with sound and/or MMP9 inhibitors.
4. Identified the effects of minocycline on adult EEG phenotypes with a 10day treatment paradigm (Lovelace et al., 2020). This paper provides the data to compare the same EEG phenotypes in adults after developmental treatment with sound and/or minocycline. This applies aims 2 and 3 to provide the baseline for how effective minocycline is in the adults.
5. Identified and tested a more specific inhibitor of MMP9, called SB-3CT, and found that it is effective in reversing multiple phenotypes (cellular, electrophysiological and behavioral) even with a single treatment during early development (Pirbhoy et al., 2020). This applies aims 2 and 3 to provide the baseline for how effective MMP9 inhibition is when given during early development. This provides added justification to our major hypothesis that early treatment is beneficial. The major goal of the next years is to build on this finding to identify longevity of effects and to test if combining sound exposure improves effects and longevity. This will be extremely beneficial in future clinical trial designs for FXS work.
6. The 5 Hz repetition rate experiments have been completed and published (Kulinich et al., 2018). We have started exposing mice to 14 kHz tone with 1 Hz repetition rate (Specific aim 1) and collected 10 (6 WT and 4 Fmr KO) auditory cortex tissue samples for biochemical analysis. All collected tissue samples for the protein analysis have been processed already and are being analyzed now. We will compare three different repetition rates in year 2 of the project.
7. The publications listed below and included in full in the Appendix provides a detailed description of our methods, data and analyses.

When examined in relation to the approved SOW, we accomplished the following:

Major task 1

Sub-task 1 – accomplished

Sub-task 2 - accomplished. We have recruited and trained a team of post-docs, graduate students and technicians to perform all experiments proposed.

Sub-tasks 3 and 4 – We have completed the 5 Hz rate experiments and published the data showing benefits (Kulinich et al., 2018) and are now collecting data on the 1 Hz exposure rate. Data collected include brain tissue for biochemical analysis and MEA EEG data for electrophysiological analysis.

Sub-task 5 – We have trained a graduate student to design and conduct behavioral experiments and this will be the main thrust in year 2 to complement our published findings on electrophysiology, spine analysis and PV/PNN/MMP-9 analysis.

Major Tasks 2 and 3 are on schedule as proposed to be accomplished in years 2 and 3 of the grant. The required personnel and resources (mice, MEA analysis pipeline and equipment) are all ready to go for the next reporting year.

Key Outcomes:

Publications (full papers included in Appendix)

1. Kulinich AO¹, Reinhard SE¹, Rais M, Lovelace JW, Scott V*, Binder DK, Razak KA[#] and Ethell IM[#]. Beneficial effects of sound exposure on auditory cortex development in a mouse model of Fragile X Syndrome. *Neurobiology of Disease*. 2020. ¹ co-first authors, [#] co-corresponding authors. 2020 Feb;134:104622. doi: 10.1016/j.nbd.2019.104622.
2. Lovelace JW, Binder DK, Ethell IM and Razak KA. Minocycline treatment reverses sound evoked EEG abnormalities in a mouse model of Fragile X Syndrome. *Frontiers in Neuroscience: Neuropharmacology* 14:771. doi: 10.3389/fnins.2020.00771. eCollection 2020.
3. Pirbhoy P, Lovelace JW, Razak KA, Binder DK and Ethell IM. Acute pharmacological inhibition of matrix metalloproteinase-9 activity during development restores perineuronal net formation and

normalizes auditory processing in *Fmr1* KO mice. J. Neurochemistry 2020 May 6. doi: 10.1111/jnc.15037. Online ahead of print

4. Nguyen AO, Binder DK, Ethell IM and Razak KA. Abnormal development of auditory responses in the inferior colliculus of a mouse model of Fragile X Syndrome. J Neurophysiol 2020 Apr 22. doi: 10.1152/jn.00706.2019.
5. Jonak CR, Lovelace JW, Ethell IM, Razak KA and Binder DK. Multielectrode array analysis of EEG biomarkers in a mouse model of Fragile X Syndrome. Neurobiol Dis 2020 May; 138:104794. doi: 10.1016/j.nbd.2020.104794. Epub 2020 Feb 6.

Reviews (included in Appendix):

1. Razak KA, Dominick K and Erickson CA. Developmental studies in Fragile X Syndrome. J Neurodev Disord. 2020 May 2;12(1):13. doi: 10.1186/s11689-020-09310-9.
2. McCullagh EA, Rotschafer SE, Auerbach BD, Klug A, Kaczmarek LK, Cramer KS, Kulesza RJ Jr., Razak KA, Lovelace JW, Lu Y, Koch U, Wang Y. Mechanisms Underlying Auditory Hypersensitivity in Fragile X Syndrome. The FASEB Journal, 2020 Mar;34(3):3501-3518. doi: 10.1096/fj.201902435R. Epub 2020 Feb 10.

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 3 post-docs, 3 graduate students and 2 research technicians on the project aims. Post-docs, Jonathan Lovelace, 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 MEA EEG data and writing papers. Drs. Lovelace and Pirbhoy wrote and received their 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 has been trained in EEG, histological and behavioral analysis. She also successfully wrote and obtained an NIH F31 training grant. This prestigious and competitive fellowship will be a major aspect of professional development for Maham. Two other new graduate students, Mawaheb Kassir and Xin Tao have been recruited to be trained on these aims, particularly to develop behavioral analysis and MEG EEG analysis tools. Research technician, Carrie Jonak, is trained to conduct MEA 30 channel EEG recordings. Her first-author publication in 2020 identified major region specific EEG differences in WT and KO mice. 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 reviews during the reporting period (see above). We were scheduled to present at the Gordon Research Conference on FXS in June 2020, but that meeting was cancelled.

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 of year 2 is to identify the optimal rate of sound presentation and then begin the combined sound exposure + minocycline treatment during early development.

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).

Our project is the first to test the idea that modifying sensory environment during development may impact autism specific behaviors and electrophysiological correlates. Although this is just the first reporting period, our publications on this topic, and the pilot data from the grant have generated a novel way to think about therapeutics in FXS. The MEA EEG recording methods we have developed has developed considerable interest in the field from both academic and pharma side to test drugs. Our discovery that MMP9 targets can have an acute impact on neural correlates of sensory hypersensitivity has an impact on how we think about altered E/I balance in autism spectrum disorders.

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. Our identification of a narrow developmental window to target therapies will impact clinical trial designs in autism. Our data on how minocycline impacts some, but not all, EEG outcome measures will provide guidance on using this treatment option and EEG biomarkers across a number of neurodevelopmental and neurodegenerative conditions.

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. We fully expect the companies to absorb these techniques in their own future experimental designs. We are engaged in identifying multiple analytical tools to enable researchers to take full advantage of this powerful and big data technique.

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 first 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.

None

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 (full papers included in Appendix)

1. Kulinich AO¹, Reinhard SE¹, Rais M, Lovelace JW, Scott V*, Binder DK, Razak KA[#] and Ethell IM[#]. Beneficial effects of sound exposure on auditory cortex development in a mouse model of Fragile X Syndrome. *Neurobiol. Dis.* 2020. ¹ co-first authors, [#] co-corresponding authors. 2020 Feb;134:104622. doi: 10.1016/j.nbd.2019.104622.
2. Lovelace JW, Binder DK, Ethell IM and Razak KA. Minocycline treatment reverses sound evoked EEG abnormalities in a mouse model of Fragile X Syndrome. *Frontiers in Neuroscience: Neuropharmacology* 14:771. doi: 10.3389/fnins.2020.00771. eCollection 2020.
3. Pirbhoy P, Lovelace JW, Razak KA, Binder DK and Ethell IM. Acute pharmacological inhibition of matrix metalloproteinase-9 activity during development restores perineuronal net formation and normalizes auditory processing in *Fmr1* KO mice. *J. Neurochemistry* 2020 May 6. doi: 10.1111/jnc.15037. Online ahead of print
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2. McCullagh EA, Rotschafer SE, Auerbach BD, Klug A, Kaczmarek LK, Cramer KS, Kulesza RJ Jr., Razak KA, Lovelace JW, Lu Y, Koch U, Wang Y. Mechanisms Underlying Auditory Hypersensitivity in Fragile X Syndrome. *The FASEB Journal*, 2020 Mar;34(3):3501-3518. doi: 10.1096/fj.201902435R. Epub 2020 Feb 10.

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).*

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.*

None

- **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.

None

- **Technologies or techniques**

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

Multielectrode array EEG mouse skull recordings. The details are described in:

Jonak CR, Lovelace JW, Ethell IM, Razak KA and Binder DK. Multielectrode array analysis of EEG biomarkers in a mouse model of Fragile X Syndrome. Neurobiol Dis. 2020 May;138:104794. doi: 10.1016/j.nbd.2020.104794. Epub 2020 Feb 6.

- **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

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: *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 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: 8
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: 12

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 designing the sound exposure chamber and controlling exposure and drug treatment across the different ages of mice in this project and analyzing protein levels.
Funding Support: DOD

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

Contribution to Project: Dr. Pirbhoy is involved with recording EEGs and performing behavioral experiments following early treatment of MMP9 inhibitors including SB-3CT and minocycline.
Funding Support: FRAXA Foundation and Univ. of California Office of the President

Name: Maham Rais
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6

Contribution to Project: Ms. Rais is a doctoral student involved with IHC, behavior and EEG recordings of all aims.
Funding Support: DOD and NIH F31

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 and EEG recordings of all aims particularly after drug injection

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: Dr. Jonathan Lovelace

Project Role: Post-doc

Researcher Identifier (e.g. ORCID ID):

Nearest person month worked: 2

Contribution to Project: Dr. Lovelace completed data analyses and publication of the paper that evaluated effects of minocycline on EEG responses in the adult as the baseline data for the developmental work that will be completed in year 2

Funding Support: FRAXA Foundation

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

New Active Grants – none have overlap with aims of the present grant

W9126G-19-2-0032 (PI: Rankin, co-PI: Razak, Yanega) 09/20/2019 - 8/31/2021 0.24 academic

DOD/Edwards Airforce Base \$663,624 (total)

Natural Resources Support Pollinators, Edwards Air Force Base, California

This grant is to conduct bat behavior and distribution field studies in the Mojave Desert. No overlap.

NIH/UTSW, Dallas (Huber, Binder, Razak) 08/15/2020 – 07/14/2025 1.0 summer

\$3,180,658.00 (total)

Sex-specific mechanisms of cortical circuit dysfunction in a mouse ASD model

The goal of this project is to identify sex-specific mechanisms of hyperexcitability in a PTEN mouse model of autism. No overlap.

Ethell IM

Nothing to report

Binder DK

NIH/UTSW, Dallas (Huber, Binder, Razak)

08/15/2020 – 07/14/2025

1.0 calendar

\$3,180,658.00 (total)

Sex-specific mechanisms of cortical circuit dysfunction in a mouse ASD model

The goal of this project is to identify sex-specific mechanisms of hyperexcitability in a PTEN mouse model of autism. No overlap.

R01NS111552 (Tiwari-Woodruff, Binder)

02/01/2020-01/31/2025

0.72 calendar

NIH NINDS

\$511,043 (Dr. Binder's portion)

Demyelination is coupled to elevated hyperexcitability leading to seizures

The long-term goal of our research is to identify mechanisms linking demyelination to neuronal hyperexcitability and neurodegeneration. No overlap.

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

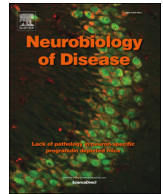
8. SPECIAL REPORTING REQUIREMENTS

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.*

Attached following this page.



Beneficial effects of sound exposure on auditory cortex development in a mouse model of Fragile X Syndrome

Anna O. Kulinich^{a,1}, Sarah M. Reinhard^{b,1}, Maham Rais^{a,d}, Jonathan W. Lovelace^b,
Veronica Scott^a, Devin K. Binder^{a,c,d}, Khaleel A. Razak^{b,c,d,*}, Iryna M. Ethell^{a,c,d,*}

^a Division of Biomedical Sciences, School of Medicine, University of California, Riverside, CA, USA

^b Psychology Department, University of California, Riverside, CA, USA

^c Neuroscience Graduate Program, University of California, Riverside, CA, USA

^d Biomedical Sciences Graduate Program, University of California, Riverside, CA, USA

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ABSTRACT

Background: Fragile X syndrome (FXS) is the most common genetic cause of autism and intellectual disability. *Fragile X mental retardation gene* (*Fmr1*) knock-out (KO) mice display core deficits of FXS, including abnormally increased sound-evoked responses, and show a delayed development of parvalbumin (PV) cells. Here, we present the surprising result that sound exposure during early development reduces correlates of auditory hypersensitivity in *Fmr1* KO mice.

Methods: *Fmr1* KO and wild-type (WT) mice were raised in a sound-attenuated environment (AE) or sound-exposed (SE) to 14 kHz tones (5 Hz repetition rate) from P9 until P21. At P21-P23, event-related potentials (ERPs), dendritic spine density, PV expression and phosphorylation of tropomyosin receptor kinase B (TrkB) were analyzed in the auditory cortex of AE and SE mice.

Results: Enhanced N1 amplitude of ERPs, impaired PV cell development, and increased spine density in layers (L) 2/3 and L5/6 excitatory neurons were observed in AE *Fmr1* KO compared to WT mice. In contrast, developmental sound exposure normalized ERP N1 amplitude, density of PV cells and dendritic spines in SE *Fmr1* KO mice. Finally, TrkB phosphorylation was reduced in AE *Fmr1* KO, but was enhanced in SE *Fmr1* KO mice, suggesting that BDNF-TrkB signaling may be regulated by sound exposure to influence PV cell development.

Conclusions: Our results demonstrate that sound exposure, but not attenuation, during early developmental window restores molecular, cellular and functional properties in the auditory cortex of *Fmr1* KO mice, and suggest this approach as a potential treatment for sensory phenotypes in FXS.

1. Introduction

Auditory hypersensitivity is commonly associated with Autistic Spectrum Disorders (ASDs) and when present early in development can impede cognitive development, which relies on human interactions with the outside world through sensory inputs (Kern et al., 2006; Marco et al., 2011; Horder et al., 2014). While sound attenuation was beneficial in controlling behavior problems related to hyper-reactivity to sound in children with ASD (Ikuta et al., 2016), habitual use of sound attenuating devices may affect linguistic communication and result in exacerbation of sound sensitivity over time (Jastreboff and Hazell, 2008; Morris, 2009). Indeed, developmental reduction of auditory input can affect inhibitory synapses in auditory cortex (Takesian et al., 2012)

and impair BDNF-dependent long-lasting inhibitory potentiation (Xu et al., 2010). Modulation of GABAergic circuits during development can result in long-lasting reversal of synaptic deficits following hearing loss in development (Kotak et al., 2013).

Fragile X syndrome (FXS) is a leading known genetic cause of autism (Harris et al., 2008) with symptoms that include intellectual disability, delayed language, social communication deficits, anxiety, seizures and sensory abnormalities (Rogers et al., 2001; Darnell and Klann, 2013), including auditory hypersensitivity (Rotschafer and Razak, 2013; Sinclair et al., 2017) and abnormal sensorimotor gating (Frankland et al., 2004). Enhanced N1 amplitude of auditory event-related potentials (ERPs; (Castren et al., 2003; Van der Molen et al., 2012)), impaired network synchronization (Ethridge et al., 2017) and

* Corresponding author at: Division of Biomedical Sciences, School of Medicine, University of California, Riverside, CA, USA.

E-mail addresses: Khaleel@ucr.edu (K.A. Razak), iryna.ethell@medsch.ucr.edu (I.M. Ethell).

¹ These authors contributed equally.

altered brain responses to stimulus repetition (Schneider et al., 2013; Knoth et al., 2018) were also observed in FXS. FXS arises from a mutation in *fragile X mental retardation 1 gene* (*Fmr1*; (Liu et al., 2018) leading to its epigenetic silencing (Mor-Shaked and Eiges, 2018), a loss of FMR protein (FMRP) (Pieretti et al., 1991)) and impaired FMRP-dependent protein translation (Darnell et al., 2011; Ferron, 2016), contributing to altered neuronal development and function (Contractor et al., 2015).

Fmr1 knock out (KO) mouse model of FXS recapitulates many features of FXS pathophysiology (Kazdoba et al., 2014), including an increased density of immature dendritic spines (Rudelli et al., 1985; Irwin et al., 2001), elevated auditory cortical responses (Rotschafer and Razak, 2013; Wen et al., 2018), reduced habituation of ERP to auditory stimuli (Lovelace et al., 2016; Wen et al., 2018), increased EEG gamma band power (Lovelace et al., 2018; Wen et al., 2019), abnormal sensorimotor gating (Olmos-Serrano et al., 2011; Kokash et al., 2019), audiogenic seizures (Musumeci et al., 2000) and impaired auditory experience-dependent plasticity (Kim et al., 2013). Our previous studies suggest a novel cellular mechanism for auditory hypersensitivity in *Fmr1* KO mice, showing delayed development of parvalbumin (PV) cells and perineuronal nets (PNN), which often enwrap PV cells and regulate their excitability (Balmer, 2016). Neurons in the auditory cortex of *Fmr1* KO mice develop hyper-responsiveness during P14-P21 window (Wen et al., 2018), when their synaptic and intrinsic properties mature (Oswald and Reyes, 2008, 2011), coinciding with the critical period plasticity window (CPP) in the mouse auditory cortex (Kim et al., 2013). These studies suggest that cortical responses in *Fmr1* KO mice become abnormal within 10–12 days of hearing onset and may be driven by abnormal sensitivity to auditory input.

The purpose of this study was to examine the effects of sound-attenuated environment (AE) or passive sound exposure (SE) during early postnatal developmental period (P9-P21). We found reduced density of PV cells, increased dendritic spine density and larger N1 amplitude of ERPs in the auditory cortex of AE *Fmr1* KO mice compared to their (wild-type) WT counterparts. In contrast, exposing *Fmr1* KO mice to trains of pure 14 kHz tones during P9-P21 period significantly increased the number of PV cells, normalized dendritic spine density and restored N1 response amplitude to WT levels.

2. Materials and methods

2.1. Ethics statement

All experiments and animal care/use protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside and were carried out in accordance with NIH “Guide for the Care and Use of Laboratory Animals”.

2.2. Mice and sound exposure

The FVB-129P2-*Fmr1*^{tm1Cgr/J} (*Fmr1* KO) and their control strain FVB-129P2-Pde6b Tyr^{c-h}/AntJ (wild-type, WT) were originally obtained from the Jackson Laboratories (Sacramento, CA, USA). Animals were maintained in an AAALAC accredited facility under a 12-h light/dark cycle with water and food provided ad libitum. For the normal exposure (NE) group, mice were raised in an accredited vivarium from birth to the day of experiment (background ~65–70 dB SPL; BK Precision, model 732A). For the sound attenuated exposure (AE), age matched WT and *Fmr1* KO mouse litters with their mothers were housed together in a custom-built sound-shielded chamber lined with anechoic foam (105 cm × 105 cm × 63 cm, background ~37–40 dB SPL; BK Precision, model 732A sound level meter) from P4–7 until the time of testing (P20-P22). The chamber was placed in a room (275 cm × 420 cm) with background ~45 dB SPL, which was accessed only once daily for animal health checks. Although we used different litters of WT and *Fmr1* KO mice, both WT and KO mice were reared

together in the same chamber at the same time. In the sound exposure (SE) group, age matched WT and *Fmr1* KO mouse litters were placed together in the same sound-shielded chamber and room with their mothers at P4–7 and exposed to pure tone pips (65 dB SPL; 14 kHz; 30 ms duration, 5 ms on/off ramp; 6 pips at a 5 Hz rep. Rate; 2 s ITI) for 24 h/day from P9 to P21. The sound was generated (Avisoft-SASLab Pro) and delivered through a speaker (Ultra Sound Gate Player BL Light; RECORDER USGH) placed in the center of the chamber mounted to the ceiling (~30 cm above the floor). No harmonics were detected during the sound delivery (Sokolich Probe Microphone System; Spectra DAQ-200). To prevent any tone exposure of the AE group, SE and AE groups did not temporally overlap and only one sound attenuation chamber was present in the room. Therefore, different WT and *Fmr1* KO mouse litters were used for SE versus AE experiment.

2.3. Immunofluorescence

Male WT and *Fmr1* KO mice were euthanized with isoflurane at P21–22 and perfused transcardially first with cold phosphate-buffered saline (PBS, 0.1 M) and then with 4% paraformaldehyde (PFA) in PBS. Brains were removed, post-fixed for 2 h in 4% PFA and 100 µm coronal sections containing auditory cortex were obtained using a vibratome (Campden Instruments) with a speed of 0.5–0.65 mm/s. Identification of auditory cortex was performed using hippocampal landmarks (Wen et al., 2018). This method was previously validated using tonotopic mapping, dye injection (Martin del Campo et al., 2012) and Paxinos mouse brain atlas and other publications on mouse auditory cortex (Anderson et al., 2009).

On average, 5–6 slices containing auditory cortex were obtained from each brain. Immunolabeling of sections was performed using the following protocol. Brain slices were post-fixed with 4% PFA in 0.1 M PBS for an extra 2 h and then washed in 0.1 M PBS. Then, brain tissues were incubated with 50 mM ammonium chloride for 15 min to quench residual PFA and washed with PBS. Next, 0.1% Triton X-100 in PBS was applied to permeabilize slices, which was followed by incubation in 5% Normal Goat Serum (NGS; Sigma, catalog# G9023–10 mL) and 1% Bovine Serum Albumin (BSA; Fisher Scientific, catalog# 9048468) in 0.1 M PBS solution to block tissue nonspecific staining. Sections were further incubated with primary antibodies and fluorescein-tagged *Wisteria floribunda* agglutinin (WFA, 4 µg/mL; Vector Laboratories, catalog# FL-1351, RRID:AB_2336875) in 0.1 M PBS containing 1% NGS, 0.5% BSA, and 0.1% Tween-20 for 24 h. Mouse anti-PV antibody (1:1000; Sigma, catalog# P3088, RRID:AB_477329) was used to visualize PV+ interneurons. WFA, a lectin that recognizes glycosaminoglycan side chains of chondroitin sulfate proteoglycans present in PNNs, primarily but not exclusively aggrecan, was used to label PNNs, referred here as WFA + PNNs (Pizzorusso et al., 2002). Next, slices were washed in 0.1 M PBS containing 0.5% Tween-20 and incubated with secondary antibodies, donkey anti-mouse Alexa 594 (4 µg/mL; Thermo Fisher Scientific, catalog# A-21203, RRID:AB_2535789), in 0.1 M PBS for 1 h. Brain tissues were further washed with 0.1 M PBS containing 0.5% Tween-20, mounted with Vectashield (Vector Labs, catalog# H-1200) and sealed with Cytoseal (ThermoScientific, catalog# 8310–16). Each slice was imaged with a Leica TCS SP5 confocal microscope using a series of 10 high-resolution optical sections (1024 × 1024-pixel format) and a 10×, or a 63× water-immersion objective (1.2 numerical aperture), 1 × zoom at 1 µm step (z-stack). For the analysis, each z-stack was collapsed into a single image, converted to a TIFF file using Image J and encoded for blind analysis. Image J was also used to analyze density of PNN + cells, PV+ cells and their colocalization in cortical layers 2–6. *t*-test was used to determine genotype specific differences for each experiment. Statistical analyses were performed using Microsoft Excel and GraphPad Prism 7.

2.4. Dendritic spine labeling and analysis

Brain slices were collected as described above and dendritic spines were labeled using DiO-listic approach as previously described (Staffend and Meisel, 2011). Tungsten particles were coated with 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; catalog# D282, Invitrogen), delivered to the brain slices by helium-powered ejection (Bio-Rad Helios Gen Gun System) and further incubated in PBS for 72 h at 4 °C before being mounted onto slides in PBS and sealed with Cytoseal (ThermoScientific, catalog# 8310–16). Labeled neurons were first identified in layers (L) 1–6 of auditory cortex using confocal microscopy with 10× objective (model LSM 510, Carl Zeiss MicroImaging) and images of basolateral dendrites of L2–6 neurons in respective layers were captured with a 63× water-immersion objective (1.2 numerical aperture) and 1× zoom using a series of high-resolution optical sections (1024 × 1024-pixel format) at 0.5 μm step intervals (z-stack). Each z-stack was collapsed into a single image by projection (LSM Image Browser, Zeiss), converted to a TIFF file and encoded for blind analysis. Analysis of the spine density (spines per 10 μm dendrite) and volume (μm³) was performed using Neurolucida 360 software (MicroBrightField; RRID: [SCR_001775](#)) (Koeppen et al., 2018). At least five animals (AE WT, *n* = 5; AE *Fmr1* KO, *n* = 7; SE WT, *n* = 5; SE *Fmr1* KO, *n* = 7) were analyzed per group, and dendrites from at least 5 neurons were imaged per animal (AE WT, *n* = 33; AE *Fmr1* KO, *n* = 39; SE WT, *n* = 28; AE *Fmr1* KO, *n* = 38).

2.5. Electrophysiology

Auditory ERPs were recorded to quantify P1, N1, P2 amplitudes and latencies across the different groups of mice. Mice were anesthetized with ketamine-xylazine (K/X) (80/10 mg/kg) with half dose supplements as needed. They were secured on a bite bar, and placed on a stereotaxic apparatus (model 930; Kopf, CA). To monitor the anesthetic state, a toe pinch reflex was assessed every 20–30 min throughout the experiment. Once the animal was anesthetized, a midline sagittal incision along the scalp was made to expose the skull, and the right temporalis muscle was displaced. A dental drill was used to remove the skull over the right auditory cortex (Martin del Campo et al., 2012). Location of core auditory cortex was identified using vasculature, and confirmed with extracellular local field potential recordings using broadband sound (0.25 Hz repetition rate) and a tungsten microelectrode (FHC Inc., diameter 125 μm). At the end of the experiment, mice were euthanized with sodium pentobarbital (i.p. 125 mg/kg).

ERP recordings were obtained in a sound-attenuated chamber lined with anechoic foam (Gretsch-Ken Industries, Oregon) as previously described (Lovelace et al., 2016). Acoustic stimuli were generated using TDT RZ6 system and presented through a free-field speaker (LCY-K100 ribbon tweeters; Madisound, WI) located 6 in. away at a 45° angle from the animal's left ear. Sound level was modified using programmable attenuators (PA5; Tucker-Davis Technologies, Florida). The speaker output was flat within +/− 3 dB for frequencies between 5 and 35 kHz as measured using a 1/4 in. B&K microphone. The stimulus consisted of trains of 10 repeating pure tones (14 kHz or 23 kHz, 100 ms duration), 5 ms rise/fall time, 8 s inter-train interval 70 dB SPL presented at 0.25 Hz repetition rate. After 5 min of baseline recordings with no auditory stimulation, at least 50 repetitions (5 trains with 10 tone pips each) of the tone were presented. Auditory ERP recordings were obtained using the BioPac system (BIOPAC Systems, Inc.). A tungsten microelectrode (FHC Inc.) was advanced into the brain using a Kopf direct drive 2660 micropositioner (depth 350–600 μm). A stainless steel wire (California Fine Wire Company) was attached to the neck muscle for grounding purposes. At each recording location, testing began only after sound-driven ERPs with an N1 component with ~30–80 ms latency could be reliably produced. The BioPac MP150 acquisition system was connected to an EEG 100C amplifier unit to which the recording electrode and ground were attached. Signals were filtered (high-pass

(0.5 Hz) and low-pass (35 Hz)). Data were sampled at a rate of either 5 or 20 kHz using Acqknowledge software, and sound delivery was synchronized with ERP recording using a 5 ms digital TTL pulse to mark the onset of each sound in a train. ERP traces were extracted from Acknowledge and converted to files compatible with Brain Vision Analyzer 2.1. Artifact rejection was done using several criteria such as amplitude, gradient, max-min and low activity. After that, recorded traces were subdivided into 250 ms segments based on a TTL pulse that marked the sound stimulus onset. P1, N1 and P2 were detected based on maximal positive or negative deflections in a certain time window: P1 (10–50 ms), N1 (30–80 ms) and P2 (75–150 ms).

2.6. Western blotting

Mice were euthanized at P21 with isoflurane and cervical dislocation was performed. Auditory cortex was identified based on methods described earlier and dissected. Tissue samples were immediately homogenized in cold HEPES buffer (4 mM HEPES buffer, pH 7.5; 300 mM sucrose; 150 mM NaCl; 0.1 mM phenylmethylsulfonyl fluoride (PMSF); protease inhibitor cocktail (Sigma, P8340); 0.5 mM sodium orthovanadate) to extract soluble cytoplasmic proteins for PV detection. Another subset of tissue samples were collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA, pH 8.0; 1% Triton X-100; 0.1% SDS; protease inhibitor cocktail; 0.5 mM sodium pervanadate) to extract membrane-bound proteins for detection of tropomyosin kinase B (TrkB). In both cases, samples were rotated at 4 °C for 1 h to allow for complete cell lysis and then cleared by centrifugation at 13,200 rpm for 20 min at 4 °C. Samples were boiled in reducing sample buffer (Laemmli 2× concentrate, S3401, Sigma), and separated on 8–16% Tris-glycine SDS-PAGE precast gels (Invitrogen). Proteins were transferred onto Protran BA 85 Nitrocellulose membrane (GE Healthcare), washed 2 times (5 min each) with milliQ water and further blocked for 1 h at room temperature in 5% BSA (BSA; Fisher Scientific, catalog# 9048468) or skim milk (catalog #170–6404, Bio-Rad), based on the manufacture's staining protocol. Incubation with primary antibody diluted in TBS/0.1% Tween-20/5% BSA was performed overnight at 4 °C. The following primary antibodies for protein detection were used: mouse anti-PV (1:1000; Millipore, catalog# MAB1572, RRID:AB_2174013), rabbit anti-β-actin (1:2000; Abcam, catalog# Ab8227, RRID:AB_2305186), mouse anti-total TrkB (1:2000; BD Transduction Laboratories, catalog# 610101, RRID:AB_397507), rabbit anti-phospho-TrkB (Tyr816) (1:2000; Millipore, catalog# ABN1381, RRID:AB_2721199) and rabbit anti-phospho-TrkB (Tyr515) (1:1000; Bioworld, catalog# AP0236). Blots were washed 3 × 10 min with TBS/0.1% Tween-20 and incubated with the appropriate HRP-conjugated secondary antibodies for 1 h at room temperature in a TBS/0.1% Tween-20/5% BSA solution. The secondary antibodies used were α-rabbit-HRP (Thermo Fisher Scientific, catalog #G-21234, RRID: [AB_2536530](#)) at 1:10000 and α-mouse-HRP at 1:10000 (Jackson ImmunoResearch Labs, catalog #715–035-150, RRID:AB_2340770). Next, blots were washed 3 × 10 min in TBS/0.1% Tween-20 and 1 × 10 min TBS and developed with ECL Detection reagent (catalog# 80196, Thermo Scientific). For reprobing, membrane blots were washed in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 6.8) for 30 min at 55 °C, then rinsed repeatedly with TBS/0.1% Tween-20 (5 × 5 min), blocked with 5% skim milk, and then re-probed. X-ray film (Thermo Scientific) was used for the protein bands signal detection, which was further processed using mini-medical X-ray processor (AFP Imaging). Developed films were then scanned, and band density was analyzed by measuring band and background intensity using Adobe Photoshop CS5.1 software (RRID: [SCR_014199](#)). Samples from *Fmr1* KO and their counterpart WT mice (NE, AE or SE groups) were run on the same blot, and precision/tolerance (P/T) ratios for *Fmr1* KO samples were normalized to averaged P/T ratios of WT samples.

2.7. Data analysis

Statistical analysis was performed using unpaired *t*-test to determine genotype differences within the same sound exposure group (NE WT vs NE *Fmr1* KO, AE WT vs AE *Fmr1* KO or SE WT vs SE *Fmr1* KO). In order to compare the effect of sound exposure, two-way ANOVA was performed (sound exposure, genotype) followed by Bonferroni's post hoc pair-by-pair comparisons between AE and SE groups. In all cases, GraphPad Prism 7 was used for the analysis and data represent mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. AE *Fmr1* KO mice show enhanced auditory ERP N1 amplitudes

Tone-evoked single unit responses are increased in auditory cortex of P21 *Fmr1* KO mice compared to WT mice (Wen et al., 2018). These mice were raised in a regular vivarium with a variety of environmental sounds (NE group). We hypothesized that developmental attenuation of environmental sounds might normalize cortical development in the KO mice. To test this hypothesis, we raised mice in a sound-attenuating box (AE) with the background noise averaging ~ 37 – 40 dB SPL (compared to ~ 65 – 70 dB SPL in the vivarium) during early postnatal (P9–P21) period. We next recorded ERPs from auditory cortex of P21–P23 mice in response to 14 kHz or 23 kHz tones and measured peak amplitudes and latencies of P1, N1 and P2 components (Fig. 1A–H, Fig.S1A–F). 14 kHz and 23 kHz were chosen based on previously published results, which demonstrate that higher percentage of cortical neurons respond to 11–15 kHz tones compared to 21–25 kHz (Rotschafer and Razak, 2013).

The P1 and P2 amplitudes evoked by 14 kHz and 23 kHz tones were not significantly different between AE WT and AE *Fmr1* KO mice (Fig. 1A–C, 1E–F, 1H). However, higher N1 amplitude was detected in response to 14 kHz tone ($p = .0496$; Fig. 1A, D; Table S1), but not to 23 kHz tone (Fig. 1B, G; Table S2), in AE *Fmr1* KO compared to AE WT mice.

No significant differences were detected in P1 and N1 latencies in response to 14 kHz and 23 kHz between AE *Fmr1* KO and WT mice (Fig.S1A, B, D, E; Table S1–S2), whereas a longer P2 latency was found in AE *Fmr1* KO mice compared to AE WT in response to 14 kHz ($p = .0093$; Fig.S1C) but not 23 kHz (Fig.S1F; Table S1–S2).

In summary, enhanced responses were observed in P21–P23 auditory cortex of *Fmr1* KO mice raised in sound-attenuated environment compared to their WT counterparts, similar to our previous findings in *Fmr1* KO mice raised in the standard vivarium.

3.2. SE *Fmr1* KO mice exposed to 14 kHz tone during development exhibit normal sound-evoked responses

We next tested whether passive developmental sound exposure of mice would normalize sound-evoked responses in *Fmr1* KO mice. Because we observed a difference in N1 amplitude between AE *Fmr1* KO and AE WT in response to 14 kHz tone, we chose this frequency for exposure with ~ 65 dB SPL (de Villiers-Sidani et al., 2008; Kim et al., 2013; Zhu et al., 2014). WT and *Fmr1* KO mice were placed in a sound-attenuated chamber at P5 and exposed to 14 kHz tone pip trains 24 h/day from P9 to P21–23 when analysis was performed.

Analysis of average peak amplitude and latency of raw ERP P1, N1 and P2 components in auditory cortex of SE WT and SE *Fmr1* KO mice showed no genotype differences for both 14 kHz and 23 kHz tones

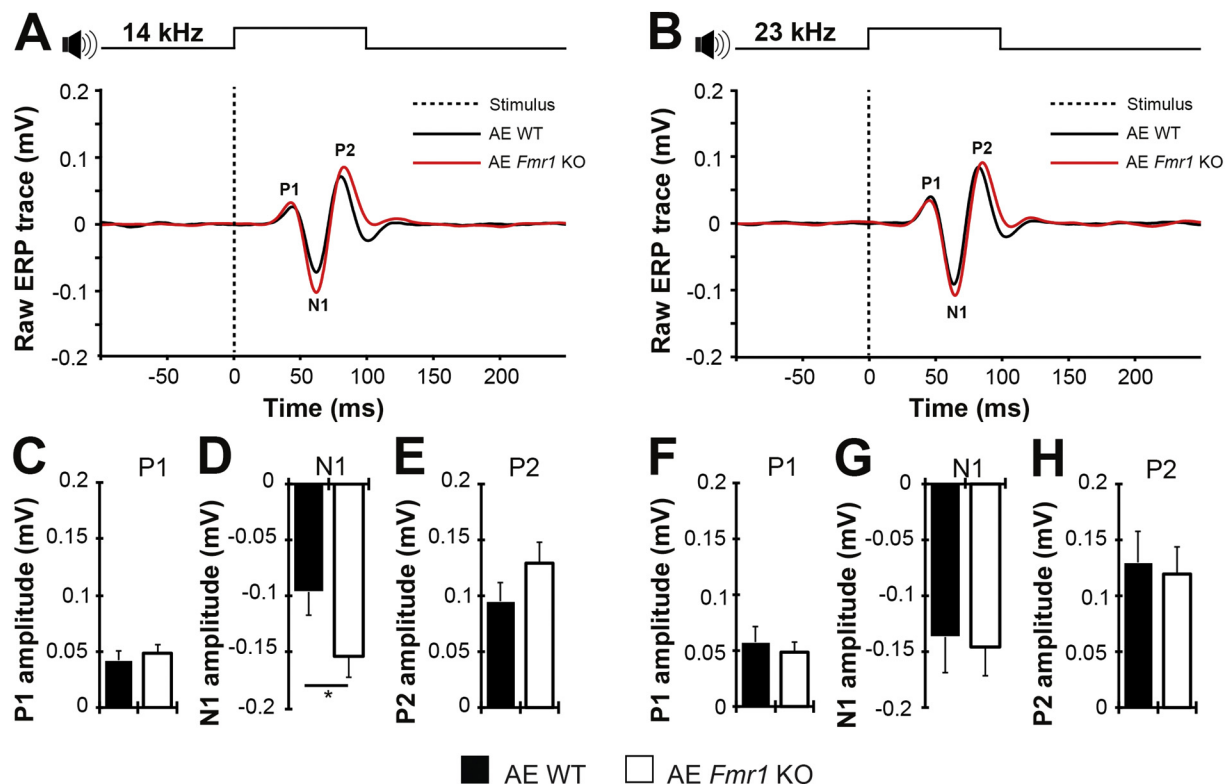


Fig. 1. Sound evoked event-related potentials (ERPs) in the auditory cortex of AE WT and *Fmr1* KO mice. ERPs were evoked by 14 kHz tones (A, C, D, E) or 23 kHz tones (B, F, G, H) in auditory cortex of AE WT and AE *Fmr1* KO (AE WT, $n = 14$ recording sites/6 animals vs AE *Fmr1* KO, $n = 19$ recording sites/8 animals). (A, B) Graphs show average raw ERP traces from all recording sites evoked by 14 kHz (A) or 23 kHz (B) tones in auditory cortex of AE WT and AE *Fmr1* KO mice. (C–H) Quantitative analysis of amplitudes of P1 (C, F), N1 (D, G) and P2 (E, H) ERP responses evoked by 14 Hz (C–E) and 23 Hz (F–H) tones. N1 amplitude of ERP evoked by 14 Hz tone was higher (D, $p < .05$) in auditory cortex of AE *Fmr1* KO compared to AE WT. No differences were detected in P1, N1 or P2 amplitudes of ERP evoked by 23 Hz tone in auditory cortex of AE *Fmr1* KO compared to AE WT mice. We presented 5 trains of 10 pure tones at 0.25 Hz repetition rate. To exclude the effects of habituation, only the responses to the first train (10 responses) were averaged to calculate amplitude and latency of P1, N1 and P2 components. Statistical analysis was done using unpaired *t*-test: *, $p < .05$; **, $p < .01$.

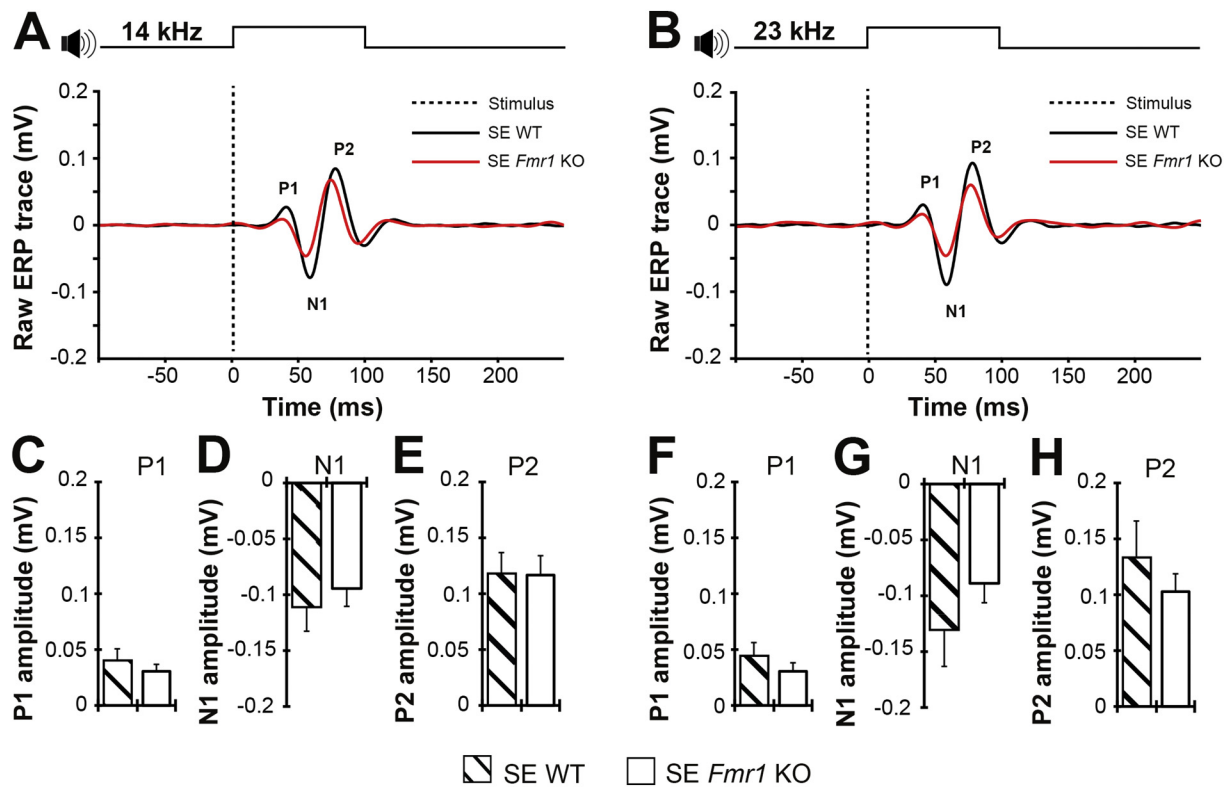


Fig. 2. Sound-evoked ERPs in the auditory cortex of SE WT and *Fmr1* KO mice. ERPs were evoked by 14 kHz tone (A, C, D, E) and 23 kHz tone (B, F, G, H) in auditory cortex of SE WT and SE *Fmr1* KO (SE WT, $n = 11$ recording sites/5 animals; SE *Fmr1* KO, $n = 13$ recording sites/6 animals). (A, B) Graphs shows average raw ERP traces from all recording sites evoked by 14 kHz (A) or 23 kHz (B) tones (E) in auditory cortex of SE WT and SE *Fmr1* KO mice. (C-H) Quantitative analysis of amplitudes of P1 (C, F), N1 (D, G) and P2 (E, H) ERP responses evoked by 14 Hz (C-E) and 23 Hz (F-H) tones. No differences were detected in P1, N1 or P2 amplitudes and latencies of ERP evoked by 14 kHz and 23 Hz tones in auditory cortex of SE *Fmr1* KO compared to SE WT mice. Statistical analysis was done using unpaired *t*-test.

(Fig. 2A-H; Fig.S1G-L, Table S1-S2). Specifically, N1 peak amplitude evoked by 14 kHz tone in SE *Fmr1* KO mice was not significantly different from SE WT group (Fig. 2D). Interestingly, two-way ANOVA analysis of P1, N1 or P2 latencies evoked by 14 kHz with exposure (AE, SE) and genotype (WT, *Fmr1* KO) as factors showed exposure effect (P1: $F_{(1,53)} = 4.101$, $p = .0479$; N1: $F_{(1,53)} = 6.369$, $p = .0146$; P2: $F_{(1,53)} = 7.626$, $p = .0079$) and interaction (N1: $F_{(1,53)} = 5.502$, $p = .0228$; P2: $F_{(1,53)} = 7.609$, $p = .0080$) with significant differences between SE *Fmr1* KO mice and AE *Fmr1* KO mice with Bonferroni's multiple comparisons test (P1: $p = .0148$; N1: $p = .0012$, P2: $p = .0002$). Further, two-way ANOVA analysis of P1, N1 or P2 latencies evoked by 23 kHz with exposure (AE, SE) and genotype (WT, *Fmr1* KO) as factors also showed exposure effect (N1: $F_{(1,52)} = 6.096$, $p = .0169$; P2: $F_{(1,52)} = 12.15$, $p = .0010$) with significant differences between SE *Fmr1* KO mice and AE *Fmr1* KO mice with Bonferroni's multiple comparisons test (P1: $p = .0246$; N1: $p = .0071$; P2: $p = .0007$). No differences in P1, N1 and P2 amplitudes and latencies were observed between SE WT and AE WT mice (Fig.S1G-L).

Our results show that developmental sound exposure restored sound evoked N1 amplitude and P2 latency in auditory cortex of *Fmr1* KO mice to WT levels.

3.3. Developmental exposure of SE *Fmr1* KO mice to 14 kHz tone reverses impaired development of PV cells in the auditory cortex of AE *Fmr1* KO mice

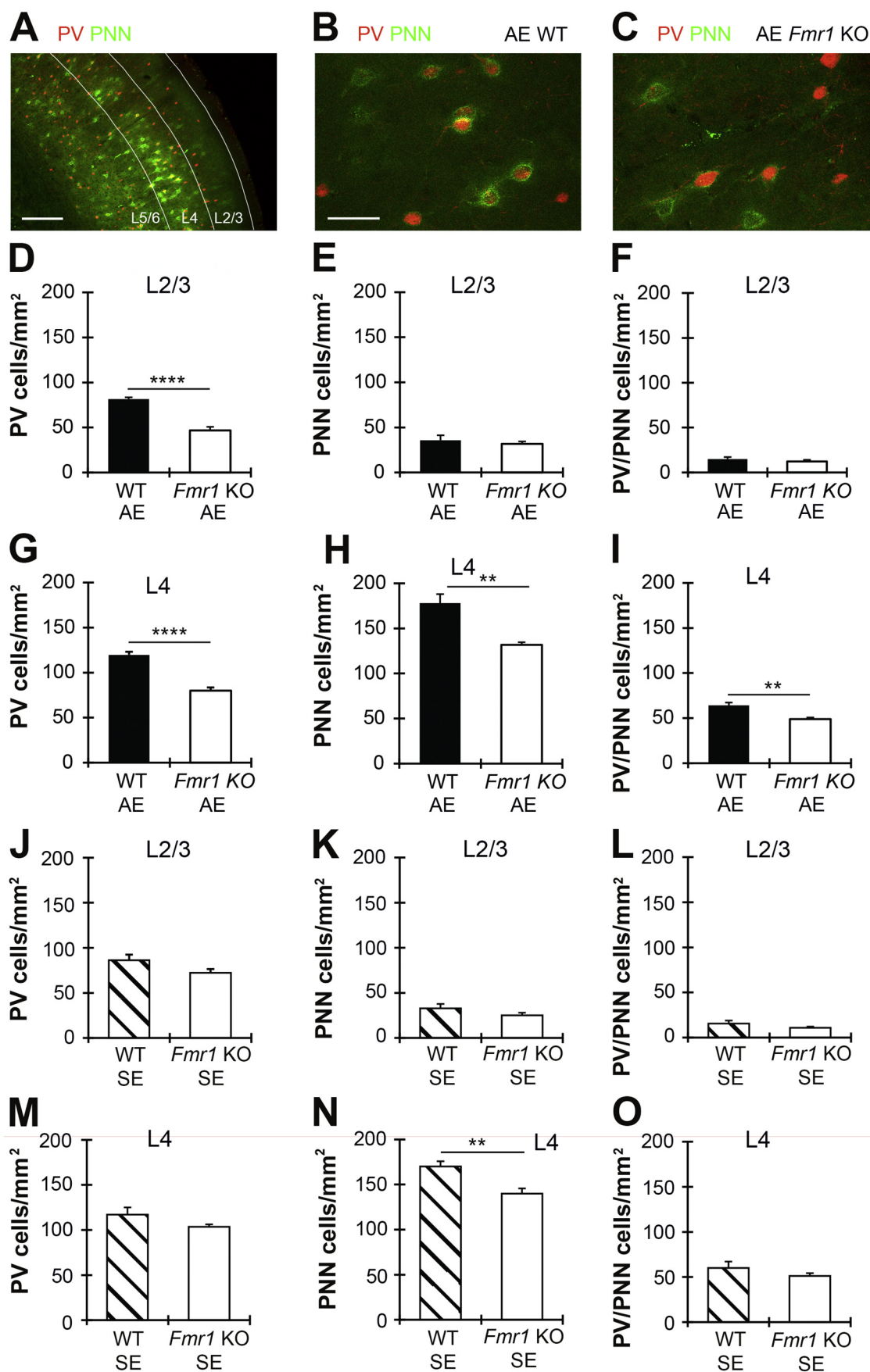
A reduced density of PV interneurons and PNNs were previously demonstrated in the auditory cortex of P21 *Fmr1* KO compared to WT mice, which may underlie abnormal responses to sound (Wen et al., 2018). Here we examined the density of PV + interneurons and PNNs in the auditory cortex of AE and SE *Fmr1* KO mice at P21 (Fig. 3A-O).

We found that PV cell density was significantly reduced in L2/3

($p < .0001$; Fig. 3D) and L4 ($p < .0001$; Fig. 3G) of AE *Fmr1* KO compared to AE WT mice. Similar changes were observed in L5/6 (Table S3). We also observed decreased density of PNNs in L4 ($p = .0025$; Fig. 3H) and L5/6 ($p = .0240$; Table S3) in *Fmr1* KO compared to WT mice. No differences in PNN density were observed in L2/3 *Fmr1* KO and WT mice (Fig. 3E), likely due to low density of PNNs in L2/3. Analysis of PV and PNN co-localization showed lower density of PV cells with PNN in L4 ($p = .0031$; Fig. 3I) and L5/6 ($p = .0140$; Table S3) in *Fmr1* KO compared to WT mice. Interestingly, we also observed a reduction in the density of PV cells lacking PNNs in L2/3 ($p < .0001$; Table S3), L4 ($p = .0024$; Table S3) and L5/6 ($p = .0001$; Table S3) in *Fmr1* KO compared to WT mice, which was not previously reported in NE mice (Wen et al., 2018). Thus, auditory cortex of *Fmr1* KO mice raised in sound-attenuated environment exhibit a decreased density of PV cells and PNNs compared to sound-attenuated WT counterparts, as previously seen in *Fmr1* KO mice raised in the standard vivarium.

In contrast, PV cell density in SE *Fmr1* KO was similar to SE WT level (Fig. 3J, M; Table S3), suggesting that the sound exposure restored PV cell development to normal levels in *Fmr1* KO mice. However, density of PNNs remained lower in L4 auditory cortex of SE *Fmr1* KO as compared to SE WT mice ($p = .0026$; Fig. 3N), but no differences were observed between SE *Fmr1* KO and SE WT mice in L5/6 ($p = .56$; Table S3). The density of PV cells containing PNNs was also not significantly different between SE WT and SE *Fmr1* KO mice (Fig. 3L, O).

A two-way ANOVA analysis was used to compare SE and AE groups that were raised in the same chamber with (SE) or without (AE) sound exposure. The analysis with exposure (AE, SE) and genotype (WT, *Fmr1* KO) as factors showed no exposure effect ($F_{(1,23)} = 3.655$, $p = .0685$), but showed a significant genotype effect ($F_{(1,23)} = 23.44$, $p < .0001$) and interaction ($F_{(1,23)} = 5.549$, $p = .0274$). However, pairwise comparison with Bonferroni's multiple comparisons test revealed an



(caption on next page)

Fig. 3. Density of PV cell, but PNNs, is normalized in P21 auditory cortex of SE *Fmr1* KO mice. (A) Confocal image shows PV immunoreactivity and PNN-labeling in auditory cortex of AE WT mice. Scale bar, 250 μm . (B, C) High magnification confocal images show representative examples of PV immunoreactivity and PNN-labeling in AE WT and AE *Fmr1* KO auditory cortex. Scale bar, 50 μm .

(D-I) Quantitative analysis of the density of PV cells (D, G), PNN-containing cells (E, H) and PNN-containing PV cells (F, I) in L2/3 and L4 auditory cortex of AE WT ($n = 6$) and AE *Fmr1* KO ($n = 5$) mice. There was reduced PV cell density in L2/3 (D, AE WT vs AE *Fmr1* KO, $p < .0001$) and L4 (G, AE WT vs AE *Fmr1* KO, $p < .0001$) of AE *Fmr1* KO mice compared to AE WT. PNN-containing cell density was lower in L4 of AE *Fmr1* KO mice compared to AE WT (H, AE WT vs AE *Fmr1* KO, $p < .01$). PNN-containing PV cell density was reduced in L4 of AE *Fmr1* KO compared to AE WT mice (I, AE WT vs AE *Fmr1* KO, $p < .01$). Statistical analysis was performed using unpaired *t*-test: **, $p < .01$; ****, $p < .0001$.

(J-O) Quantitative analysis of density of PV cells (J, M), PNNs (K, N) and PNN-containing PV cells (L, O) in L2/3 (J-L) and L4 (M-O) of P21 auditory cortex in SE WT and SE *Fmr1* KO mice (SE WT, $n = 8$; SE *Fmr1* KO, $n = 8$). PNN-containing cell density remained lower in L4 of SE *Fmr1* KO mice compared to SE WT (N, $p < .01$). No differences were observed in the density of PV cells in L2/3 (J) and L4 (M). PNN-containing PV cell density was also similar between SE WT and SE *Fmr1* KO in L2/3 (L) and L4 (O) auditory cortex. Statistical analysis was done using unpaired *t*-test: **, $p < .01$.

increased PV cell density in SE *Fmr1* KO compared to AE *Fmr1* KO ($p < .05$), but no difference between SE WT and AE WT ($p > .9999$) in all layers.

Taken together, the results demonstrate that exposing mice to 14 kHz tone during development results in increased density of PV cells in all layers of *Fmr1* KO mice to WT levels, without an effect on PNNs.

3.4. AE but not SE *Fmr1* KO mice auditory cortex neurons show abnormal increase in dendritic spine density

Dendritic spine density and morphology were analyzed in L2–6 auditory cortex of AE and SE mice using DiOlistic approach (Fig. 4A–L). A significantly higher dendritic spine density was observed in basolateral dendrites of L2/3 and L5/6 excitatory neurons in AE *Fmr1* KO

compared to WT mice ($p = .0061$, Fig. 4E; $p = .0090$, Fig. 4I), but L4 neurons were not different (Table S4). Dendritic spine morphology was evaluated based on spine volume, where spines with small volume ($< 0.5 \mu\text{m}^3$) represent immature spines with small heads and spines with large volume represent mature spines (stubby- or mushroom-like spines with large heads (Koeppen et al., 2018)). No differences in spine morphology were detected in L2/3 and L5/6 auditory cortex between WT and *Fmr1* KO (Fig. 4F, J). Thus, *Fmr1* KO mice raised in sound-attenuated environment show increased spine density in L2/3 and L5/6 neurons, but no significant changes in dendritic spine morphology.

In contrast, dendritic spine density in basolateral dendrites of L2/3 and L5/6 excitatory neurons was similar in SE *Fmr1* KO and WT mice (Fig. 4C–D, 4G–H, 4K–L; Table S4). There was one significant difference in spine morphology detected in L2/3 auditory cortex of SE *Fmr1* KO

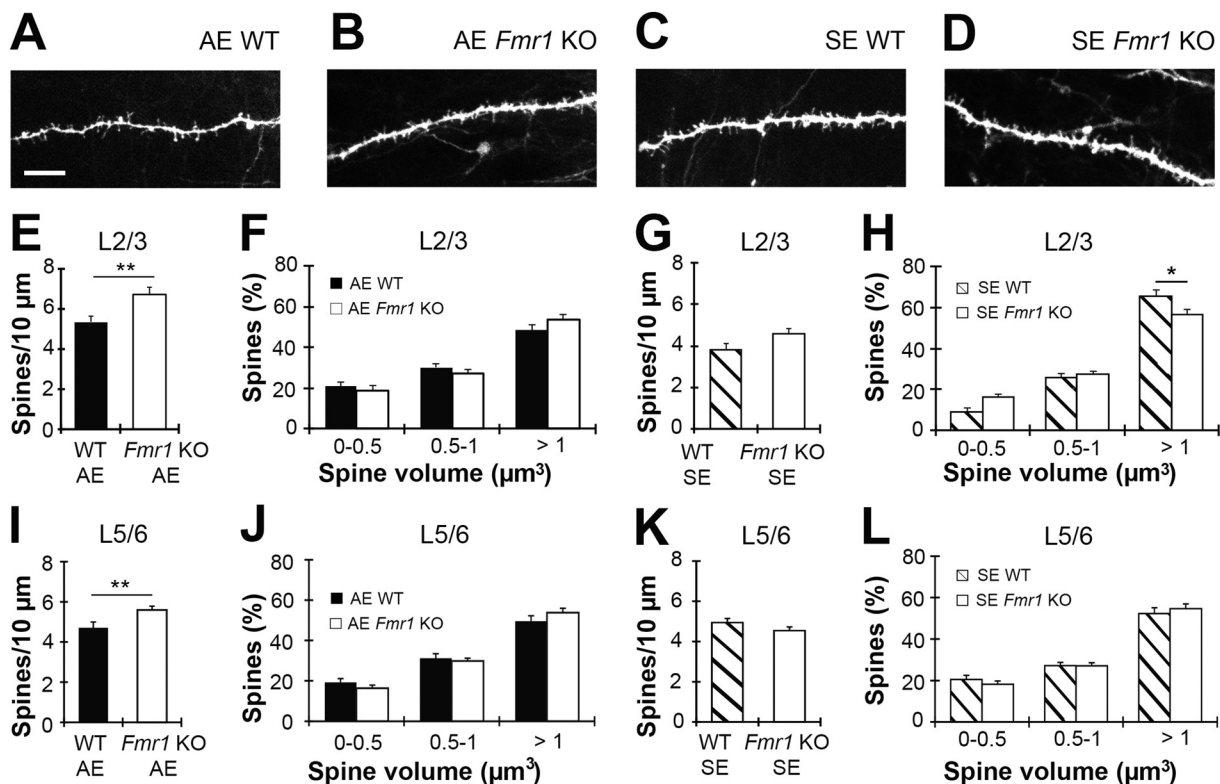


Fig. 4. Analysis of dendritic spine density and morphology in the auditory cortex of AE and SE mice showed beneficial effects of passive sound exposure. (A–D) Confocal images showing DiI-labeled dendritic spines in P21 auditory cortex of AE WT (A), AE *Fmr1* KO (B), SE WT (C) and SE *Fmr1* KO (D) mice. Scale bar, 10 μm . (E, F, I, J) Quantitative analysis of spine density (E, I) and spine morphology (F, J) in L2/3 (E, F) and L5/6 (I, J) auditory cortex of AE WT and AE *Fmr1* KO mice. Spine density was higher in AE *Fmr1* KO mice compared to AE WT in L2/3 (E, AE WT, $n = 43$ dendrites vs AE *Fmr1* KO, $n = 25$ dendrites, $p < .01$) and in L5/6 (I, AE WT, $n = 29$ dendrites vs AE *Fmr1* KO, $n = 46$ dendrites, $p = .0090$). No differences in spine morphology were found between AE WT and AE *Fmr1* KO in L2/3 (F) and L5/6 (J). Statistical analysis was done using unpaired *t*-test (E, I) or two-way ANOVA (for spine volume, F and J): *, $p < .05$; **, $p < .01$; ****, $p < .0001$. (G, H, K, L) Quantitative analysis of spine density (G, K) and spine morphology (H, L) in L2/3 and L5/6 auditory cortex of SE WT and SE *Fmr1* KO mice. There were no differences in spine density between SE *Fmr1* KO and SE WT in L2/3 (G, SE WT, $n = 23$ dendrites vs SE *Fmr1* KO, $n = 35$ dendrites) and L5/6 (K, SE WT, $n = 47$ dendrites vs SE *Fmr1* KO, $n = 43$ dendrites). There were fewer spines with volume $> 1 \mu\text{m}^3$ in L2/3 auditory cortex of SE *Fmr1* KO compared to SE WT (H, $p < .05$). Statistical analysis was done using unpaired *t*-test (G, K) or two-way ANOVA (for spine volume, H and L): *, $p < .05$.

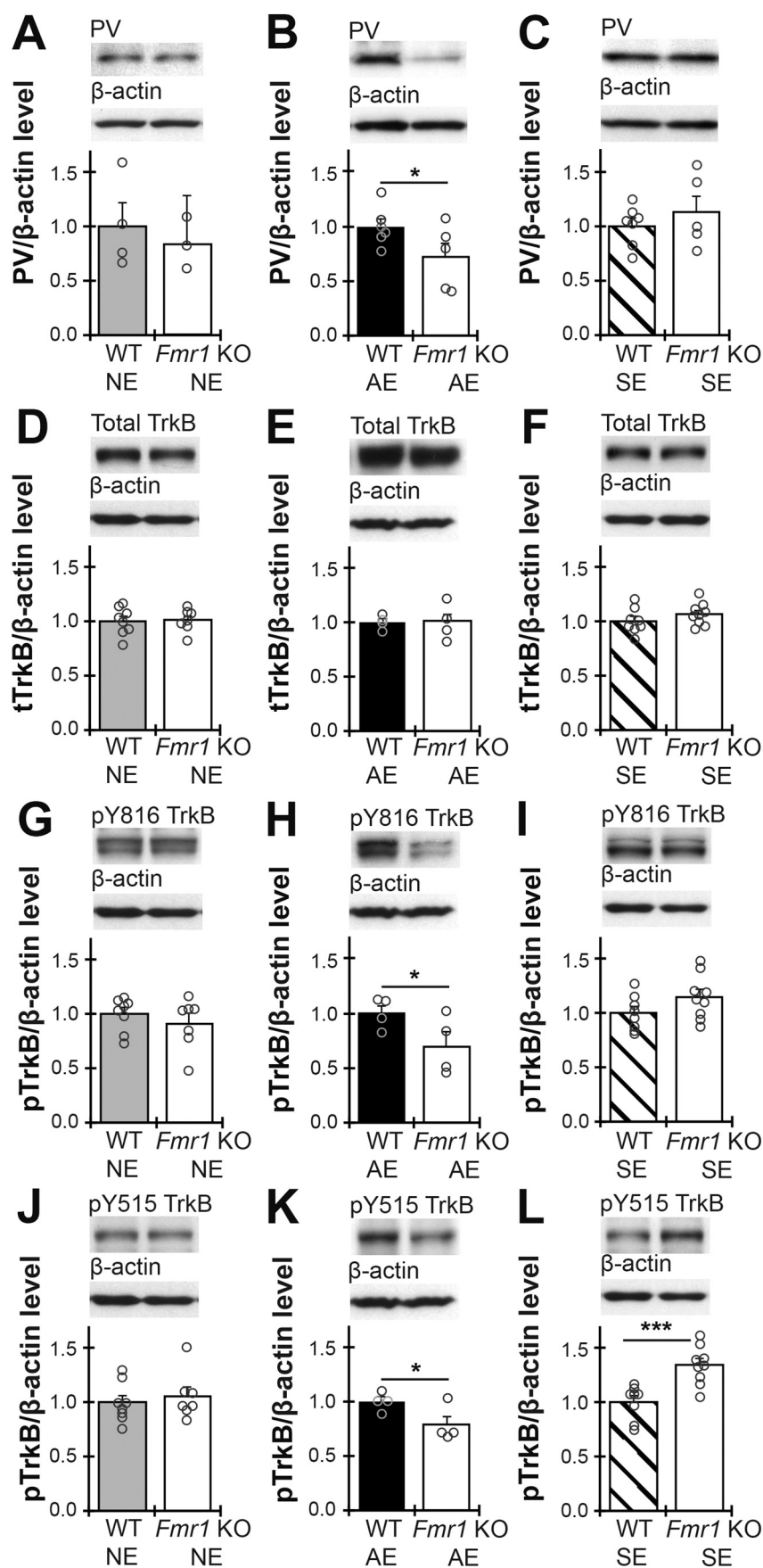


Fig. 5. Passive sound exposure during development normalized PV protein levels and enhanced TrkB phosphorylation in SE *Fmr1* KO mice. PV protein levels (A-C), total full-length TrkB (D-F), TrkBpY816 (G-I) and TrkBpY515 (J-L) were normalized to β -actin levels in P21 auditory cortex of NE (A, D, G, J), AE (B, E, H, K) and SE (C, F, I, L) WT and *Fmr1* KO mice. There was reduced PV level in AE *Fmr1* KO compared to AE WT (B, AE WT: $n = 6$ vs AE *Fmr1* KO: $n = 5$, $p = .0403$) and no differences were detected in NE (A, NE WT: $n = 4$ vs NE *Fmr1* KO: $n = 3$) and SE *Fmr1* KO (C, SE WT: $n = 7$ vs SE *Fmr1* KO: $n = 5$) compared to their WT counterparts. No differences in total TrkB protein level were found for NE *Fmr1* KO (D, NE WT: $n = 8$ vs NE *Fmr1* KO: $n = 7$), AE *Fmr1* KO (E, AE WT: $n = 4$ vs AE *Fmr1* KO: $n = 4$) and SE *Fmr1* KO (F, SE WT: $n = 8$ vs SE *Fmr1* KO: $n = 9$) compared to their WT counterparts. pTrkB-Y816 level was decreased in AE *Fmr1* KO compared to AE WT (H, $p = .0479$). No differences were detected in SE *Fmr1* KO compared to SE WT (I). pTrkB-Y515 level was decreased in AE *Fmr1* KO (K, $p = .0306$) and increased in SE *Fmr1* KO (L, $p = .0004$) compared to their WT counterparts. Statistical analysis was done using unpaired t -test: *, $p < .05$; ***, $p < .001$.

compared SE WT mice, showing a decrease in the proportion of large spines ($p < .05$; Fig. 4H). A two-way ANOVA with exposure (AE, SE) and genotype (WT, *Fmr1* KO) as factors showed an effect of sound exposure on spine density in L2/3 auditory cortex ($F_{(1,122)} = 35.47$, $p < .0001$) with significant changes in both WT (AE WT vs SE WT, $p = .0011$) and *Fmr1* KO mice (AE *Fmr1* KO vs SE *Fmr1* KO, $p < .0001$) with Bonferroni's multiple comparisons test. In L5/6, there was only a significant effect of sound exposure in *Fmr1* KO (AE *Fmr1* KO vs SE *Fmr1* KO, $p = .0006$, Bonferroni's multiple comparisons test).

In summary, developmental sound exposure restored dendritic spine density in auditory cortex of *Fmr1* KO mice to WT levels.

4. SE *Fmr1* KO mouse auditory cortex shows normal PV protein levels and increased TrkB phosphorylation

To assess whether the reduction in the density of PV cells in the auditory cortex of AE *Fmr1* KO mice is a result of reduced PV levels, we examined PV protein levels in the auditory cortex using Western blotting. While there was no difference in PV levels between NE *Fmr1* KO and WT (Fig. 5A), PV levels were significantly decreased in AE *Fmr1* KO mice compared to AE WT ($p = .0403$; Fig. 5B). In contrast, SE *Fmr1* KO mice showed similar PV levels as SE WT (Fig. 5C; Table S5).

As TrkB signaling is implicated in PV cell development and survival (Nomura et al., 2017; Xenos et al., 2018) we also evaluated total and phosphorylated levels of TrkB in WT and *Fmr1* KO mice. We found that TrkB levels (145 kDa) were similar between *Fmr1* KO and WT in all groups (Fig. 5D-F). We further examined activation of TrkB by evaluating its phosphorylation (p) on the tyrosine (Y)816 and Y515. No differences in pTrkB-Y816 and pTrkB-Y515 were observed in NE *Fmr1* KO compared to NE WT (Fig. 5G, J). However, we observed a significant decrease in pTrkB-Y816 and pTrkB-Y515 in AE *Fmr1* KO compared to AE WT (pY816: $p = .0479$ and pY515: $p = .0306$; Fig. 5H, K). Interestingly, a significant increase in pTrkB-Y515, but not pTrkB-Y816, levels was observed in SE *Fmr1* KO mice compared to SE WT ($p = .0004$; Fig. 5I, L, Table S5). A two-way ANOVA analysis with exposure (AE, SE) and genotype (WT, *Fmr1* KO) as factors showed the effect of sound exposure on both pTrkB-Y816 (exposure effect: $F_{(2,34)} = 4.005$, $p = .0274$ and interaction effect: $F_{(2,34)} = 4.023$, $p = .0270$) and pTrkB-Y515 levels (exposure effect: $F_{(2,34)} = 7.415$, $p = .0021$ and interaction effect: $F_{(2,34)} = 7.427$, $p = .0021$). Pairwise comparison with Bonferroni's multiple comparisons test revealed an increase of pTrkB-Y816 levels ($p = .0015$) and pTrkB-Y515 levels ($p < .0001$) in SE *Fmr1* KO compared to AE *Fmr1* KO. This observation parallels the increased PV cell density we observed in SE *Fmr1* KO mice.

Together, our results indicate that developmental sound exposure increased PV level and TrkB phosphorylation in auditory cortex of *Fmr1* KO mice.

5. Discussion

We demonstrate differential effects of auditory experience on auditory cortex development in WT and *Fmr1* KO mice. First, we found enhanced sound-evoked responses in *Fmr1* KO mice compared to WT mice raised in sound-attenuated environment that are associated with increased dendritic spine density, and reduced PV expression and TrkB phosphorylation. This suggests that developmental sound attenuation may not be feasible to reduce auditory hypersensitivity in FXS. In contrast, we observed beneficial effects of developmental tone exposure in restoring molecular, cellular and functional properties in the auditory cortex of *Fmr1* KO mice to WT levels. Specifically, comparison of sound-exposed *Fmr1* KO mice to *Fmr1* KO mice reared in sound-attenuated environment show: (1) reduced N1 amplitude of ERPs; (2) increased PV expression; (3) decreased dendritic spine density; and (4) enhanced TrkB phosphorylation (Fig. 6).

One major finding of this study is the modulation of PV cell density in auditory cortex of *Fmr1* KO following passive developmental sound

exposure. PV cells are GABAergic interneurons that regulate network oscillations associated with sensory processing and contribute to the maintenance of E/I balance (Sohal et al., 2009; Hu and Jonas, 2014). Our previous work showed delayed PV cell development in the auditory cortex of *Fmr1* KO mice (Wen et al., 2018). This is consistent with studies of somatosensory and visual cortices of *Fmr1* KO mice, suggesting a common role of PV interneurons in abnormal sensory processing and hypersensitivity in FXS (Selby et al., 2007; Gibson et al., 2008; Goel et al., 2018). In this study, we found that developmental sound attenuation did not restore normal PV cell development, suggesting that sensory input is probably not the primary cause of the deficit in *Fmr1* KO mice. In contrast, developmental exposure to 14 kHz sound increased PV cell density and PV/PNN co-localization in auditory cortex of *Fmr1* KO mice. Given the consistent deficits in PV cell density and function across sensory systems, our data suggest the possibility of passive sound exposure during development as a potential approach to reduce hypersensitivity. However, SE *Fmr1* KO mice still show impaired PNN formation in L4 auditory cortex suggesting possible benefits of combining pharmacological interventions to restore PNN levels and acoustic exposure during cortical development. Our previous studies implicated enhanced MMP-9 activity in impaired PNN formation and abnormal cortical responses in *Fmr1* KO mice (Sidhu et al., 2014; Wen et al., 2018). Genetic reduction of MMP-9 in *Fmr1* KO mice resulted in normal development of PNNs and normal response magnitudes in auditory cortical neurons (Wen et al., 2018), providing a justification for targeting MMP-9 pharmacologically. It is possible that enhanced MMP-9 activity may result in excessive cleavage of aggrecan and a reduction in aggrecan-containing PNNs detected with WFA (Roughley and Mort, 2014; Miyata and Kitagawa, 2016; Lovelace et al., 2019). Future studies will determine whether acute or chronic MMP-9 inhibition during development will restore PNN formation and normalize cortical responses in *Fmr1* KO mice.

Another finding of this study is that developmental sound exposure normalized density of dendritic spines, major postsynaptic sites of excitatory synapses, in auditory cortex of *Fmr1* KO mice. An abundance of immature dendritic spines is seen in the brains of humans with FXS (Irwin et al., 2001) and *Fmr1* KO mice (Comery et al., 1997). However, there are controversial results on spine density in developing and adult *Fmr1* KO that are influenced by mouse genetic background, brain area and developmental period (He and Portera-Cailliau, 2013). In this study, we demonstrate that developmental sound exposure reduces dendritic spine density on basolateral dendrites of L2/3 and L5/6 excitatory neurons in P21 *Fmr1* KO auditory cortex to WT levels. Our data show that the acoustic manipulations during early postnatal development influence developing synaptic circuits in *Fmr1* KO auditory cortex by affecting both inhibitory and excitatory neurons.

In AE *Fmr1* KO mice, decreased PV cell density and increased spine density is predicted to favor excitation over inhibition. Indeed, when ERPs were recorded from the auditory cortex, AE *Fmr1* KO mice showed larger N1 amplitudes in response to 14 kHz, but not 23 kHz, tone than their WT counterparts. Although a large number of adult auditory cortical neurons respond to frequencies ~20–25 kHz range (Zhou et al., 2008; Barkat et al., 2011; Carrasco et al., 2013), the tonotopic maps develop high frequency selectivity later in development (Zhang et al., 2001; Carrasco et al., 2013). Therefore, at P21 there may be more neurons responding to frequencies < 20 kHz in the auditory cortex. Abnormal synchrony, which is a key feature in developing *Fmr1* KO mice (Goncalves et al., 2013), may be also responsible for a larger ERP evoked by the 14 kHz tone in *Fmr1* KO mice, compared to WT. This is consistent with larger tone-evoked ERP amplitudes in humans with FXS (Castren et al., 2003; Schneider et al., 2013; Knoth et al., 2018) and our previous studies showing enhanced auditory cortical single-unit responses and impaired habituation to repeated sounds in *Fmr1* KO mice (Rotschafer and Razak, 2013; Lovelace et al., 2016; Wen et al., 2018). Interestingly, developmental sound exposure resulted in a decrease of P1, N1 and P2 latencies of ERP evoked by both 14 kHz and 23 kHz tones

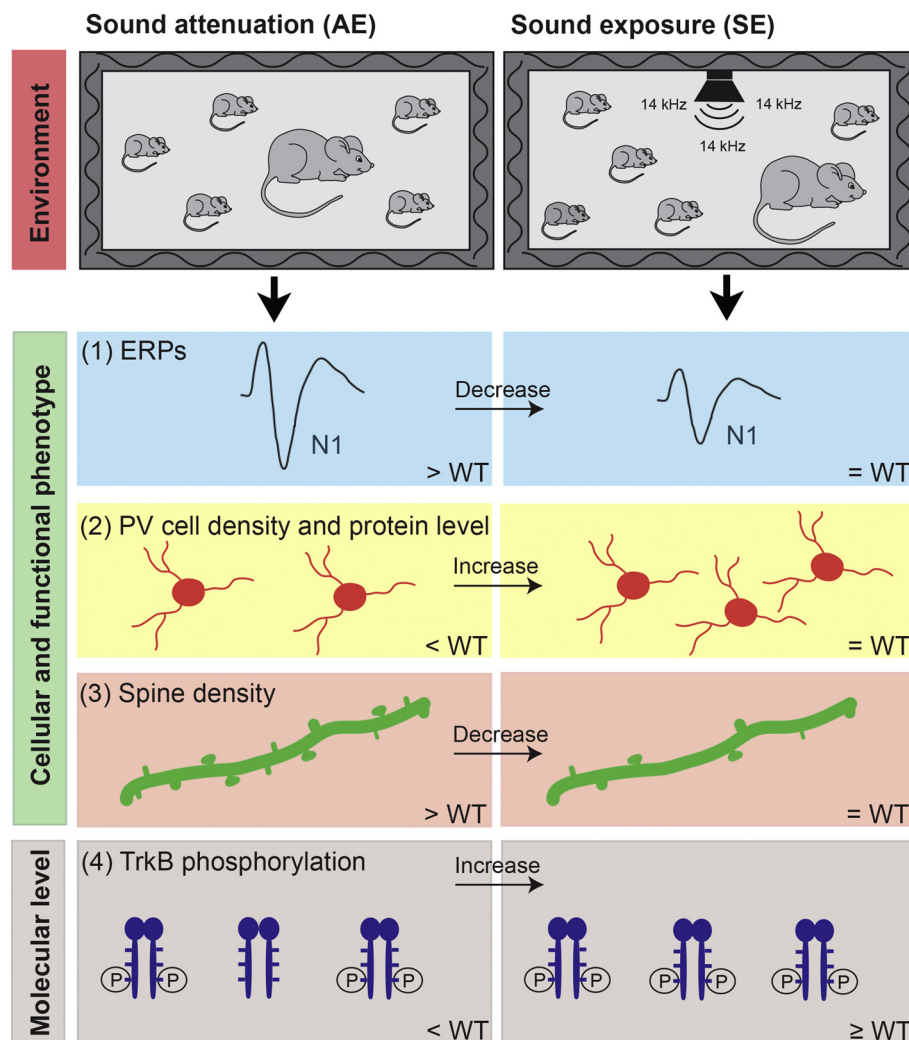


Fig. 6. Acoustic exposure during early postnatal developmental window restores molecular, cellular and functional alterations observed in the auditory cortex of *Fmr1* KO mice to WT levels. Schematic depiction of the molecular, cellular and functional changes in *Fmr1* KO mice compared to WT mice and the effects of the sound exposure. Left panels, AE *Fmr1* KO mice reared in sound-attenuated environment (AE) during early development showed (1) decreased PV cell density and PV protein levels; (2) increased dendritic spine density; (3) enhanced ERP N1 amplitude; and (4) reduced TrkB phosphorylation on Y816 and Y515 in the auditory cortex compared to WT mice. Right panels, SE *Fmr1* KO mice exposed to 14 Hz tone during development (P9-P21) showed (1) increased PV cell density and PV levels; (2) decreased dendritic spine density; (3) decreased N1 amplitude; and (4) increased phosphorylation of TrkB on Y816 and Y515 in auditory cortex compared to AE *Fmr1* KO mice reared in a sound attenuated environment. PV levels, spine density and N1 amplitude were not different between SE WT mice and SE *Fmr1* KO mice. Moreover, phosphorylation of TrkB on Y816 and Y515 was respectively similar or higher in auditory cortex of SE *Fmr1* KO mice compared to SE WT mice.

in auditory cortex of SE *Fmr1* KO mice compared to AE *Fmr1* KO, which indicate maturation of these ERP components. Kim et al., 2013 showed that exposure of mice to 16 kHz during development increased the tonotopic representation of 16 kHz in WT, but not in *Fmr1* KO mice, suggesting abnormal critical period plasticity in the *Fmr1* KO mice. This tonotopic plasticity is unlikely to contribute to our ERP findings as ERP amplitude was not different between SE WT and SE *Fmr1* KO mice in spite of larger 16 kHz representation observed previously in WT mice (Kim et al., 2013). We also observed a reduction of average ERP N1 amplitude in *Fmr1* KO mice following developmental exposure to sound without changes in the WT mice. Future experiments should more closely explore relationships between tonotopic maps and ERPs in order to understand the underlying mechanisms. Taken together, our data suggest that passive exposure of mice to pure tones during the auditory critical period normalizes the cellular and functional properties of the auditory cortex in *Fmr1* KO mice.

While the observed increase in the density of PV cells may underlie the changes in auditory cortical responses of sound-exposed *Fmr1* KO mice, the mechanism of the up-regulation is still not clear. BDNF-TrkB signaling is implicated in the development and function of GABAergic neurons (Nakahara et al., 2004; Berghuis et al., 2006). TrkB signaling supports PV cells and influences gamma-band synchronization in hippocampus (Zheng et al., 2011; Lucas et al., 2014). In cortex, TrkB deletion from PV interneurons resulted in dysregulation of patterned high-frequency cortical activity and disinhibition of local excitatory neurons (Xenos et al., 2018). In *Fmr1* KO mice, BDNF-TrkB signaling was implicated in FXS-associated alterations (Castren and Castren, 2014).

Impaired BDNF-TrkB signaling may cause delayed development of fast spiking interneurons in P5 *Fmr1* KO somatosensory cortex, which was restored following the administration of the TrkB agonist LM22A-4 (Nomura et al., 2017). In contrast, reduced density of PV interneurons with increased TrkB expression was demonstrated in adult *Fmr1* KO mice (Selby et al., 2007). We did not observe changes in total TrkB levels, but TrkB phosphorylation on Y816 and Y515 was reduced in auditory cortex of AE *Fmr1* KO mice compared to AE WT. Interestingly TrkB phosphorylation was increased in *Fmr1* KO auditory cortex after sound exposure that was concurrent with the increase in PV expression, suggesting a role of TrkB signaling in regulating PV cells in the auditory cortex of sound-exposed *Fmr1* KO mice. Genetic reduction of BDNF levels in neural precursor cells derived from *Fmr1* KO mice increased pTrkB-Y816, but not pTrkB-Y515 levels, suggesting changes that are compensatory to BDNF signaling deficit (Danesi et al., 2018). Interestingly, hyperactivity and deficits in startle responses were ameliorated in BDNF(+/-)/*Fmr1* KO mice, suggesting a possible role of BDNF/trkB signaling in these behaviors (Uutela et al., 2012).

6. Conclusions

Alterations of the acoustic environment during early postnatal developmental window lead to changes of PV expression, spine density and auditory responses in *Fmr1* KO mice. Our data suggest that sensory manipulations during a critical developmental period might influence neuronal circuit development and E/I balance in FXS providing a basis for future studies of sensory manipulations in addition to

pharmacological interventions. These results also make the intriguing suggestion that some deficits observed during early postnatal developmental period in *Fmr1* KO mouse auditory system may trigger compensatory mechanisms to restore responses (Antoine et al., 2019). More studies are necessary to investigate which parameters of the sound exposure are key to observed changes and to understand long-lasting effects of the early developmental exposure. In addition, our data suggest the need to perform high-resolution developmental studies to measure cellular and circuit changes across different brain areas with a stringent control of sensory input to understand the relative contributions of direct genetic effects and changes that arise due to altered experience.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2019.104622>.

Declaration of Competing Interest

We report no conflict of interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2019.104622>.

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Minocycline Treatment Reverses Sound Evoked EEG Abnormalities in a Mouse Model of Fragile X Syndrome

Jonathan W. Lovelace¹, Iryna M. Ethell^{2,3}, Devin K. Binder^{2,3} and Khaleel A. Razak^{1,2*}

¹ Department of Psychology and Neuroscience Graduate Program, University of California, Riverside, Riverside, CA, United States, ² Neuroscience Graduate Program, University of California, Riverside, Riverside, CA, United States, ³ Division of Biomedical Sciences, School of Medicine, University of California, Riverside, Riverside, CA, United States

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*Correspondence:

Khaleel A. Razak
Khaleel@ucr.edu

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Fragile X Syndrome (FXS) is a leading known genetic cause of intellectual disability. Many symptoms of FXS overlap with those in autism including repetitive behaviors, language delays, anxiety, social impairments and sensory processing deficits. Electroencephalogram (EEG) recordings from humans with FXS and an animal model, the *Fmr1* knockout (KO) mouse, show remarkably similar phenotypes suggesting that EEG phenotypes can serve as biomarkers for developing treatments. This includes enhanced resting gamma band power and sound evoked total power, and reduced fidelity of temporal processing and habituation of responses to repeated sounds. Given the therapeutic potential of the antibiotic minocycline in humans with FXS and animal models, it is important to determine sensitivity and selectivity of EEG responses to minocycline. Therefore, in this study, we examined if a 10-day treatment of adult *Fmr1* KO mice with minocycline (oral gavage, 30 mg/kg per day) would reduce EEG abnormalities. We tested if minocycline treatment has specific effects based on the EEG measurement type (e.g., resting versus sound-evoked) from the frontal and auditory cortex of the *Fmr1* KO mice. We show increased resting EEG gamma power and reduced phase locking to time varying stimuli as well as the 40 Hz auditory steady state response in the *Fmr1* KO mice in the pre-drug condition. Minocycline treatment increased gamma band phase locking in response to auditory stimuli, and reduced sound-evoked power of auditory event related potentials (ERP) in *Fmr1* KO mice compared to vehicle treatment. Minocycline reduced resting EEG gamma power in *Fmr1* KO mice, but this effect was similar to vehicle treatment. We also report frequency band-specific effects on EEG responses. Taken together, these data indicate that sound-evoked EEG responses may serve as more sensitive measures, compared to resting EEG measures, to isolate minocycline effects from placebo in humans with FXS. Given the use of minocycline and EEG recordings in a number of neurodegenerative and neurodevelopmental conditions, these findings may be more broadly applicable in translational neuroscience.

Keywords: fragile X syndrome, autism, forebrain, sensory hypersensitivity, EEG, minocycline, MMP-9

INTRODUCTION

Fragile X Syndrome (FXS) is a genetic cause of intellectual disability (Crawford et al., 2001) that results from a mutation in the *Fragile X Mental Retardation 1* (*Fmr1*) gene and down-regulation of Fragile X Mental Retardation Protein (FMRP) (Yu et al., 1991). FMRP is an RNA binding protein, and one of the fundamental cellular effects of FMRP loss is abnormal protein synthesis including proteins that regulate synaptic function (Darnell et al., 2011). FXS symptoms include intellectual disability, anxiety, repetitive behaviors, social communication deficits, delayed language development and abnormal sensory processing (Wisniewski et al., 1991; Abbeduto and Hagerman, 1997; Miller et al., 1999; Musumeci et al., 1999; Roberts et al., 2001; Sabaratnam et al., 2001; Berry-Kravis, 2002; Hagerman and Hagerman, 2002; Hagerman et al., 2009; Van der Molen et al., 2010; Sinclair et al., 2017b). Diagnosis of autism in children with FXS ranges between 18–30% (Rogers et al., 2001; Belmonte and Bourgeron, 2006). The overlap in symptoms of FXS and autism suggest overlapping cellular and circuit mechanisms.

Abnormal sensory processing in humans with FXS includes hypersensitivity and reduced habituation to repeated sensory stimuli (Castrén et al., 2003; Schneider et al., 2013). These symptoms affect multiple sensory systems (Rais et al., 2018), are seen from early in development, and may lead to cognitive deficits and anxiety (Orefice et al., 2016). Both humans with FXS (Castrén et al., 2003; Schneider et al., 2013; Van der Molen and Van der Molen, 2013; Ethridge et al., 2016) and *Fmr1* knockout (KO) mice (Rotschafer and Razak, 2013, 2014; Lovelace et al., 2016; McCullagh et al., 2020) show debilitating auditory hypersensitivity. Recent EEG recordings from humans show altered cortical oscillatory activity in FXS. Wang et al. (2017) compared age-matched healthy controls (mean age = 26.4 years) and FXS patients (mean age = 25.6) and showed enhanced resting state gamma frequency power and greater spatial spread of phase-synchronized gamma band activity in FXS patients that may mark abnormally activated cortex and hypersensitivity (Merker, 2013). When neural oscillations were induced with sounds, inter-trial phase coherence (phase locking factor) was reduced in FXS patients, particularly at gamma frequencies (Ethridge et al., 2017, 2019). Van der Molen et al. (2012) studied age-matched control and FXS patients (mean age = 29 years) and showed enhanced N1 amplitude of the ERPs in response to tones. These results, consistent with Castrén et al. (2003), indicate abnormally enhanced tone evoked responses and/or enhanced synchrony of responding population of cells. Ethridge et al. (2016) studied age-matched control (mean age = 28.8 years) and patients with FXS (mean age = 28.5) and recorded ERPs in response to repetitions (2 Hz repetition rate) of the same tone (1000 Hz, 65 dB). They found that the N1 amplitude showed reduced habituation across repeated stimuli in humans with FXS. They also reported decreased phase locking and increased single-trial power in the gamma band. The phase locking deficits in the gamma range suggest an inability of underlying circuits to generate appropriately timed inhibitory responses. Importantly, the EEG phenotypes were correlated with abnormal sensory sensitivity reports and deficits in social

communication as measured with the social and communication questionnaire. These correlations suggest clinical significance of EEG phenotypes in humans with FXS.

Remarkably similar EEG differences are observed in *Fmr1* KO, compared to wild type (WT), mice. Lovelace et al. (2018) showed enhanced resting EEG gamma band power, reduced sound evoked inter-trial phase coherence and increased single trial gamma power in both auditory and frontal cortex (AC, FC) of *Fmr1* KO mice. Increased amplitude and reduced habituation of auditory ERPs are also seen in the *Fmr1* KO mice (Lovelace et al., 2016; Sinclair et al., 2017a; Wen et al., 2019; Kulinich et al., 2020). These data are consistent with *in vivo* cortical single cell recordings that show auditory hyper-responsiveness and broader frequency tuning curves (Rotschafer and Razak, 2013; Wen et al., 2018) and *in vitro* cortical slice preparations that show enhanced gamma band power in auditory cortex and synchrony across cortical layers (Goswami et al., 2019). Sinclair et al. (2017a) showed that racemic baclofen, an agonist of GABA-b receptors, reduced abnormalities in sound evoked gamma oscillations in the ERP recorded from *Fmr1* KO mice. Kulinich et al. (2020) showed that controlled sound exposure during early development reduced ERP N1 amplitude in adult *Fmr1* KO mice. These data indicate that ERP parameters are sensitive to drug treatment and developmental acoustic experience. The similarities in human and mouse EEG phenotypes suggest multiple translation relevant and objective biomarkers that could be used for both development of treatments and to understand cellular mechanisms of drug action in FXS.

One promising therapeutic pathway to reduce sensory hypersensitivity in FXS patients is the reduction of matrix metalloprotease-9 (MMP-9). MMP-9 is an endopeptidase involved in activity-dependent modifications of the extracellular matrix (Reinhard et al., 2015). MMP-9 is a target of FMRP-dependent translation regulation, and MMP-9 activity is upregulated in the auditory cortex (Wen et al., 2018) and inferior colliculus (Kokash et al., 2019) of *Fmr1* KO mice. Genetic reduction of MMP-9 ameliorated correlates of auditory hypersensitivity in the *Fmr1* KO mice including normalization of auditory ERP habituation (Lovelace et al., 2016), pre-pulse inhibition of auditory startle (Kokash et al., 2019) and calling rate of ultrasonic vocalizations (Toledo et al., 2019). Single cell hyper-responsiveness and abnormal development of parvalbumin (PV) expressing inhibitory neurons in the auditory cortex of *Fmr1* KO mice were also reversed by genetic reduction of MMP-9 in *Fmr1* KO mice (Wen et al., 2018). These data suggest that treatments that reduce MMP-9 may be effective in reducing sensory symptoms in FXS. Consistent with this notion, minocycline has received considerable attention in treating FXS symptoms. Minocycline is a tetracycline antibiotic with multiple targets of action that include inflammation, apoptosis and microglial clearance. Minocycline also reduces MMP-9 levels (Dziembowska et al., 2013), and this effect may be associated with beneficial effects of minocycline treatment on neurobehavioral symptoms in multiple studies of animal models (Bilousova et al., 2009; Siller and Broadie, 2011; Rotschafer et al., 2012; Dansie et al., 2013; Toledo et al., 2019). These improvements include improvements in anxiety-like behavioral outcomes, ultrasonic

vocalizations, pre-pulse inhibition of startle, dendritic spine density measurements and audiogenic seizures. Changes to MMP-9 with minocycline may also underlie beneficial effects reported in humans with FXS (Paribello et al., 2010; Leigh et al., 2013; Schneider et al., 2013). Paribello et al. (2010) conducted an open label study of minocycline treatment on FXS patients (mean age = 18 years) and found improvements in the irritability subscale of the Aberrant Behavior Checklist-Community, the Clinical Global Improvement Scale and the Visual Analog Scale for behavior. Benefits were seen in both adolescents and adults. Leigh et al. (2013) conducted a randomized, placebo controlled, double blind study of minocycline treatment (3 months) in children and adolescents with FXS (mean age = 9.2 years). They also found an improvement in the Clinical Global Improvement Scale and in the mood-related behaviors on the Visual Analog Scale (minocycline 5.26 ± 0.46 cm, placebo 4.05 ± 0.46 cm; $p = 0.0488$).

Despite the evidence for beneficial effects of minocycline in FXS, how EEG responses are impacted by treatment is less clear. Only one study, done in humans with FXS, showed that minocycline treatment alters EEG responses. Schneider et al. (2013) used a double-blind, placebo controlled study of the effects of minocycline treatment in children (mean age = 10.5 years) with FXS. They showed that 3 months of minocycline treatment reduced N1 and P2 amplitudes and increased ERP N1 habituation to repeated tone presentation in humans with FXS, compared to placebo. But whether resting EEGs are differentially impacted by treatment compared to sound-evoked responses is not known in either mice or humans. Within resting and evoked EEGs, whether different oscillation frequencies are differentially impacted is also not known. These data are crucial to move EEG responses forward as translation-relevant outcome measures. Given the impact of MMP-9 on extracellular matrix and the functionality of parvalbumin positive inhibitory interneurons, we hypothesized that minocycline treatment of adult *Fmr1* KO mice would reduce resting state gamma power, improve gamma band phase locking and reduce ERP abnormalities, compared to vehicle treatment. Our data replicates a number of EEG/ERP phenotypes reported previously (Lovelace et al., 2018) and adds evidence for decreased temporal fidelity of 40 Hz auditory steady state responses (ASSR) in the *Fmr1* KO mice. We report that minocycline treatment reduces sound evoked, but not resting state, EEG abnormalities in *Fmr1* KO mice compared to vehicle treatment.

MATERIALS AND METHODS

Mice

Male C57BL/6 *Fmr1* KO mice (B6.129P2-*Fmr1*^{tm1Cgr}/J, stock #003025) and congenic male WT mice (stock # 000664) were obtained from Jackson Laboratories. All mice were housed in our vivarium for at least 72 h before experiments were carried out. PCR analysis of genomic DNA isolated from mouse tails were used to confirm genotypes of all the mice in this study. Mice were maintained in an AAALAC-accredited vivarium under 12-h light/dark cycles. Water and standard mouse chow were provided for *ad libitum* consumption. NIH guidelines

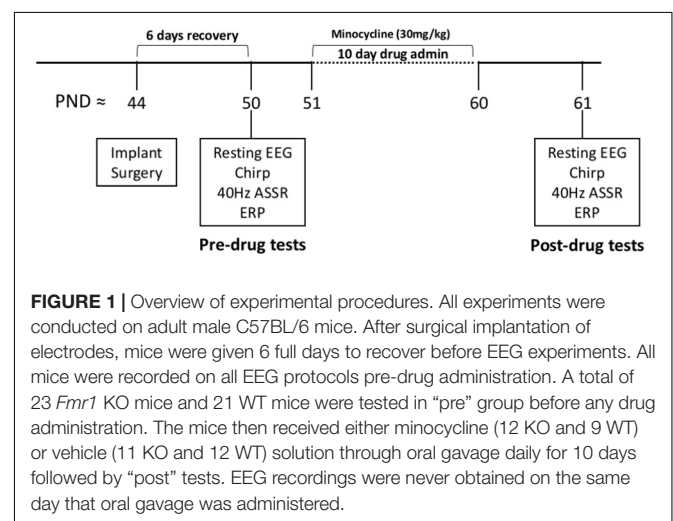
were followed for all procedures with Institutional Animal Care and Use Committee approval of animal use procedures. EEG recordings were obtained from 21 WT and 23 *Fmr1* KO mice, with ages between postnatal day (P) 44 and P80. A 2×2 (genotype $\times$ treatment) experimental design was used with 12 *Fmr1* KO and 9 WT mice receiving minocycline treatment and the remaining mice receiving vehicle.

Overview of Experimental Procedures

Figure 1 provides an overview of the procedure. After 6 days of recovery from surgery to implant the EEG recording electrodes, EEG responses were recorded from both auditory and frontal cortex (AC, FC). These recordings served as pre-drug baseline responses to obtain genotype specific differences. After 10 days of daily minocycline (30 mg/kg) or vehicle treatment, the same mice were recorded for post drug/vehicle effects. The minocycline dose was chosen based on Bilousova et al. (2009), who showed improvement in spine maturation and performance on behavioral tests in the *Fmr1* KO mice. Dansie et al. (2013) and Toledo et al. (2019) used the same dose and found improvements in hyperactivity and ultrasonic vocalization phenotypes in the *Fmr1* KO mice.

Surgery for EEG Electrode Implantation

Surgical procedures were similar to those used in Lovelace et al. (2018, 2019), and are only briefly described here. Following a brief period of isoflurane exposure (0.2–0.5%), mice were given an i.p. injection of ketamine and xylazine (K/X, 80/10 mg/kg). Mice were secured in a bite bar and placed on a stereotaxic apparatus (model 930; Kopf, CA, United States). Anesthetic plane was monitored through periodic (10 min interval) inspection of the toe pinch reflex and Supplementary doses of K/X was administered if required. Once a stable anesthetic plane was achieved, a midline sagittal incision was made along the scalp to expose the skull. Small holes (1 mm diameter) were drilled (Foredom dental drill) in the skull overlying the right auditory cortex (−1.6 mm, +4.8 mm), right frontal lobe (+3.0 mm, +1.6 mm), and left occipital (−4.8 mm, −2.95 mm) (coordinates



relative to Bregma: anterior/posterior, medial/lateral). Three channel electrode posts (Plastics One, MS333-2-A-SPC) were attached to 1 mm stainless steel screws (Plastics One, 00-96 X1/16). The screws were advanced into the skull holes until secure and to the point of contact with the dura. Dental cement was applied to additionally secure the screws to the skull. Triple antibiotic ointment was applied along the edges of the dental cement. Mice were placed on a heating pad for recovery after a subcutaneous injection of Buprenorphine (0.1 mg/kg). A second Buprenorphine injection was administered between 6 and 10 h after surgery. Mice were then singly housed and monitored daily in the vivarium until the day of first EEG recordings, 6 days after surgery. At least 4 days separated last injection of Buprenorphine and EEG recordings.

Acoustic Stimulation for EEG Recordings

EEG recordings were done using similar procedures as Lovelace et al. (2018, 2019). Recordings were obtained in a sound-attenuated chamber lined with anechoic foam (Gretsch-Ken Industries, Oregon). Sound stimuli were generated using RVPdsEx software and RZ6 hardware (Tucker Davis Technologies, FL, United States) and presented through a free-field speaker (MF1, Tucker-Davis Technologies, FL, United States) located directly above the cage (12 inches). The speaker output was ~ 70 dB SPL at the floor of the recording chamber with fluctuation of ± 3 dB for frequencies between 5 and 35 kHz as measured using a 1/4 inch B&K microphone.

To quantify the ability of neural generators to produce synchronized oscillations to time varying stimuli, we employed two types of auditory stimuli. The first type of stimulation is called the auditory chirp modulated sound (henceforth, “chirp”). The chirp is a tone or noise whose amplitude is modulated using a sinusoid with increasing or decreasing frequency in the 1–100 Hz range (Artieda et al., 2004; Purcell et al., 2004; Pérez-Alcázar et al., 2008). The chirp facilitates a rapid measurement of evoked phase synchrony to auditory stimuli of varying frequencies and can be used to compare temporal processing in different groups in clinical and pre-clinical settings (e.g., Purcell et al., 2004). Ethridge et al. (2017) used a chirp to induce synchronized oscillations in their EEG recordings and identified a gamma synchronization deficit in FXS. Inter-trial phase coherence analysis (ITPC, Tallon-Baudry et al., 1996) can then be used to determine the fidelity with which neural generators synchronize oscillations to the stimulus frequencies on a trial-by-trial basis. Both humans with FXS and the *Fmr1* KO mice show deficits in phase locking factor in the gamma band frequencies (~ 40 Hz) (Ethridge et al., 2017; Lovelace et al., 2018).

We used a chirp stimulus to evaluate phase synchronization in the mouse EEG recordings to identify the effects of minocycline treatment. The chirp stimulus was a 2 s duration broadband noise stimulus that was amplitude modulated (100% depth) by a sinusoid whose frequency increased from 1 to 100 Hz (up-chirp). We only assessed an up-chirps in this study since no differences were found across chirp directions in our previous work (Lovelace et al., 2018). To avoid the influence of onset responses in our calculation of ITPC to the chirp, we ramped the stimulus in sound level from 0 to 100% over 1 s (rise time)

which then smoothly transitioned into the chirp. Trains of chirps were repeated 300 times, with the interval between each train randomly generated to be between 1 and 1.5 s.

Second, we used a click train to assess the auditory steady state response (ASSR). The purpose of this type of stimulus is to drive steady brain oscillations at only one specific frequency of interest. The ASSR stimulus trains consisted of 0.5 ms clicks repeated at a rate of 40 Hz over 1 s period. Each train was presented 200 times with an inter-train interval of 2 s. ASSR is a widely used diagnostic biomarker for disorders like schizophrenia (O'Donnell et al., 2013). The ASSR facilitates an assessment of the function of parvalbumin (PV) positive (PV+) GABAergic cells at their resonance frequency (~ 40 Hz). Abnormal PV cell function is central to our working hypothesis on mechanisms of hyperactivity and the mechanistic action of minocycline through the inhibition of MMP-9. In addition to the chirp responses and ASSR, we recorded ERPs with broadband noise stimulus trains. Each train consisted of 10 repetitions (1 or 2 Hz repetition rate) of broadband noise, with 100 trains being presented at each repetition rate. Each noise burst was presented at 70 dB SPL and was 100 ms in duration, including a 5 ms rise/fall time. The interval between each stimulus train was 8 s.

Electrophysiology

The BioPac system (BIOPAC Systems, Inc) was used for resting and sound evoked EEG signal acquisition from awake mice inside a custom-built Faraday cage. A 15 min habituation period was provided to each mouse when first placed in the EEG recording chamber before being connected to the BioPac system. Under brief isoflurane anesthesia, a three-channel tether was connected to the three-channel electrode post (implanted in the mouse during surgery). This tether was connected to a commutator located above the cage. An additional 15 min of habituation to being connected to the tether was provided before EEG recordings commenced.

The BioPac MP150 acquisition system was connected to two EEG 100C amplifier units (one for each channel) to which the commutator was attached. The lead to the occipital cortex was used as reference for both FC and AC screw electrodes. Signals were recorded with filters set to high-pass (> 0.5 Hz) and low-pass (< 100 Hz). The gain was maintained the same ($10,000\times$) between all recordings. Data were sampled at 2.5 kHz (Acqknowledge software) and down sampled to 1024 Hz post hoc using Analyzer 2.2 (Brain Vision Inc.). TTL pulses were used to synchronize stimulus onset in each train with EEG recording. Five minutes of resting EEG was recorded first. Resting EEG is defined as recordings obtained with no specific stimuli. This was followed by ERP recordings in response to trains of broadband noise, chirps and ASSR.

Drug and Vehicle Treatment Procedures

Drug solutions were prepared using a vehicle of 0.9% NaCl + 0.3% Tween 80, targeting an administration volume of 6 mL/kg and minocycline concentration of 30 mg/kg. Stock solutions of minocycline (MP Biomedicals) were prepared regularly and kept frozen until time of use, when they were thawed, vortexed, and then administered. All drugs and vehicle control solutions were

administered daily for 10 days through a stainless steel curved oral gavage tip (Cadence Science, product #7910). After each use, gavage tips were soaked in ethanol, rinsed with DI water and autoclaved before being used again.

Data Analysis

Data files extracted from Acqknowledge software were saved in a format compatible with Analyzer 2.2 (Brain Vision Inc). The resting EEG recordings were notch filtered at 60 Hz to remove any residual line frequency power. A semi-automatic procedure implemented in Analyzer 2.2 was used for artifact rejection after visual inspection of all EEG files. Less than 20% of data were rejected due to artifacts from any single animal recording.

The resting EEG data were divided into 2 s segments and each segment was subjected to Fast Fourier Transforms (FFT) analysis using a 10% Hanning window at 0.5 Hz bin resolution. The average power density ($\mu\text{V}^2/\text{Hz}$) was calculated for each mouse from 1 to 100 Hz. Power was binned according to spectral bands: Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8–13 Hz), Beta (13–30 Hz), Low Gamma (30–55 Hz), and High Gamma (65–100 Hz). Chirp, ASSR, and ERP traces were processed with Morlet wavelets linearly spaced from 1 to 100 Hz using voltage (μV). Wavelet coefficients were exported as complex values for use with Inter Trial Phase Coherence (ITPC) analysis. Wavelets were run with a Morlet parameter of 10. To measure phase synchronization at each frequency across trials, ITPC was calculated as follows:

$$\text{ITPC}(f, t) = \frac{1}{n} \sum_{k=1}^n \frac{F_k(f, t)}{|F_k(f, t)|}$$

where f is the frequency, t is the time point, and k is trial number. Thus, $F_k(f, t)$ refers to the complex wavelet coefficient at a given frequency and time for the k th trial.

We also measured baseline corrected single trial power for ERP analysis. Real values of spectral power (μV^2) were derived from Morlet wavelets. Baseline correction was done by taking the average power from –250 ms through –150 ms of the ERP window for each frequency layer and directly subtracting that average power from all values for their respective frequency layer in the window on a trial by trial basis. After baseline was corrected for each trial, the trials were averaged together. This is done to isolate changes in sound evoked power from background.

Statistical Analysis

Statistical group comparisons of ITPC and baseline corrected single trial power for the various stimulation paradigms were quantified using a Monte Carlo permutation approach. The details of the analysis were similar to Lovelace et al. (2018, 2019). Time-frequency analysis was conducted by binning time into 256 parts and frequency into 100 parts, resulting in a 100×256 matrix. Non-parametric analysis was used to determine contiguous regions in the matrix that were significantly different from a distribution of 2000

randomized Monte Carlo permutations based on previously published methods (Maris and Oostenveld, 2007). If the cluster sizes of the real genotype assignments (both positive and negative direction, resulting in a two-tailed alpha of $p = 0.025$) were larger than 97.25% of the random group assignments, those clusters were considered significantly different between experimental conditions.

Fmr1 KO mice are hyperactive compared to WT mice (Dansie et al., 2013) and movement can alter cortical gain (Niell and Stryker, 2010; Fu et al., 2015). To determine if movement was a factor in creating genotype differences in power spectral density, a piezoelectric transducer was placed underneath the recording cage to detect when a mouse was moving during EEG recordings. The term “resting” refers to EEGs recorded without any specific stimuli. The term “still” is used to describe resting EEG when the mouse was stationary. The term “moving” is used to describe resting EEG when the mouse was moving based on a threshold criterion for the piezoelectric signal. Movement was confirmed by analyzing the video recording (under IR light) taken throughout the EEG recording procedure. The differences between experimental conditions in resting power were analyzed on five dependent variables using one-way MANCOVA with movement as the covariate. The independent variables were genotype + drug condition and the dependent variables were five frequency bins (theta to high gamma). A $2 \times 2 \times 2$ design was used Genotype (WT or KO) $\times$ Drug (minocycline or vehicle) $\times$ Pre/Post. Three main comparisons were done: (1) comparing WT to KO in the “Pre” condition to establish phenotypes between genotypes; (2) comparing WT (vehicle) to KO (minocycline) to assess the level of rescue minocycline treatment had on KO mice compared to controls; and (3) comparing KO (vehicle) to KO (minocycline) to determine if minocycline had an effect that was greater than vehicle alone in KO mice. The proportion of time spent moving during the 5 min recording session was used as a covariate to identify genotype effects and control for potential effects of hyperactivity on cortical responses. Genotype comparisons were corrected for multiple comparisons using Bonferroni adjustments. Bonferroni adjusted p -values are reported by multiplying the p -value for each pairwise comparison by five (for five frequency bands), so all reported p -values can be interpreted using a $p < 0.05$ cutoff, while maintaining an effective $\alpha = 0.01$ for each multiple comparison. If data distributions did not meet assumptions of normality or homogeneity of variance, a $\log_{10}$ transform was applied to the data to be compared. Plots and figures that show measures of power are in their untransformed original format, but all statistics reporting is done on original or transformed data that meets parametric assumptions of MANCOVA.

RESULTS

The main goal of this study was to determine if 10 days of daily minocycline treatment, compared to vehicle, reversed resting state and sound-evoked EEG phenotypes in the auditory and frontal cortex (AC, FC) of adult *Fmr1* KO mice. In addition, we

quantified how different oscillation bands in EEG responses were affected by the treatment.

Minocycline and Vehicle Treatment Reduced Resting State Gamma Power in *Fmr1* KO Mice

Before EEG recordings were made, mice were allowed to habituate to the recording chamber as well as being attached to the recording tether for a total of ~30 min. After mice were habituated, 5 min of resting EEG was recorded in the absence of any specific auditory stimulation. Movement state was monitored by a piezo electric transducer located on the floor of the recording chamber. Power spectral density was calculated for both genotypes in the pre drug condition and in both AC and FC (**Figures 2A,C**). For statistical comparisons, average power in each frequency band was compared using canonical frequency cutoffs (**Figures 2B,D**). Statistical comparisons are the result of MANCOVA, using percentage movement for individual mice as a covariate, in order to isolate genotype and drug effects from hyperactivity phenotypes. Assumptions of equality of covariance were confirmed using Box's M, $p = 0.274$, and Levene's test of equality of error variances. Error variance was not different across genotypes (all $p > 0.05$). We report an effect of genotype (Pillai's Trace = 0.646, $p = 1.6308 \times 10^{-7}$) across all 5 of the combined frequency variables, which include movement as a covariate. We then determined that the only frequency band that was not different between genotypes in the AC of *Fmr1* KO mice was in the alpha range, $F(1,41) = 6.505$, $p = 0.0730$, $\eta^2 = 0.137$ (**Figure 2B**). All other frequency bands in the AC were significantly increased in *Fmr1* KO mice: Theta $F(1,41) = 9.485$, $p = 0.0185$, $\eta^2 = 0.188$, Beta $F(1,41) = 8.551$, $p = 0.0280$, $\eta^2 = 0.173$, Low Gamma $F(1,41) = 33.366$, $p = 4.4988 \times 10^{-6}$, $\eta^2 = 0.449$, High Gamma $F(1,41) = 17.156$, $p = 0.0008$, $\eta^2 = 0.295$.

The same pattern of differences were also observed in the FC: Box's M, $p = 0.497$, Levene's, all $p > 0.05$, Pillai's Trace = 0.753, $p = 4.4855 \times 10^{-5}$, with no observed difference in alpha $F(1,41) = 6.570$, $p = 0.0707$, $\eta^2 = 0.138$, and significant differences in all other bands, Theta $F(1,41) = 13.657$, $p = 0.0032$, $\eta^2 = 0.250$, Beta $F(1,41) = 16.085$, $p = 0.0013$, $\eta^2 = 0.282$, Low Gamma $F(1,41) = 23.240$, $p = 9.9619 \times 10^{-5}$, $\eta^2 = 0.362$, High Gamma $F(1,41) = 21.727$, $p = 0.0002$, $\eta^2 = 0.346$. In general, *Fmr1* KO mice showed an increase in power, but the most statistically robust differences were in the Gamma range. Significant differences in the gamma ranges replicate our original report (Lovelace et al., 2018) in this new cohort of mice, with a very similar "U" shape across the frequency profile.

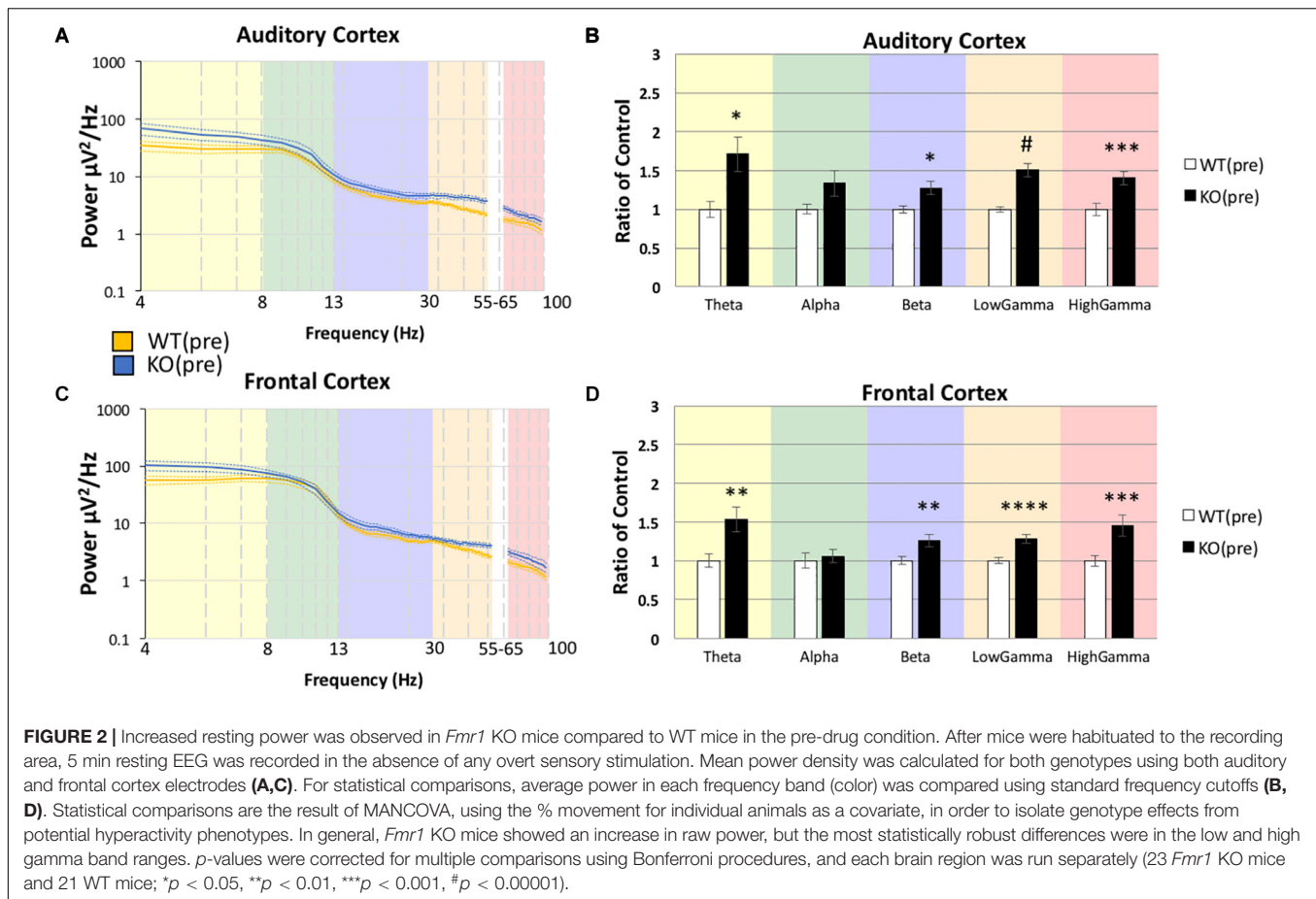
Using the same statistical approach as described above, we tested if the abnormally high resting EEG power in *Fmr1* KO mice (**Figure 2**) was responsive to minocycline treatment. Minocycline treated *Fmr1* KO mice were compared to vehicle treated WT mice (**Figure 3**) using MANCOVA in the AC (**Figure 3B**): Box's M, $p = 0.032$, Levene's, all $p > 0.05$, Pillai's Trace = 0.789, $p = 6.0748 \times 10^{-5}$, with the only observed difference in Theta $F(1,20) = 19.906$, $p = 0.0012$, $\eta^2 = 0.499$, and no significant

differences in all other bands, Alpha $F(1,20) = 1.485$, $p = 1.1858$, $\eta^2 = 0.069$, Beta $F(1,20) = 2.931$, $p = 0.5118$, $\eta^2 = 0.128$, Low Gamma $F(1,20) = 6.599$, $p = 0.0916$, $\eta^2 = 0.248$, High Gamma $F(1,20) = 0.740$, $p = 1.999$, $\eta^2 = 0.036$.

The same pattern was also observed in the FC (**Figure 3D**): Box's M, $p = 0.219$, Levene's, all $p > 0.05$, Pillai's Trace = 0.662, $p = 0.0021$, with the only observed difference in Theta $F(1,20) = 17.589$, $p = 0.0022$, $\eta^2 = 0.468$, and no significant differences in all other bands, Alpha $F(1,20) = 0.787$, $p = 1.927$, $\eta^2 = 0.038$, Beta $F(1,20) = 1.477$, $p = 1.1920$, $\eta^2 = 0.069$, Low Gamma $F(1,20) = 0.182$, $p = 3.3711$, $\eta^2 = 0.009$, High Gamma $F(1,20) = 0.754$, $p = 1.977$, $\eta^2 = 0.036$. There were no significant differences in the gamma band power in either AC or FC, but theta power remained elevated even after minocycline treatment.

To determine if minocycline had a greater therapeutic effect than vehicle control we compared *Fmr1* KO mice treated with minocycline to *Fmr1* KO mice treated with vehicle (**Figure 4B**) in the AC (Box's M, $p = 0.458$, Levene's, all $p > 0.05$, Pillai's Trace = 0.514, $p = 0.05$). No differences were observed in any frequency band, Theta $F(1,18) = 0.167$, $p = 3.4385$, $\eta^2 = 0.009$, Alpha $F(1,18) = 0.640$, $p = 2.1706$, $\eta^2 = 0.034$, Beta $F(1,18) = 0.427$, $p = 2.6089$, $\eta^2 = 0.023$, Low Gamma $F(1,18) = 0.452$, $p = 2.5500$, $\eta^2 = 0.024$, High Gamma $F(1,18) = 3.185$, $p = 0.4560$, $\eta^2 = 0.150$. In the FC as well (**Figure 4D**, Box's M, $p = 0.089$, Levene's, all $p > 0.05$, Pillai's Trace = 0.383, $p = 0.1909$), no differences were observed in any frequency band, Theta $F(1,18) = 0.726$, $p = 2.0266$, $\eta^2 = 0.039$, Alpha $F(1,18) = 0.292$, $p = 2.9790$, $\eta^2 = 0.016$, Beta $F(1,18) = 1.812$, $p = 0.9747$, $\eta^2 = 0.091$, Low Gamma $F(1,18) = 1.361$, $p = 1.2926$, $\eta^2 = 0.070$, High Gamma $F(1,18) = 4.353$, $p = 0.2572$, $\eta^2 = 0.195$. These data indicate that both treatment types (vehicle and minocycline) reduce gamma band power in the *Fmr1* KO mice. **Supplementary Figure S1** shows additional comparisons of resting EEG power to indicate that there were no effects of minocycline treatment compared to vehicle controls in either genotype, and that *Fmr1* KO mice have a significantly increased resting EEG power compared to WT, regardless of drug administration. **Supplementary Figure S2** shows that if the drug effects were compared to pre-drug conditions, then minocycline does show a significant reduction of gamma power in the *Fmr1* KO mice but this effect is obscured when comparing only KO(veh) to KO(mino) as shown in **Figure 4**. One explanation is that the drug administration protocol itself and the direct effect of minocycline both have statistically trending effects given the sample sizes. By directly comparing pre-drug conditions to minocycline treatment combines both trending effects into a single significant observation. Considering the different comparisons possible, our overall interpretation is that minocycline does not have an additional impact on resting EEG than vehicle in the *Fmr1* KO mice in our study.

Delta power was analyzed separately because the data either did not meet assumptions of homogeneity of variance, normality, or both. Using non-parametric Mann-Whitney *U*-tests for each comparison on raw untransformed data, we observed that resting EEG delta power was different between the genotypes in the pre-drug condition in AC ($U = 95$, WT(pre) $n = 21$, KO(pre)

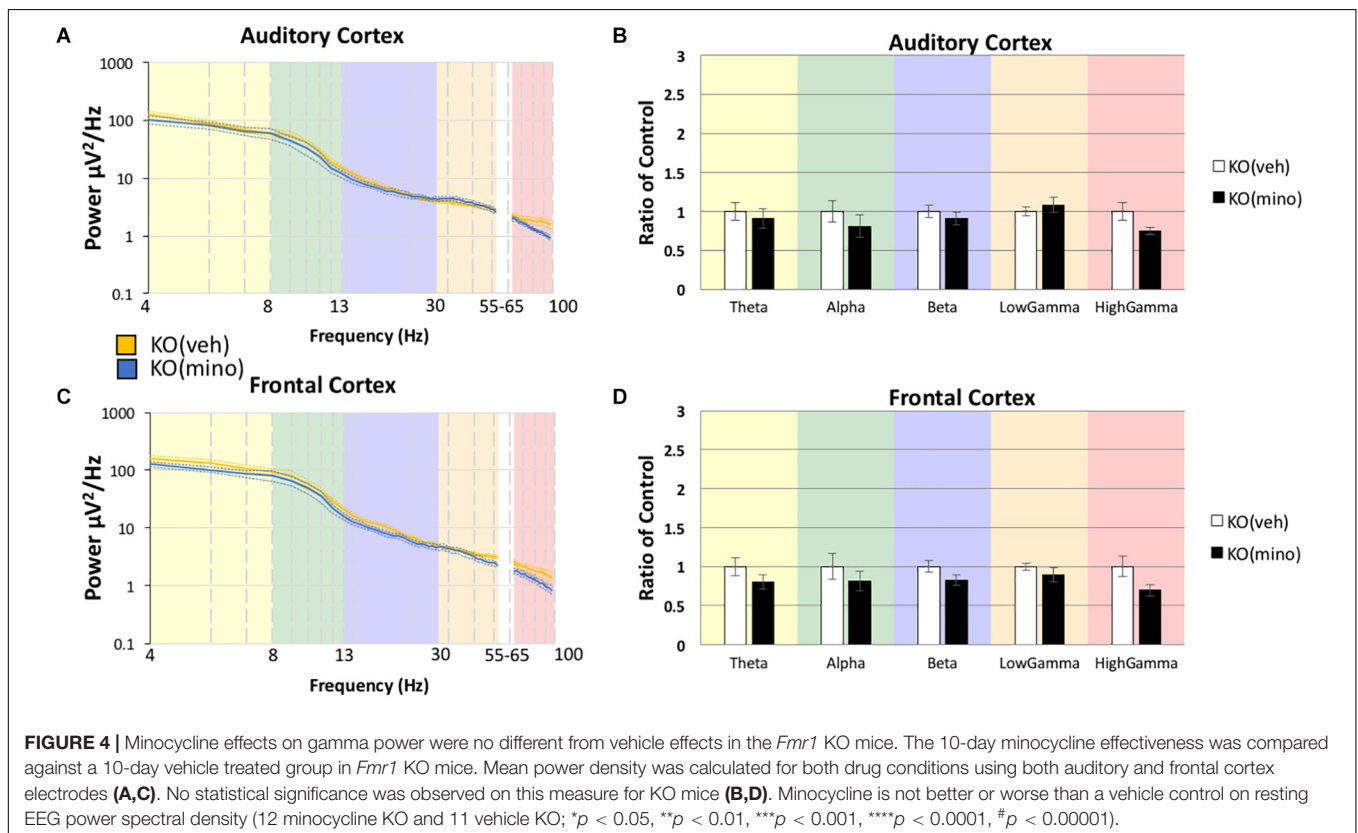
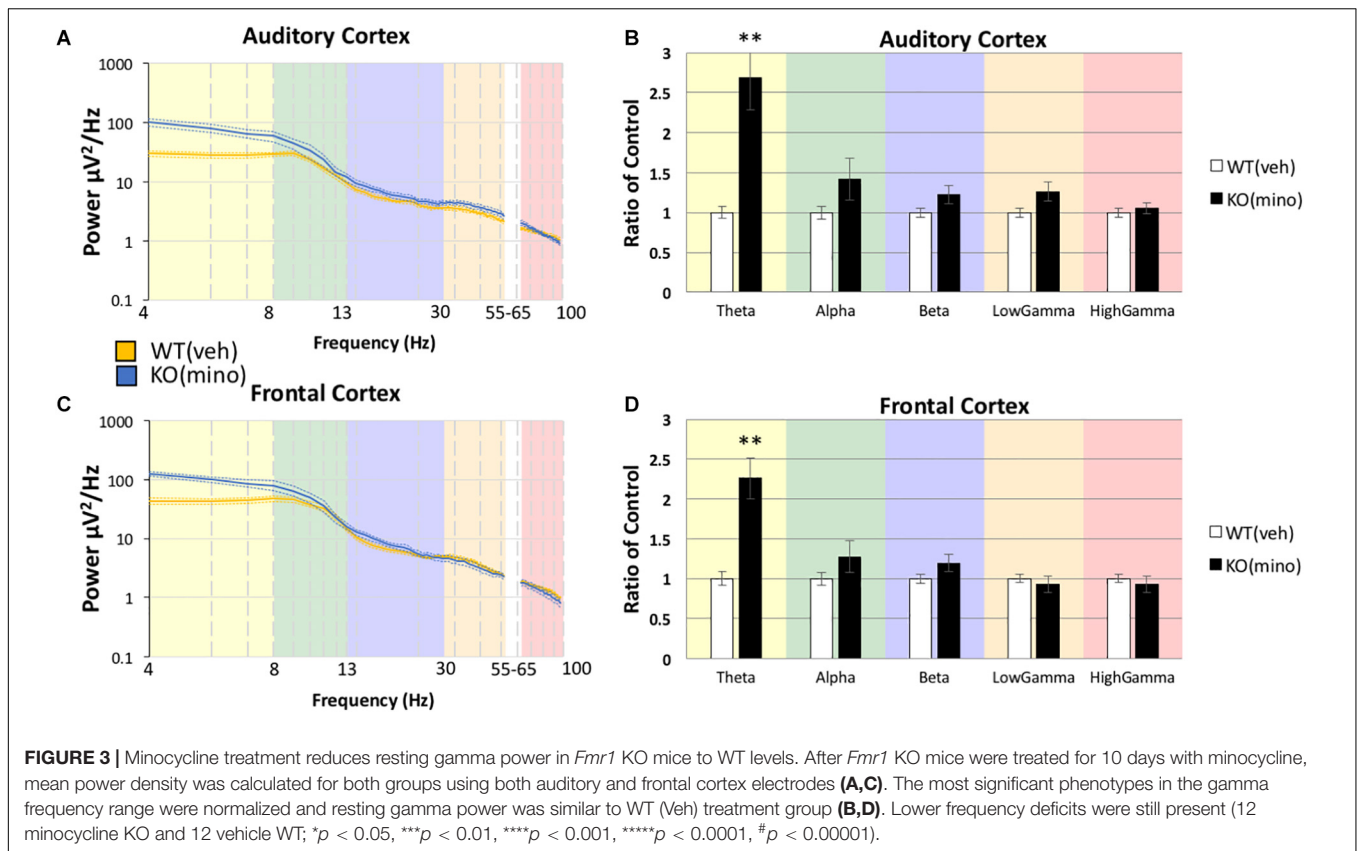


$n = 23$, $p = 0.0006$) and FC ($U = 90$, WT(pre) $n = 21$, KO(pre) $n = 23$, $p = 0.0004$) (Supplementary Figure S3), and these differences were not altered by minocycline compared to WT vehicle controls: AC ($U = 13$, WT(veh) $n = 12$, KO(mino) $n = 11$, $p = 0.0011$) FC ($U = 7$, WT(veh) $n = 12$, KO(mino) $n = 11$, $p = 0.0003$) (Supplementary Figure S3). There was no difference between minocycline and vehicle treated KO mice: AC, $U = 34$, KO(veh) $n = 10$, KO(mino) $n = 11$, $p = 0.1392$; FC, $U = 35$, KO(veh) $n = 10$, KO(mino) $n = 11$, $p = 0.1590$ (Supplementary Figure S3). Thus, delta power is increased in *Fmr1* KO mice compared to WT, but minocycline did not correct this phenotype.

Minocycline Improves ITPC in *Fmr1* KO Mouse Cortex Compared to Vehicle

The genotype differences in chirp response ITPC in the pre-drug condition (Figures 5A,D) were mostly consistent with our previous report (Lovelace et al., 2018). In the AC, ITPC was lower in the beta and low-gamma bands in the KO mice. In the FC, ITPC was lower from beta to high-gamma frequencies in the KO mice. One difference between the present data and our published work was the increase in ITPC in *Fmr1* KO mice at high gamma frequencies (~60–80 Hz) in the AC. Minocycline treatment of *Fmr1* KO mice significantly increased ITPC (Figures 5B,E) compared to vehicle treated *Fmr1* KO mice

across beta to gamma bands (~20–100 Hz) in the FC, and in the high gamma band (~80–100 Hz) in the AC. A comparison of *Fmr1* KO mice treated with minocycline to vehicle treated WT mice (Figures 5C,F) shows no difference in ITPC in the AC between ~20–40 Hz (where prominent reductions were observed in pre-drug condition). But since minocycline did not significantly increase ITPC over vehicle in this region (~20–40 Hz) (Figure 5B), this normalization of the phenotype does not appear to be better than vehicle treatment. However, in the FC, minocycline treatment significantly increased ITPC across beta-gamma frequencies compared to vehicle treatment in *Fmr1* KO mice (Figure 5E) and no significant differences in ITPC were observed across beta-low gamma frequencies when comparing minocycline treated *Fmr1* KO mice to vehicle treated WT controls (Figure 5F). Supplementary Figure S4 (A–F) shows a different method of plotting the same data to better illustrate the effects of minocycline on chirp ITPC only for the frequencies present in the chirp at any given time (i.e., along the diagonal of time-frequency response in Figure 5). Plots in S4 show the mean and standard error of ITPC of chirp response across the drug conditions, and allow the variability within groups to be visualized. These data indicate that minocycline was more effective in increasing phase coherence to time-varying stimuli when compared to vehicle treatment.



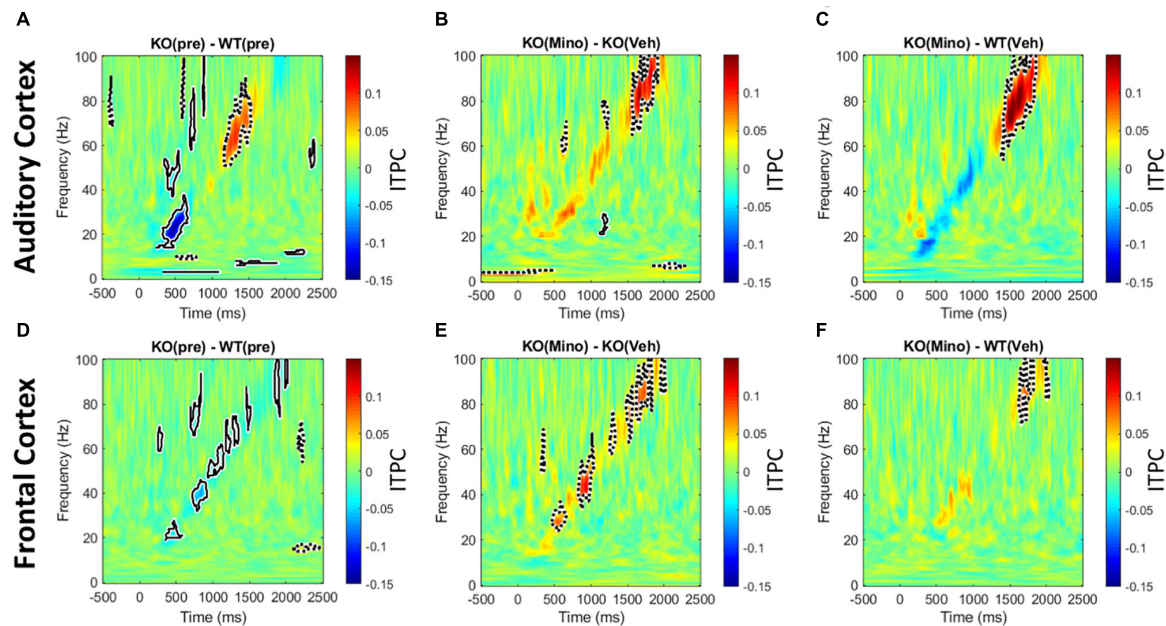


FIGURE 5 | Minocycline reverses phase locking deficits tested with chirp stimuli. **(A–F)** Colored regions are group mean differences between different genotype and drug conditions. Black-outlined regions are significantly reduced (blue with solid black outline) or increased (red with dotted black outline) as a result of a statistical permutation approach. **(A, D)** In the AC, there is a decrease in ITPC at beta to low-gamma bands, and an increase in ITPC in the high gamma range in the *Fmr1* KO mice. In the FC, there is a decrease in ITPC between beta to high gamma frequencies. **(B, E)** Ten days of minocycline treatment increases ITPC in *Fmr1* KO mice compared to vehicle in the FC, but not in the AC, suggesting a differential response of the two regions to this treatment. **(C, F)** The normalizing effect of minocycline in *Fmr1* KO mice can be seen. In both the AC and FC, there is no difference between the minocycline treated KO and vehicle treated WT groups at beta/low-gamma bands.

Minocycline Improves ASSR ITPC in *Fmr1* KO Mouse Cortex Compared to Vehicle

The 40 Hz auditory steady state response (ASSR) was compared between the *Fmr1* KO and WT mice in the pre-drug condition. There is a significant reduction of ITPC in the FC of the *Fmr1* KO mice (**Figure 6D**), but not in the AC (**Figure 6A**). After treating *Fmr1* KO mice with minocycline for 10 days, ITPC values in the FC were significantly increased over vehicle treated KO mice (**Figure 6E**). Finally, direct comparison of the *Fmr1* KO mice treated with minocycline to WT vehicle treated group show no statistical difference (**Figure 6F**). To complement the cluster analysis plots in **Figure 6** and **Supplementary Figure S4** (G–L) show the group mean and standard error of ITPC in the ASSR using the same data from **Figure 6**. These plots allow the variability within groups to be visualized. Together, these data reveal a novel genotype and cortical region specific ITPC deficit in the 40 Hz ASSR, and that minocycline can reverse these deficits compared to vehicle treatment.

Minocycline Reduces Sound-Induced Baseline Corrected Gamma Power in *Fmr1* KO Mice Compared to Vehicle

Trains of 100 ms duration broad band noise bursts were presented to mice (200 repetitions). After correcting for baseline (–250 ms through –150 ms) single trial power was averaged over

all trials. This allows for the analysis of sound-induced power that is normalized to the baseline for each animal. Consistent with previous report (Wen et al., 2019), we found a genotype difference with the *Fmr1* KO mice showing on-going increase in power in the delta and 60–80 Hz gamma band in the 100–400 ms time window after stimulus onset (**Figures 7A,D**). This was seen in both regions, with the FC also showing an ongoing decrease in beta/low gamma band power. Minocycline treatment drastically reduced onset power in *Fmr1* KO mouse responses over vehicle treatment in both FC and AC (**Figures 7B,E**). There was also a reduction in the ongoing 60–80 Hz gamma power. In the AC, however, there was increased ongoing power in 2 distinct lower-frequency bands at ~20 Hz and ~40 Hz. Finally, comparison of the minocycline-treated *Fmr1* KO mice to WT vehicle treated group reveals a shift in power in AC from high gamma to low gamma bands. Additionally, in the FC, ongoing gamma power and reduced beta – low gamma power were normalized (**Figures 7C,F**). We interpret these data to indicate a beneficial effect of minocycline in both AC and FC by reducing stimulus-evoked onset power and longer latency induced power in the high gamma frequencies.

Additional Analysis of Movement During EEG Recordings

While the FFT analysis of resting EEG used movement as a covariate, it is possible that movement itself was affected across recording sessions and/or within sessions due to auditory

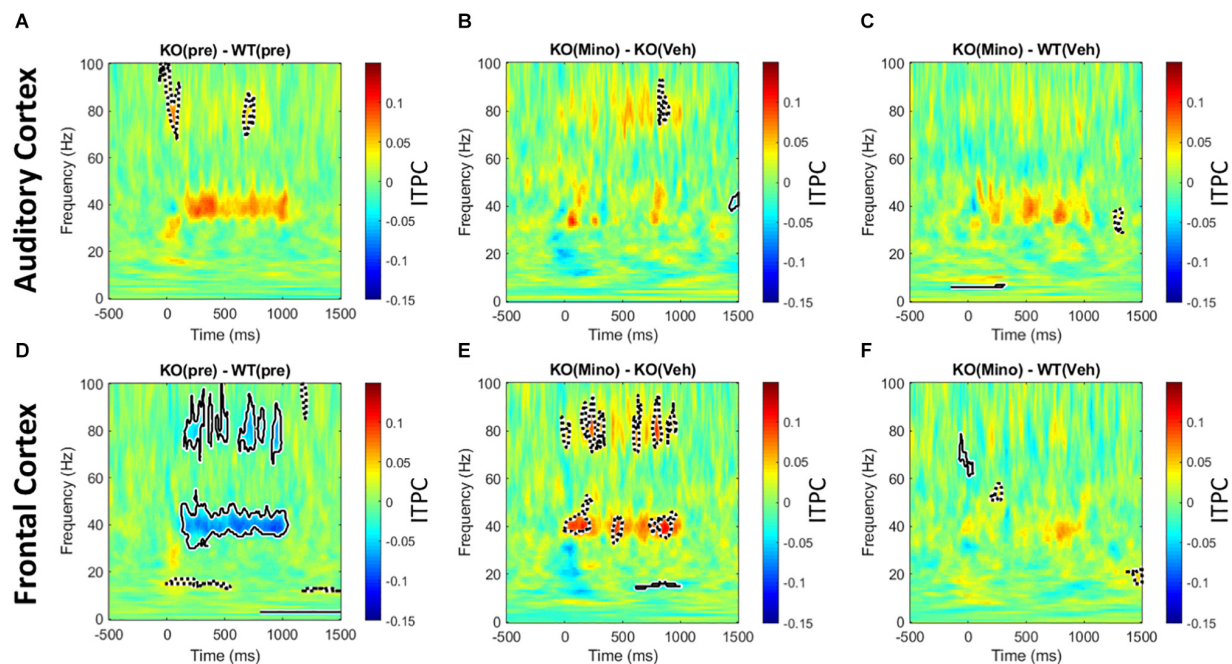


FIGURE 6 | Minocycline reverses phase locking deficits in the 40 Hz ASSR in the FC. An auditory steady state response is the response to a series of 40 Hz train of clicks (1 s duration). The analysis and interpretation are the same as in **Figure 5**. Comparing genotype differences in ITPC before drug treatment reveals a robust reduction in the *Fmr1* KO mice compared to WT in the FC (**D**), but not in the AC (**A**). After treating KO mice with minocycline for 10 days, ITPC values in the FC increased significantly compared to vehicle-treated controls in matching time x frequency regions (**E**), with no effect in the AC (**B**). Direct comparison of ASSR in *Fmr1* KO mice treated with minocycline to vehicle-treated WT mice show virtually no difference, suggesting a rescue of the phenotype in the FC (**F**), and again no effect found in the AC (**C**).

stimulus presentation. **Supplementary Figure S5A** shows the percentage of time mice moved during resting EEG across genotypes and treatment. First, we determined that KO mice moved less than WT during pre-drug treatment using a *t*-test, $t(42) = 2.741$, $p = 0.0090$. Then to determine drug effects, we used time spent moving during pre-drug treatment as a co-variate in a two-way ANCOVA on Genotype X Drug, this controls for the original movement differences found in the pre drug condition. There was no effect of Genotype $F(1,37) = 1.187$, $p = 0.2829$, Drug $F(1,37) = 0.676$, $p = 0.4163$, or Genotype X Drug interaction $F(1,37) = 0.013$, $p = 0.9105$. Therefore, the EEG changes due to minocycline are unlikely to be due to the drug's effect on movement or due to changes in movement across recording conditions.

To determine if exposure to sound altered movement of the animals, we recorded the % time mice spent moving in 5 s windows of silence between presentations of 100 trains of sounds over a period of sound evoked recordings (~52 min). KO mice also move less than WT mice during sound presentation, $t(42) = 5.051$, $p = 0.000009$, two-way ANCOVA on Genotype X Drug using movement during pre as a co-variate shows no effect of Genotype $F(1,37) = 0.263$, $p = 0.6114$ or Drug $F(1,37) = 3.319$, $p = 0.0765$, but there was a significant Genotype $\times$ Drug interaction $F(1,37) = 4.8790$, $p = 0.0335$. Further analysis of simple effects shows that minocycline had a trending effect on reducing WT movement during sound presentation $F(1,18) = 4.346$, $p = 0.0516$, but had no effect

on KO mice $F(1, 18) = 0.016$, $p = 0.8993$. Therefore, the effects of minocycline during sound presentation in the KO mice is unlikely to be due to altered movements by the sound. To identify if auditory stimulation within a recording session affected movement, we analyzed movement in the silent intervals during acoustic stimulation for the first 10 and the last 10 trains of sounds (**Supplementary Figures S5C,D**). Yet again, the data does not show any effects of minocycline on movement within a recording session in the *Fmr1* KO mice.

DISCUSSION

We studied the effects of 10-day minocycline treatment in adult mice on resting EEG power, phase locking in the response to auditory chirp stimuli and 40 Hz ASSR, and on the induced power in response to noise burst stimuli. These responses provide insight into abnormalities in background cortical activity, temporal fidelity in evoked synchronization and sound-induced power, respectively, in *Fmr1* KO mice, and serve to identify neural correlates of abnormal auditory processing in FXS. We replicated many of the previously reported phenotypes (Lovelace et al., 2018) in this new cohort of *Fmr1* KO mice. This includes increased gamma band resting EEG power, reduced ITPC to chirp and increased ongoing induced gamma power (Lovelace et al., 2018; Wen et al., 2019). We also report a novel 40 Hz ASSR deficit with a reduction in ITPC in the FC, but not AC, of *Fmr1*

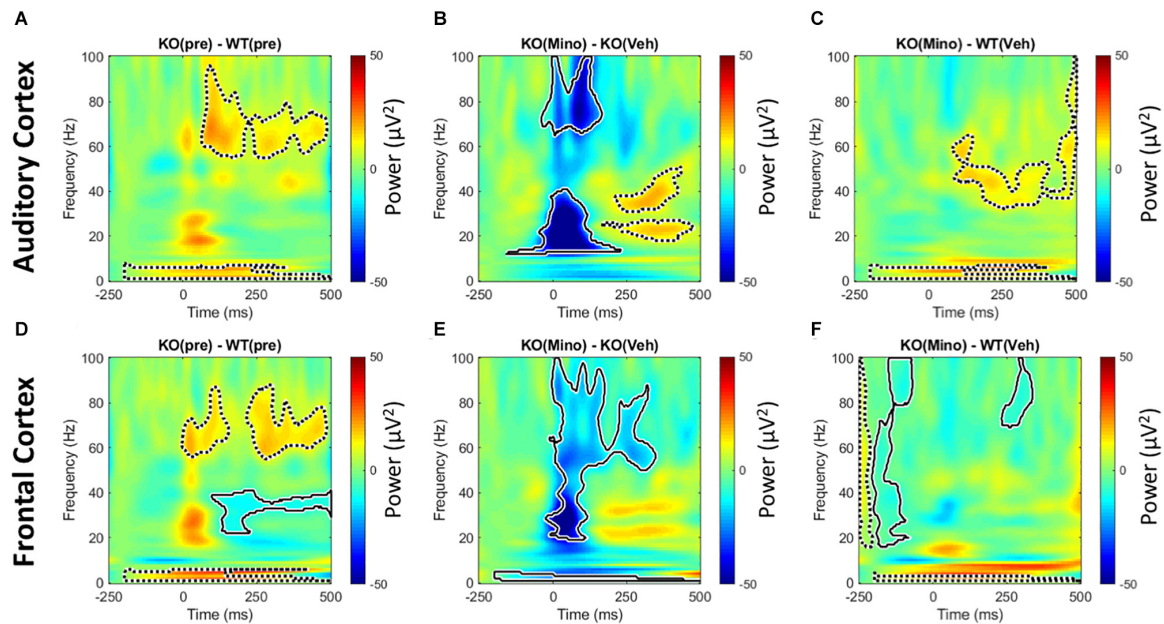


FIGURE 7 | Minocycline reduces sound-induced gamma power in *Fmr1* KO mice. Trains of 100 ms duration broad band noise were used to generate event related potentials (200 repetitions) for time × frequency analyses. After correcting for baseline (−250 ms through −150 ms) single trial power was averaged over all trials. This allows for the analysis of power that is normalized to individual animal baselines. In the pre-drug condition, *Fmr1* KO mice showed increased on-going power after the auditory stimulus in the delta and high gamma range in both AC and FC (A,D). In addition, the FC showed a significant reduction in power the beta to low gamma frequency range (D). Minocycline treatment significantly reduced onset power in *Fmr1* KO animals over vehicle treatment in both FC and AC (B,E), but also increased power in the AC in two distinct bands at ~20 and ~40 Hz that occurred well after sound onset. Finally, comparing minocycline treated *Fmr1* KO mice to vehicle-treated WT group, reveals a shift in power in AC from high gamma in (A), to low gamma in (C). Additionally in the FC, ongoing gamma power and modulation of beta – low gamma power in (D) are significantly reduced (F).

KO mice compared to WT mice. The major finding in terms of minocycline treatment is that sound-evoked, but not resting, EEG phenotypes in adult *Fmr1* KO mice are more sensitive to minocycline treatment than vehicle. This includes improved fidelity of evoked synchronization with chirps and ASSR, and reduced induced power.

Minocycline does not change resting EEG power distribution in a manner that is different from vehicle treatment in the *Fmr1* KO mice. Even though the drug reduced resting EEG gamma power in the *Fmr1* KO mice to vehicle treated WT levels, vehicle treatment in the KO mice had the same effect. This effect was seen in both AC and FC. This suggests that other factors, such as the treatment protocol (including stress and arousal of daily handling and oral gavage protocols), can influence resting EEG responses in the *Fmr1* KO mice. Even though the *Fmr1* mice were given 15 min of habituation in the recording chamber after the electrodes were connected, it is possible that their baseline arousal state is higher when resting EEGs were recorded compared to WT mice. Resting EEG rhythms can be altered by neuromodulators that promote arousal such as acetylcholine and influence drug × genotype interactions (Babiloni et al., 2013). Sound evoked oscillations may have a larger magnitude, compared to high frequency resting EEG components, to reveal sensitivity to minocycline compared to vehicle.

In terms of evoked responses, a significant effect of minocycline treatment in *Fmr1* KO mice compared to vehicle treatment was the improvement in ITPC for chirp response.

Ethridge et al. showed that ITPC is reduced in humans with FXS. Likewise, Lovelace et al. (2018) showed a reduction in ITPC to the chirp in the *Fmr1* KO mice. ITPC measures the ability of evoked oscillations to phase lock with fidelity from one trial to the next. Reduced ITPC indicates that the neural generators show higher jitter in their phase locking to repeated temporally modulated stimuli. In both humans with FXS and *Fmr1* KO mice, the reduction was robust in the 30–50 Hz range suggesting a gamma band deficit.

Gamma band ITPC deficit in chirp response is further substantiated by the observed 40 Hz ASSR ITPC deficits in *Fmr1* KO mice. ASSR may be a useful translational biomarker as robust differences are seen in neurodevelopmental disorders including schizophrenia and autism. In schizophrenia, patients show reduced phase locking to 40 Hz stimulation (Light et al., 2006). Studies in children with autism, and their unaffected first degree relatives, showed a reduced 40 Hz ASSR power and phase locking compared to age-matched controls (Wilson et al., 2007; Rojas et al., 2008). Consistent with this, we observed a reduction in ITPC in the 40 Hz ASSR in *Fmr1* KO mice. Whether children with FXS also show abnormal ASSR has not been tested, but the mouse results suggest that this may be a potentially useful avenue for clinical applications in humans with FXS.

The ITPC deficits for both the chirp response and ASSR are reversed by minocycline treatment. In both cases, minocycline-treated KO mice showed improved responses compared to vehicle-treated KO group suggesting a true

drug effect. The gamma band deficits in ITPC suggests that minocycline may act by affecting PV cell function in *Fmr1* KO mice. There is converging evidence for dysfunction of PV neurons in *Fmr1* KO mice, and this has been seen across auditory, somatosensory and visual cortices (Selby et al., 2007; Nomura et al., 2017; Goel et al., 2018; Wen et al., 2018). Abnormal PV cell function may be related to reduced integrity of perineuronal nets (PNN). PNNs are specialized assemblies of extracellular matrix components that preferentially form around PV neurons (Brewton et al., 2016; Lensjø et al., 2017; Nguyen et al., 2017; Ueno et al., 2018; Wen et al., 2018). Loss of PNNs around PV cells is predicted to reduce PV expression, as well as excitability of these interneurons (Balmer, 2016). MMP-9 cleaves components of extracellular matrix and increased activity of MMP-9 in FXS may lead to reduced PNN expression and abnormal function of PV cells. Indeed, genetically reducing MMP-9 in *Fmr1* KO mice normalizes PV/PNN development and electrophysiological responses in the auditory cortex of *Fmr1* KO mice. One known effect of minocycline is the inhibition of MMP-9 (Dziembowska et al., 2013). However, minocycline has other effects in the nervous system including modulation of cell apoptosis, inflammation and microglial clearance (Dean et al., 2012). EEG data obtained using SB-3CT, a specific inhibitor of MMP-9, show reversal of multiple EEG phenotypes (Pirbhoy et al., 2020). This adds strength to the notion that reduction of neural correlates of auditory hypersensitivity by minocycline involves MMP-9 inhibition. Therefore, the reduction of MMP-9 and normalization of PV neuron function may underlie beneficial effects of minocycline treatment, which manifests as normalized gamma band synchrony in both ASSR and chirp responses.

Another major effect of minocycline treatment was reduction in stimulus evoked and induced ERP power. With time/frequency analysis of auditory ERPs, two types of gamma oscillations can be distinguished. The short latency (<100 ms) response is tied to stimulus onset, while the longer latency (150–400 ms) gamma oscillation contains induced power that is not stimulus locked (Franowicz and Barth, 1995; Crone et al., 2001; Brosch et al., 2002; Jeschke et al., 2008). Using a cortical slice preparation, Metherate and Cruikshank (1999) showed that thalamic stimulation caused an early glutamatergic potential in the input layer of auditory cortex. This was followed by long-latency gamma-band fluctuations driven by polysynaptic activity (akin to induced power). This long-latency gamma band activity was dominant in the middle and superficial layers and propagated intracortically. This may facilitate formation of transient neural assemblies by synchronizing activity across rapidly changing pools of neurons. The gamma fluctuations consisted of both fast depolarization and GABAergic inhibition. Given the broader tuning curves of cortical neurons in the *Fmr1* KO mice, and increased synchrony across neurons (Goswami et al., 2019), the transient neural assemblies formed in the 150–400 msec window after stimulus onset may incorporate the activity of a larger number of neurons with overlapping tuning. This would produce the observed increase in induced gamma power.

Minocycline treatment in KO mice reduced both sound-evoked onset and ongoing induced power when compared to vehicle control. One interpretation is that by reducing the

magnitude of the onset response, the intracortical spread of polysynaptic activity and synchronized neural pool recruitment is reduced. Additionally, there were no phase-locking differences in sound-evoked ERPs between WT and KO pre-drug, indicating that changes in sound-evoked power were primarily driven by increased magnitudes of individual responses, and not inter-trial synchrony. However, therapeutic reduction in sound-evoked power by minocycline was associated with a reduction in ITPC, which indicates that even though there was no difference in ITPC pre-drug, the mechanism of minocycline action seems to be through desynchronization of neural populations, which may contribute to a smaller evoked response, and subsequently preventing the network from entering a persistently active state.

In general, FC appeared to be more malleable in response to minocycline than AC. It is possible that AC requires earlier time points of treatment compared to the FC. Indeed, Pirbhoy et al. (2020) showed that the treatment of young (P21–22) mice with SB-3CT, a selective MMP-9 inhibitor, increases ITPC of chirp responses around 40 Hz, and increases the density of PV and PNN expressing cells in the AC. Regional differences may also arise because the neural generators that give rise to gamma oscillations in the AC are different from the FC. Sound-evoked oscillations recorded from the AC are likely driven by a mixture of primary cortical neural generators and from multiple subcortical structures (Bidelman, 2018), while FC gamma oscillations are less likely to be directly influenced by auditory subcortical sites. However, FC gamma oscillations can be strongly modulated by projection PV cells in the basal forebrain, in a state-dependent manner associated with arousal and attention (Kim et al., 2015). Evidence of such a possibility can be seen by comparing induced power to sound bursts in *Fmr1* KO mice treated with minocycline in this study, to transgenic mice which have a specific cortical KO of *Fmr1* (Lovelace et al., 2019). The correlation between these 2 groups on this measure is striking, including increased power in distinct bands at ~20 and ~40 Hz post stimulation, in addition to a reduction in ITPC during sound onset in the AC. One interpretation is that minocycline could have its therapeutic effect at treating local cortical circuits or basal forebrain structures, and less of an effect on subcortical auditory pathways. Future studies will more systematically examine the role of treatment age on regional brain effects, and the longevity of the beneficial effects.

Clinical Relevance

Sensory hypersensitivity is a debilitating and consistent symptom of FXS, and auditory hypersensitivity is a prominent manifestation noted in clinical and parent reports. Early childhood sensory processing abnormalities may lead to increased anxiety and delayed language development. In the mouse model of FXS, we have identified four neural correlates of auditory hypersensitivity: increased single neuron responses to sounds (Rotschafer and Razak, 2013; Wen et al., 2019; Nguyen et al., 2020), more neurons activated by a single sound (Rotschafer and Razak, 2013; Nguyen et al., 2020), reduced habituation of cortical responses to repeated sounds (Lovelace et al., 2016) and abnormal synchrony of sound driven responses (Lovelace et al., 2018, 2019; Wen et al., 2019). Either genetic

reduction of MMP-9 (Lovelace et al., 2016; Wen et al., 2018) or reduction of MMP-9 with a specific inhibitor (Pirbhoy et al., 2020) or with the FDA-approved minocycline normalized these abnormal responses (present study). Taken together with studies that observed improved outcomes in humans with FXS (Paribello et al., 2010; Leigh et al., 2013; Schneider et al., 2013) and the *Drosophila* model of FXS (Siller and Broadie, 2011), these data point to the strong potential of MMP inhibition as a therapeutic avenue to reduce sensory symptoms in humans with FXS.

An urgent need in neurodevelopmental disorders research and drug development is the identification of objective, robust, and translation-relevant biomarkers (Sahin et al., 2018). EEG phenotypes may serve this need, given the consistency in both type and direction of abnormalities in *Fmr1* KO mice and humans with FXS. Moreover, EEG phenotypes recorded in humans with FXS are correlated with abnormal sensory sensitivity reports and deficits in social communication as measured with the social and communication questionnaires. These correlations suggest clinical significance of EEG phenotypes in humans and point to the utility of EEG measures for both stratification and for measuring drug efficacy in clinical trials. An important aspect of biomarker development is that the specificity and sensitivity of drug effects (over placebo effects) can be quantified in both mice and humans. Our data from the *Fmr1* KO mouse suggest that minocycline has spectral band- and EEG response-specific effects. Future studies in humans with FXS should examine the effects of minocycline on a range of EEG measures in humans. So far, published human data (Schneider et al., 2013) have only examined sound evoked habituation, and showed that minocycline, compared to placebo, improved ERP N1 component habituation to repeated sounds in patients with FXS. Whether phase-locking and resting EEGs are also improved in humans remains to be tested.

Minocycline has been tested as a potential therapeutic in a number of neurodevelopmental and neurodegenerative conditions including Angelman syndrome (Grieco et al., 2014), schizophrenia (Chaudhry et al., 2012), depression (Miyaoka et al., 2012), Parkinson's disease (Thomas and Le, 2005) and Alzheimer's disease (Choi et al., 2007). EEG recordings have been used to obtain physiological markers in these conditions (Sponheim et al., 1994, 2000; Narayanan et al., 2014). Some of the phenotypes may be used as outcome measures for candidate drug testing. Our results suggest that an evaluation of how minocycline affects a broad range of EEG phenotypes in preclinical models

is required before designing outcome measures in humans. Different EEG responses and cortical regions are differentially susceptible to the specific treatment protocol used. This is an important consideration moving forward with the use of EEGs in clinical trials.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, UCR.

AUTHOR CONTRIBUTIONS

JL, DB, IE, and KR designed the study and worked together to write the manuscript. JL performed the experiments and analyzed the data. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnins.2020.00771/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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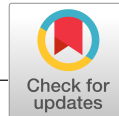
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ORIGINAL ARTICLE

Acute pharmacological inhibition of matrix metalloproteinase-9 activity during development restores perineuronal net formation and normalizes auditory processing in *Fmr1* KO mice

Patricia S. Pirbhoy¹ | Maham Rais¹ | Jonathan W. Lovelace² | Walker Woodard¹ | Khaleel A. Razak² | Devin K. Binder¹ | Iryna M. Ethell¹

¹Division of Biomedical Sciences, School of Medicine, University of California, Riverside, CA, USA

²Department of Psychology, University of California Riverside, Riverside, CA, USA

Correspondence

Iryna M. Ethell, Division of Biomedical Sciences, School of Medicine, University of California, Riverside, Riverside, CA 92521, USA.

Email: iryna.ethell@medsch.ucr.edu

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Abstract

Individuals with Fragile X Syndrome (FXS) and autism spectrum disorder (ASD) exhibit cognitive impairments, social deficits, increased anxiety, and sensory hyperexcitability. Previously, we showed that elevated levels of matrix metalloproteinase-9 (MMP-9) may contribute to abnormal development of parvalbumin (PV) interneurons and perineuronal nets (PNNs) in the developing auditory cortex (AC) of *Fmr1* knock-out (KO) mice, which likely underlie auditory hypersensitivity. Thus, MMP-9 may serve as a potential target for treatment of auditory hypersensitivity in FXS. Here, we used the MMP-2/9 inhibitor, SB-3CT, to pharmacologically inhibit MMP-9 activity during a specific developmental period and to test whether inhibition of MMP-9 activity reverses neural oscillation deficits and behavioral impairments by enhancing PNN formation around PV cells in *Fmr1* KO mice. Electroencephalography (EEG) was used to measure resting state and sound-evoked electrocortical activity in auditory and frontal cortices of postnatal day (P)22–23 male mice before and one-day after treatment with SB-3CT (25 mg/kg) or vehicle. At P27–28, animal behaviors were tested to measure the effects of the treatment on anxiety and hyperactivity. Results show that acute inhibition of MMP-9 activity improved evoked synchronization to auditory stimuli and ameliorated mouse behavioral deficits. MMP-9 inhibition enhanced PNN formation, increased PV levels and TrkB phosphorylation yet reduced Akt phosphorylation in the AC of *Fmr1* KO mice. Our results show that MMP-9 inhibition during early postnatal development is beneficial in reducing some auditory processing deficits in the FXS mouse model and may serve as a candidate therapeutic for reversing sensory hypersensitivity in FXS and possibly other ASDs.

KEYWORDS

electroencephalography, fragile X syndrome, matrix metalloproteinase-9, parvalbumin, perineuronal nets, sensory hypersensitivity

Abbreviations: AC, auditory cortex; Akt, protein kinase B; ASD, autism spectrum disorders; EEG, electroencephalography; FC, frontal cortex; FFT, fast fourier transform; *Fmr1* KO, *Fragile X mental retardation 1* gene knock-out; FXS, fragile X syndrome; ITPC, inter-trial phase coherence; MMP-2/9, matrix metalloproteinase-2/9; mTOR, mammalian target of rapamycin; PNN, perineuronal net; PV, parvalbumin; RRID, research resource identifier; STP, single trial power; TrkB, tropomyosin-related kinase B.

1 | INTRODUCTION

Fragile X Syndrome (FXS) is a neurodevelopmental disorder caused by the hypermethylation of the *Fragile X mental retardation 1* (*Fmr1*) gene, resulting in the loss of the Fragile X Mental Retardation Protein (FMRP) (Verkerk et al., 1991), a regulator of protein synthesis (Laggerbauer, 2001). FXS is the most prevalent cause of inherited intellectual disability (Hagerman, Rivera, & Hagerman, 2008) and a leading genetic cause of autism, providing a genetic model to study the neurobiological mechanisms underlying autism spectrum disorders (ASD) (Crawford, Acuna, & Sherman, 2001; Hagerman et al., 2008). Individuals with FXS exhibit cognitive dysfunctions (Freund & Reiss 1991; Kaufmann et al., 2004), increased anxiety (Talisa, Boyle, Crafa, & Kaufmann, 2014), and sensory hyperexcitability (Ethridge et al., 2016; Miller et al., 1999; Rotschafer & Razak, 2013).

Auditory hypersensitivity in individuals with FXS and *Fmr1* knock-out (KO) mice is commonly characterized by a heightened response to non-aversive stimuli (Crane, Goddard, & Pring, 2009; McDiarmid, Bernardos, & Rankin, 2017; Rais, Binder, Razak, & Ethell, 2018; Sinclair, Oranje, Razak, Siegel, & Schmid, 2017), reduced ability to habituate to sounds (Castren, Paakkonen, Tarkka, Ryynanen, & Partanen, 2003; Ethridge et al., 2016; Lovelace et al., 2016; Wang et al., 2017), and greater resting state gamma power in electroencephalography (EEG) recordings (Ethridge et al., 2017; Lovelace, Ethell, Binder, & Razak, 2018; Lovelace et al., 2019; Wang et al., 2017). Studies in the *Fmr1* KO mice also show increased sensory responses, broader receptive fields, impaired sound selectivity (Rotschafer & Razak, 2013), and abnormal network synchronization (Paluszkiwicz, Olmos-Serrano, Corbin, & Huntsman, 2011). It is hypothesized that the inability to habituate to auditory stimuli (Castren et al., 2003; Ethridge et al., 2016) including ongoing background noise, a phenotype that is also observed in *Fmr1* KO mice (Lovelace et al., 2016, 2018), may contribute to hypersensitivity to sounds and cortical hyperexcitability (Ethridge et al., 2016, 2017; Gibson, Bartley, Hays, & Huber, 2008; Lovelace et al., 2018, 2019). Notably, EEG gamma frequency band abnormalities are associated with heightened sensory sensitivities and social communication deficits in FXS individuals (Ethridge et al., 2016, 2017, 2019). Taking advantage of the link between EEG abnormalities and sensory hypersensitivity in FXS, this study used these EEG measures to test the efficacy of a candidate treatment in *Fmr1* KO mice.

Individuals with FXS (Dziembowska et al., 2013) and *Fmr1* KO mice (Bilousova et al., 2009; Gkogkas et al., 2014; Sidhu, Dansie, Hickmott, Ethell, & Ethell, 2014) exhibit increased levels of the secreted endopeptidase, matrix metalloproteinase-9 (MMP-9). Specifically, *Fmr1* KO mice show elevated levels of MMP-9 in the auditory cortex (AC) during postnatal day (P) 3–18 period. It is hypothesized that increased activity of MMP-9 may contribute to the development of auditory hypersensitivity by excessively degrading PNNs, thereby resulting in impaired PNN formation around PV interneurons and affecting PV development in the AC of *Fmr1* KO mice at P21 (Wen et al., 2018). Indeed, genetic reduction of MMP-9

levels enhanced PNN formation around PV interneurons and restored sound-evoked responses and spontaneous activity to WT levels (Wen et al., 2018). Genetic removal of MMP-9 also rescued altered event-related potential (ERP) habituation responses in the AC of adult *Fmr1* KO mice (Lovelace et al., 2016), normalized dendritic spine abnormalities in the hippocampus and improved abnormal behaviors in *Fmr1* KO mice (Sidhu et al., 2014). PV interneuron dysfunction contributes to abnormal network synchrony and alterations in gamma oscillations (Lewis, Hashimoto, & Volk, 2005; Sohal, Zhang, Yizhar, & Deisseroth, 2009; Vreugdenhil, Jefferys, Celio, & Schwaller, 2003). Overall, these studies indicate that (1) MMP-9 may influence the development of hypersensitive networks during the third and fourth postnatal weeks, a time that coincides with a critical developmental window for inhibitory and excitatory circuit maturation (Gibson et al., 2008; Oswald & Reyes, 2008) and (2) that targeted inhibition of MMP-9 may serve as a candidate therapeutic to reduce auditory hypersensitivity in FXS.

In this study, we tested the hypothesis that acute pharmacological inhibition of aberrant MMP-9 activity during the critical developmental period would ameliorate cortical processing deficits, including abnormal resting state neural oscillatory patterns, the ability to synchronize to stimulus-induced oscillations, PV/PNN deficits, and behavioral impairments. Our results show that acute inhibition of MMP-9 during the P22–23 developmental period improved evoked synchronization to auditory stimuli and enhanced PNN formation around PV cells. We also observed beneficial effects of acute MMP-9 inhibition at P27–28 on animal behaviors, such as anxiety and hyperactivity. MMP-9 inhibition at P27–28 also increased PV levels and TrkB phosphorylation, and reduced aberrant Akt phosphorylation in the developing AC of *Fmr1* KO mice.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments and animal care/use protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside (approval number 20190015 and 20190029) and were carried out in accordance with NIH 'Guide for the Care and Use of Laboratory Animals'. The study was not pre-registered.

2.2 | Animals

Experimental animals, C57BL/6 mice, were obtained from Jackson Laboratories (RRID: IMSR_JAX:003025). Age-matched wild-type (WT) and *Fmr1* KO male mice were used between postnatal day (P) 18–19 (EEG surgery $N = 79$, $N = 14$ died post-surgery), P22–23 (EEG recording [$N = 65$] and PV/PNN analysis [$N = 24$: note the same EEG animals were used for analysis] and DQ gelatin assay [$N = 16$]), and P27–29 for behavior ($N = 37$) and Western blot ($N = 8$). For a detailed description of total animals and animals per group, please

refer to Figures 1, 6, and 7. A total of 140 male mice were used in this study with an average weight of 8 g and range from 6 to 18 g. All genotypes were confirmed with the analysis of tail samples by Transnetyx, using real-time PCR-based system to probe for WT (*fmr*^{+/+} forward: 5'-TGTGATAGAATATGCAGCATGTGA-3') or KO (*fmr*^{-/-} forward: 5'CACGAGACTAGTGAGACGTG-3') target sequence in each sample. Mice were maintained in an AAALAC accredited facility in 12-hr light/dark cycles and food and water was available ad libitum. All experimental procedures were performed on C57BL/6 (WT, RRID:IMSR_JAX:000664) and *Fragile X mental retardation gene-1* (*Fmr1*) knock-out (KO) mice (RRID: IMSR_JAX:003025, The Jackson Laboratory).

2.3 | Surgery for electroencephalography recordings

Surgical procedures were performed as described in detail previously (Lovelace et al., 2018, 2019). Briefly, mice were anesthetized with isoflurane inhalation (0.2%–0.5%) and given an intraperitoneal (i.p.) injection of ketamine and xylazine (K/X: 100/20 mg/kg). Toe pinch reflex was used to measure anesthetic state every 10 min throughout the surgery, and supplemental doses, no more than half of the original dose of K/X, were administered as needed. Mice were placed in a stereotaxic frame (model 930; Kopf, CA). Artificial tear gel was applied to the eyes to prevent drying. Once the mouse was anesthetized, a midline sagittal incision was made along the scalp to expose the skull. A Foredom dental drill was used to drill three holes 1 mm in diameter in the skull overlying the right AC (−1.6 mm, +4.8 mm), right frontal lobe (+2.6 mm, +1.0 mm), and left occipital cortex (−3.5 mm, −5.2 mm) (coordinate relative to bregma: anterior/posterior, medial/lateral). Three channel electrode posts (Plastics One, MS333-2-A-SPC) were attached to 1-mm stainless steel screws (Plastics One, 8L003905201F) and screws were advanced into the drilled holes until secure. Special care was taken not to advance the screws beyond the point of contact with the dura. Dental cement was applied around the screws, on the base of the post, and exposed skull. Postoperative care included topical application of a triple antibiotic ointment along the edges of the dental cement followed by two subcutaneous injections of buprenorphine (0.05 mg/kg), one immediately after surgery, and one 6–10 hr after surgery. Mice were placed on a heating pad to aid recovery from anesthesia. Mice were group housed, returned to the vivarium, and monitored daily until the day of EEG recordings, which allowed 4–5 days recovery post-surgery before recording.

2.4 | Electrophysiology

All EEG recordings were performed during 5 a.m.–7 p.m. Mice were habituated for 20 min in an anechoic foam-lined sound-attenuating chamber (Gretch-Ken Industries Inc.) and connected to the BioPac acquisition system (BIOPAC Systems, Inc) through a three-channel tether under brief isoflurane anesthesia. The tether was connected

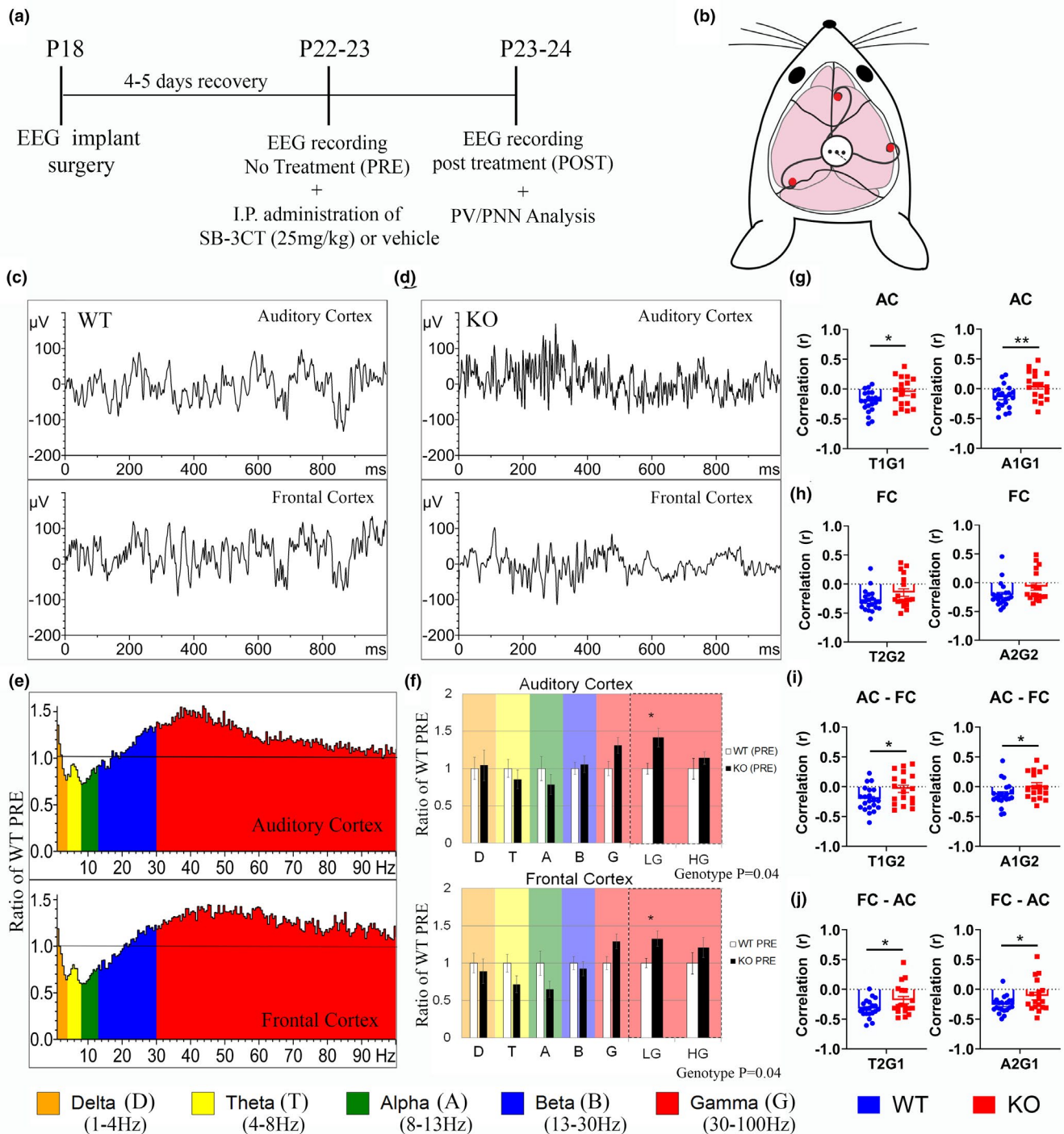
to a commutator located directly above the cage. Mice were then allowed to habituate to being connected to the tether for an additional 15 min before EEG recordings were obtained. During the recording session, a piezoelectric sensor placed under the floor of the cage detected mouse movement in the recording arena.

EEG activity was obtained using the BioPac system and Acqknowledge recording software from awake and freely moving mice. The BioPac MP150 acquisition system was connected to two EEG 100C amplifier units (one for each channel) to which the commutator was attached. The lead to the occipital cortex served as a reference for both frontal and auditory cortex screw electrodes. The acquisition hardware was set to high-pass (> 0.5 Hz) and low-pass (< 100 Hz) filters. Normal EEG output data were collected with gain maintained the same (10,000 X) between all recordings. Data were sampled at a rate of 2.5 kHz using Acqknowledge software and down sampled to 1,024 Hz post hoc using Analyzer 2.1 (Brain Vision LLC). Sound delivery was synchronized with EEG recording using a TTL pulse to mark the onset of each sound in a train. Resting EEGs were recorded for 5 min (during this time no auditory stimuli were presented) followed by recordings in response to auditory stimulation. After all EEG experiments were completed, mice were perfused for histology or used for behavioral tasks and Western blot analysis.

2.5 | Acoustic stimulation

Acoustic stimulation paradigms were similar as those previously used in *Fmr1* KO mice (Lovelace et al., 2018, 2019), which is analogous to work in humans with FXS. Acoustic stimuli were generated using RPDsEx software and RZ6 hardware (Tucker-Davis Technologies) and presented through a free-field speaker (MF1 Multi-Field Magnetic Speaker; Tucker-Davis Technologies) located 30 cm away directly above the cage. Sound pressure level (SPL) was modified using programmable attenuators in the RZ6 system. The speaker output was ~ 70 dB SPL at the floor of recording chamber with fluctuation of ± 3 dB for frequencies between 5 and 35 kHz as measured with a ¼ in Bruel and Kjaer microphone.

A chirp-modulated signal (henceforth 'chirp') to induce synchronized oscillations in EEG recordings was used to quantify fidelity of responses to time-varying stimuli. The chirp is a two-second broadband noise stimulus with amplitude modulated (100% modulation depth) by a sinusoid whose frequencies increase (Up-chirp) or decrease (Down-chirp) linearly in the 1–100 Hz range (Artieda et al., 2004; Perez-Alcazar et al., 2008; Purcell, John, Schneider, & Picton, 2004). The chirp facilitates a rapid measurement of transient oscillatory response (delta to gamma frequency range) to auditory stimuli of varying frequencies and can be used to compare oscillatory response in different groups in clinical and preclinical settings. Inter-trial phase coherence analysis (phase locking factor) can then be used to determine the ability of the neural generator to synchronize oscillations to the frequencies present in the stimulus (Tallon-Baudry, Bertrand, Delpuech, & Pernier, 1996). The chirp stimulus may be preferable over the traditional steady state stimulus



in studies of children with neurodevelopmental disorders, as it can quickly and efficiently measure multiple modulation frequencies in a shorter period of time.

To avoid onset responses contaminating phase locking to the amplitude modulation of the chirp, the stimulus was ramped in sound level from 0% to 100% over 1 s (rise time), which then smoothly transitioned into chirp modulation of the noise (see Figure 3c for example). Up- and Down-chirp trains were presented 300 times each (for a total of 600 trains). Both directions of modulation were tested to ensure any frequency-specific effects were

not due to the frequency transition history within the stimulus. Up- and Down-chirp trains were presented in an alternating sequence. The interval between each train was randomly varied between 1 and 1.5 s.

2.6 | Data analysis

All EEG data analyses were performed as described previously (Lovelace et al., 2018, 2019). Briefly, EEG data were extracted from

FIGURE 1 Developing *Fmr1* KO mice exhibit increased low gamma power. (a) Experimental timeline shows mice were implanted at P18 with two-channel EEG electrodes (WT, $N = 35$; *Fmr1* KO, $N = 30$) in the auditory cortex (AC) and the frontal cortex (FC), and with an occipital cortex reference electrode. Animals were allowed 4–5 days to recover post-surgery (WT, $N = 35$; *Fmr1* KO, $N = 30$; $N = 14$ (WT $N = 8$; KO $N = 6$) animals died post-surgery) and EEG recording was done prior to treatment (PRE) at P22–23 (WT, $N = 23$, excluded $N = 1$; *Fmr1* KO, $N = 21$, excluded $N = 1$). Immediately after the EEG recording, animals were given either an intraperitoneal injection of SB-3CT (25 mg/kg) or vehicle. At P23–24, a post-treatment EEG recording was performed. Brains were collected for PV/PNN analysis immediately after EEG recording. (b) Schematic shows placement of electrodes, which consisted of a three-channel electrode post with three 1-mm stainless steel screws (depicted by red dots). (c–j) EEG recording was done prior to treatment (PRE) at P22–23. Resting data (baseline, in the absence of auditory stimulation) were collected for 5 min and divided into 2-s segments for spectral analysis. Depicted are examples of EEG segments from individual WT (c) and *Fmr1* KO (d) mice, which include simultaneous recording from the AC and the FC. (e and f) Graphs show average spectral power ($\mu V^2/Hz$) in the AC and FC of *Fmr1* KO mice ($N = 20$) in a histogram (e) and bar graph format (f) expressed as the ratio of WT controls ($N = 22$) prior to treatment (PRE). A value of 1 indicates no mean difference in power between WT and *Fmr1* KO mice, while values above the black line indicate KO > WT, and below indicate KO < WT. (f) Statistical analysis was performed using a one-way MANCOVA with the percentage of time spent moving as a covariate. Genotype differences were compared using six spectral bands per region: Delta (D), Theta (T), Alpha (A), Beta (B), Low Gamma (LG), and High Gamma (HG). Since assumptions were not violated, we report an effect of genotype (AC: Pillai's Trace = 2.465, $p = .04$; FC: Pillai's Trace = 2.587, $p = .036$) across all six of the combined frequency variables. Low gamma was significantly increased after correction for multiple comparisons in the both the AC and FC: AC low gamma, $F(1,27) = 10$, $p = .003$, $\eta^2 = 0.204$, FC low gamma, $F(1,27) = 9.012$, $p = .005$, $\eta^2 = 0.188$ (WT $N = 22$, *Fmr1* KO $N = 20$). All graphs represent average values and the error bars indicate SEM. To determine whether there is a relationship between power across different frequencies and/or regions, a Pearson's correlation was done for resting baseline segments comparing *Fmr1* KO ($N = 21$, excluded $N = 3$) versus WT control mice ($N = 23$, excluded $N = 2$). (g) Graph shows Pearson's correlation (r) for each mouse for AC Theta/Low Gamma and AC Alpha/Low Gamma (WT, $N = 21$; *Fmr1* KO, $N = 18$; T1G1: $p = .02$; A1G1: $p = .008$). (h) Graph shows the Pearson's correlation (r) for FC Theta/Low Gamma and FC Alpha/Low Gamma. (i) Graph shows the Pearson's correlation (r) for AC Theta/FC Low Gamma and AC Alpha/FC Low Gamma (T1G2: $p = .03$; A1G2: $p = .04$). (j) Graph shows the Pearson's correlation (r) for the FC Theta/AC Low Gamma and FC Alpha/AC Low Gamma (T2G1: $p = .04$; A2G1: $p = .03$). For power coupling comparisons, animals that moved < 5% during the resting baseline were excluded from the analysis

Acqknowledge and converted to files compatible with Analyzer 2.1 software. All data were notched filtered at 60 Hz to remove residual line noise from recordings. Artifact rejection was performed using BrainVision Analyzer. Several criteria were used to search for artifacts including amplitude, gradient, max-min, and low activity. Less than 30% of data were rejected due to artifacts from any single mouse. If more than 30% of the data was rejected, the animal was excluded from analysis (Resting baseline: WT PRE, $N = 1$; *Fmr1* KO PRE, $N = 1$; vehicle-treated *Fmr1* KO, $N = 2$; vehicle-treated WT, $N = 2$; SB-3CT-treated *Fmr1* KO, $N = 1$; Chirp: *Fmr1* KO PRE, $N = 2$, vehicle-treated WT, $N = 1$; SB-3CT-treated WT, $N = 1$; *Fmr1* KO, $N = 2$, SB-3CT-treated *Fmr1* KO, $N = 1$). Notably, no outlier test was performed; instead, animals were excluded based on the exclusion criterion described above or movement percentage.

2.7 | Resting EEG analysis

Five minutes of EEG data (no auditory stimulus) were divided into 2-s segments and Fast Fourier Transforms (FFT) were calculated on each segment using 0.5 Hz bins, using a Hanning window, with no overlap, and then average power ($\mu V^2/Hz$) was calculated for each mouse from 1 to 100 Hz. Power was then further binned into standard frequency bands. We compared genotype mean differences on six bands per region: Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8–13 Hz), Beta (13–30 Hz), Low Gamma (30–55 Hz), and High Gamma (65–100 Hz). The gamma band (30–100 Hz) was divided into low (30–60 Hz) versus high (> 60 Hz) bands in gamma range because studies show that the lower gamma frequency rhythm may be associated with activity of PV cells (Balakrishnan & Pearce, 2014; Dvorak &

Fenton, 2014; Ray & Maunsell, 2011), whereas the higher gamma frequencies may be related to spiking activity near the electrodes (Buzsaki & Wang, 2012; Ray & Maunsell, 2011). The spectral bands used are based on a number of previous studies on FXS in both mice (Radwan, Dvorak, & Fenton, 2016) and humans (Ethridge et al., 2016, 2017; Wang et al., 2017).

Responses to chirp trains were analyzed using Morlet wavelet analysis. Chirp trains were segmented into windows of 500 ms before chirp onset to 500 ms after the end of the chirp sound (total of 3 s because each chirp was 2 s in duration). EEG traces were processed with Morlet wavelets from 1 to 100 Hz using complex number output (voltage density, $\mu V/Hz$) for Inter-Trial Phase Coherence (ITPC) calculations, and power density ($\mu V^2/Hz$) for non-phase locked single trial power (STP). Wavelets were run with a Morlet parameter of 10 as this gave the best frequency/power discrimination. This parameter was chosen since studies in humans found most robust difference around 40 Hz, where this parameter is centered (Ethridge et al., 2017). To measure phase synchronization at each frequency across trials, Inter-Trial Phase Coherence (ITPC) was calculated. The equation used to calculate ITPC is as follows:

$$ITPC(f,t) = \frac{1}{n} \sum_{k=1}^n \frac{F_k(f,t)}{|F_k(f,t)|}$$

where f is the frequency, t is the time point, and k is the trial number. Thus, $F_k(f, t)$ refers to the complex wavelet coefficient at a given frequency and time for the k th trial. There were no < 225 trials (out of 300) for any given mouse after segments containing artifacts were rejected.

2.8 | Statistical analysis and definition of movement states

All statistical analysis was performed as described previously (Lovelace et al., 2018, 2019). Statistical group comparisons of chirp responses (ITPC and STP) were quantified by wavelet analysis using MATLAB (MATLAB, RRID:SCR_001622). The analysis was conducted by binning time (2 s segments) into 256 parts and frequency into 100 parts, resulting in a 100×256 matrix. Non-parametric cluster analysis was used to determine contiguous regions in the matrix that were significantly different from a distribution of 2000 randomized Monte Carlo permutations based on previously published methods (Maris & Oostenveld, 2007). Briefly, if the cluster size of the real genotype assignments (both positive and negative direction, resulting in a two-tailed alpha of $p = .025$) were larger than 97.25% of the random group assignments, those clusters were considered significantly different between genotypes. This method avoids statistical assumptions about the data and corrects for multiple comparisons. In all cases, $p < .05$ was considered significant for ANOVA and Student's *t* tests. Where *t* tests were performed, *r* was calculated as an effect size. When interactions were found and multiple comparisons for ANOVA were made, data were analyzed on each factor for simple effects and corrected using Bonferroni adjustments. If assumptions of sphericity were violated for repeated measures ANOVA, the Greenhouse–Geisser correction was used.

As movement can alter cortical gain (Niell & Stryker, 2010), and *Fmr1* KO mice show hyperactivity, a piezoelectric transducer was placed under the EEG recording arena to detect when the mouse was moving. The term 'resting' is used to indicate EEGs recorded in these mice without any specific auditory stimuli. The term 'still' is used to describe resting EEG when the mouse was stationary. The term 'moving' is used to describe resting EEG when the mouse was moving based on a threshold criterion for the piezoelectric signal that was confirmed by analyzing the video recording (under IR light) that was taken throughout the EEG recording procedure. In all cases where genotype means are reported, standard error of the mean (SEM) was used. The genotype differences in resting power were analyzed on six dependent variables using one-way Multivariate analysis of co-variance (MANCOVA) with one covariate (movement), independent variables (IV): genotype (*Fmr1* KO, WT), dependent variables (DV): six frequency bins (delta to high gamma). The proportion of time spent moving during the 5-min recording session was used as a covariate to isolate effects of genotype and control for the effect movement has on cortical gain. When multiple comparisons for MANCOVA were made, genotype comparisons were corrected using Bonferroni adjustments. The divisor for Bonferroni correction for multiple comparisons (for six frequency bands) on MANCOVA was set to 6, $\alpha = 0.05/6 = 0.0083$. Data are often expressed and plotted as ratio of control group values to gauge relative differences in various factors using the same scale. Statistical analysis was done using SPSS software package (SPSS, RRID:SCR_002865).

2.9 | Treatment

The matrix metalloproteinase-2/9 inhibitor, SB-3CT, in saline containing 10% DMSO (Millipore, Cat. 444274), or vehicle (10% DMSO in saline) was administered intraperitoneally (i.p., 25 mg/kg) to WT and *Fmr1* KO mice at P22–23 (for timeline, see Figure 1a). For treatment, animals were assigned based on simple randomization. To blind experimenter to treatment, animals were given a numerical identifier with no treatment identification that was used during EEG recording and PV/PNN analysis. A second group of mice that did not undergo EEG surgery or recording received a single treatment of SB-3CT (25 mg/kg) or vehicle injections at P27–28 and were subjected to behavioral tests at P28–29 (Figure 6a). To blind experimenter to treatments during behavior, tests were performed by two experimenters, one experimenter administered treatment and second experimenter was blinded to assigned treatment of each mouse. A third group that did not undergo EEG surgery or recording received a single treatment of SB-3CT (25 mg/kg) or vehicle injections at P21–22 (Figure 7a). To blind experimenter to treatments, one experimenter prepared drugs and assigned a numerical identifier to each treatment/animal and a second experimenter administered the treatment and was blinded to each assigned treatment. Initial number of animals used per group: EEG implant surgery (WT, $N = 35$; *Fmr1* KO, $N = 30$), EEG recording (WT PRE, $N = 23$; *Fmr1* KO PRE, $N = 21$; vehicle-treated WT, $N = 18$; SB-3CT-treated WT, $N = 15$; vehicle-treated *Fmr1* KO, $N = 14$; SB-3CT-treated *Fmr1* KO, $N = 13$), PV/PNN analysis ($N = 6$ per group; vehicle-treated WT, $n = 18$; SB-3CT-treated WT, $n = 23$; vehicle-treated *Fmr1* KO, $n = 20$; SB-3CT-treated *Fmr1* KO, $n = 17$), behavior (vehicle-treated *Fmr1* KO, $N = 10$; SB-3CT-treated *Fmr1* KO, $N = 9$; vehicle-treated WT, $N = 10$; SB-3CT-treated WT, $N = 8$), Western blot (vehicle-treated *Fmr1* KO, $N = 4$; SB-3CT-treated *Fmr1* KO, $N = 4$; vehicle-treated WT, $N = 4$; SB-3CT-treated WT, $N = 4$), and DQ gelatin assay (vehicle-treated *Fmr1* KO, $N = 5$; SB-3CT-treated *Fmr1* KO, $N = 5$; vehicle-treated WT, $N = 3$; SB-3CT-treated WT, $N = 3$).

2.10 | Dye-quenched (DQ) gelatin assay and analysis

The dye-quenched (DQ) gelatinase activity assay is a hydrolytic activity assay used to measure MMP-2/9 activity. The DQ gelatin assay measures MMP-2/9 activity by measuring the fluorescent intensity of a FITC-quenched gelatin peptide that fluoresces following cleavage by MMP-2 or MMP-9 to measure MMP-2/MMP-9 proteolytic activity (Vandooren et al., 2011). To confirm that i.p. administration of SB-3CT results in the inhibition of MMP-9 activity in the brain, 1 hr after treatment with SB-3CT inhibitor or vehicle P22 WT mice ($N = 3$ per group) were euthanized with isoflurane and the auditory cortex and surrounding temporal cortex (average weight of tissue ~20 mg) were dissected based on coordinates (Paxinos and Franklin 2004) and previous electrophysiological and dye-placement studies (Martin del Campo, Measor, & Razak, 2012). For an example of

auditory cortex dissection, please see Figure S2–1. The 1-hr time point was chosen based on the ~ 45 min half-life of SB-3CT (Gooyit et al., 2012). The AC was resuspended in lysis buffer (50mM Tris-HCL [pH = 7.4] buffer containing 150mM NaCl, 5mM EDTA, 0.05% Triton X-100, 1mM PMSF) containing protease inhibitor cocktail (Sigma-Aldrich, cat. #P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, cat. #P0044). Lysates were measured for total protein concentrations using the protocol for the BCA colorimetric protein assay (Pierce, cat. #23-235).

Lysates were diluted in reaction buffer and mixed with DQ-gelatin stock, a fluorescence-labeled gelatin substrate (Molecular Probes, E12055) as described previously (Lovelace et al., 2019). Lysates were coded and processed by experimenter blinded to codes. Fluorescence intensity was analyzed using 495 nm excitation wavelength and 515 nm emission wavelength in a fluorescence microplate reader equipped with standard fluorescein filters (SpectraMax M2, Molecular Devices). The signal from the samples was measured every 20 min during a 3-hr incubation period. For each time point, background fluorescence intensity was corrected by subtracting the values derived from the blank, which consisted of only reaction buffer and DQ gelatin. A standard curve to assess gelatinase activity was generated using recombinant mouse MMP-9 activity (rmMMP-9, specific activity approx. 1,500 pmol min⁻¹ µg⁻¹, R&D Systems, Cat. # 909-MM-010). A linear regression of rmMMP-9 activity (standard curve) and relative gelatinase activity based on the average fluorescence intensity of three replicates was used to assess gelatinase proteolytic activity in the lysates.

2.11 | Immunohistochemistry

For histology, mice were euthanized with isoflurane and perfused transcardially with ice-cold phosphate-buffered saline (0.1 M PBS, pH 7.4) and 4% paraformaldehyde (PFA). Brains were sectioned in the coronal plane on a Vibratome (Leica S1000) at 100 µm and sections were stored in PBS before staining. Prior to immunostaining, free-floating sections were post-fixed for an additional 2 hr in 4% PFA and then washed in 0.1M PBS. Sections were then quenched with 50mM ammonium chloride for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked for 1 hr at 20–22°C in 5% donkey serum (DS; Sigma-Aldrich Cat# D9663, RRID:AB_2810235) and 1% bovine serum albumin (BSA; Fisher Scientific, Catalog #9048468) in 0.1 M PBS. Sections were incubated overnight in primary antibodies and a 1:500 dilution of fluorescein-tagged *Wisteria floribunda* Lectin (4 µg/ml WFA, Vector Laboratories Cat# FL-1351, RRID: AB_2336875) in a 1% DS, 0.5% BSA, and 0.1% Tween-20 solution. Primary antibodies included a 1:1,000 dilution of mouse anti-PV antibody (Sigma-Aldrich Cat#P3088, RRID:AB_477329) to label PV interneurons. Sections were incubated in a 1:500 dilution of donkey anti-mouse Alexa Fluor 594 for 1 hr. After immunostaining, sections were mounted on slides and cover slipped with Vectashield containing DAPI (Vector Laboratories Cat#H-1200, RRID:AB_2336790) and Cytoseal (ThermoScientific, Catalog #8310-16).

2.12 | Image quantification

Slices were imaged by confocal microscopy (model LSM 510, Carl Zeiss MicroImaging or Leica SP5) using a series of 10 high-resolution optical sections (1,024 × 1,024 pixel format) that were captured for each slice at 10 × magnification at 1µm step intervals (z-stack). All images were acquired under identical conditions. Each z-stack was collapsed into a single image by projection, converted to a TIFF file, and encoded for blind analysis using ImageJ (NIH software, ImageJ, RRID:SCR_003070). The AC was identified using hippocampal landmarks (Figure S2–1). This method has been previously validated using tonotopic mapping and dye injection (Martin del Campo et al., 2012) and comparison with the Paxinos mouse atlas and other publications on the mouse AC (Anderson, Christianson, & Linden, 2009). Nevertheless, the precise boundary between primary AC (A1) and anterior auditory field (AAF) of the mouse AC cannot be clearly established. Both these fields are part of the lemniscal auditory system and comprise the core AC. Therefore, we use the AC to include both A1 and AAF. ImageJ was used to identify and count PNN-positive, PV-positive cells, and PV/PNN co-localization ($N = 6$ per group; vehicle-treated WT, $n = 18$; SB-3CT-treated WT, $n = 23$; vehicle-treated *Fmr1* KO, $n = 20$; SB-3CT-treated *Fmr1* KO, $n = 17$). Cortical layers were identified (Anderson et al., 2009) and used for layer-specific counts (Layers 1–5/6 of the AC). A selection tool was used to measure and specify layers of the AC and the point tool was used to label PNNs and PV cells and added to the ROI manager.

2.13 | Behavior

2.13.1 | Open-field test

Behavioral tests were performed as described previously (Lovelace et al., 2019; Sidhu et al., 2014). Briefly, anxiety-like behavior was tested in P27–28 mice ($N = 6–7$ per group) by quantifying their tendency to travel to the center of an open field and time spent in thigmotaxis (Yan, Asafo-Adjei, Arnold, Brown, & Bauchwitz, 2004; Yan, Rammal, Tranfaglia, & Bauchwitz, 2005). A 43 × 43 cm open-field arena with 43-cm-high walls was constructed from clear acrylic sheets. The open-field arena was placed in a brightly lit room, and one mouse at a time was placed in a corner of the open field and allowed to explore for 10 min while being recorded with digital video from above. The floor was cleaned with 3% acetic acid, 70% ethanol and water between tests to eliminate odor trails. The mice were tested between the hours of 8:00 a.m. and 1:00 p.m., and this test was always performed prior to the elevated plus maze by the same experimenter. The arena was subdivided into a 4 × 4 grid of squares with the middle of the grid defined as the center. A line 4 cm from each wall was added to measure thigmotaxis. Locomotor activity was scored by the analysis of total line crosses and speed as described previously with some modifications (Yan et al., 2005) using TopScan Lite Software (Clever Sys., Inc.). A tendency to travel to the center (total number of entries into large and small center squares) and the

time in thigmotaxis were used as an indicator of anxiety-like behaviors using TopScan Lite software (CleverSys Inc). The analysis was performed in 5-min intervals for the total 10-min exploration duration. Assessments of the digital recordings were performed blind to the condition. Statistical analysis was performed with unpaired *t* test using GraphPad Prism 6 software. Data represent mean $\pm$ standard error of the mean (SEM).

2.13.2 | Elevated plus maze

The elevated plus maze consisted of four arms in a plus configuration. Two opposing arms had 15-cm tall walls (closed arms), and two arms were without walls (open arms). The entire maze sat on a stand 1 m above the floor. Each arm measured 30 cm long and 10 cm wide. Mice were allowed to explore the maze for 10 min while being recorded by digital video from above. The maze was wiped with 3% acetic acid, 70% ethanol and water between each test to eliminate odor trails. This test was always done following the open-field test. TopScan Lite software was used to measure the percent of time spent in open arms and speed. The time spent in open arm was used to evaluate anxiety-like behavior. The velocity and total arm entries were measured to evaluate overall locomotor activity. The analysis was performed in 5-min intervals for the total 10-min exploration duration. Assessments of the digital recordings were done blind to the condition using TopScan Lite software. Statistical analysis was performed with unpaired *t* test using GraphPad Prism 6 software. Data represent mean $\pm$ standard error of the mean (SEM).

2.14 | Western blot analysis

Western blot analysis was performed as described previously (Lovelace et al., 2019). The AC was removed from each mouse ($N = 3$ –4 mice per group), cooled in PBS, and homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100, and 1 mM PMSF) containing protease inhibitor cocktail (Sigma-Aldrich, cat. # P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich, cat. #P0044). The samples were rotated at 4°C for at least 1 hr to allow for complete cell lysis and then cleared by centrifugation at 10,000 *g* for 15 min at 4°C. Supernatants were isolated and boiled in reducing sample buffer (Sigma-Aldrich, Laemmli 2 $\times$ concentrate, S3401), and separated on 8%–16% Tris-Glycine SDS-PAGE precast gels (EC6045BOX, Life Technologies). Proteins were transferred onto Protran BA 85 Nitrocellulose membrane (GE Healthcare) and blocked for 1 hr at 20–22°C in 5% skim milk (catalog #170-6404, Bio-Rad). Primary antibody incubations were performed overnight at 4°C with antibodies diluted in TBS/0.1% Tween-20/5% BSA. The following primary antibodies were used: rabbit anti-Akt (Cell Signaling Technology Cat# 9272, RRID:AB_329827); rabbit anti-phospho-Akt (Ser473; Cell Signaling Technology Cat# 9271, RRID: AB_329825); mouse anti-PV (Millipore, Cat#MAB1572, RRID:AB_2174013), mouse anti-total TrkB (1:2000; BD Biosciences

Cat# 610101, RRID:AB_397507), rabbit anti-phospho-TrkB (Tyr515) (1:1,000, Bioworld, catalog #AP0236), and rabbit anti- β -actin at 1:2000 (Abcam Cat #ab8227, RRID:AB_2305186). All primary antibodies were from Cell Signaling Technology and used at a dilution of 1:1,000, unless stated otherwise.

Blots were washed 3 $\times$ 10 min with TBS/0.1% Tween-20 and incubated with the appropriate HRP-conjugated secondary antibodies for 1 hr at 20–22°C in a TBS/0.1% Tween-20/5% BSA solution. The secondary antibodies used were HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Labs Cat#715-035-150, RRID:AB_2340770) or HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Labs Cat# 111-035-003, RRID:AB_2313567). After secondary antibody incubations, blots were washed 3 $\times$ 10 min in TBS/0.1% Tween-20, incubated in ECL 2 Western Blotting Substrate (Thermo Scientific, catalog #80196) and a signal was collected with CL-XPosure film (Thermo Scientific, catalog #34090). For re-probing, membrane blots were washed in stripping buffer (2% SDS, 100 mM β -mercaptoethanol, 50mM Tris-HCl, pH 6.8) for 30 min at 55°C, then rinsed repeatedly with TBS/0.1% Tween-20, finally blocked with 5% skim milk, and then re-probed. Developed films were then scanned, and band density was analyzed by measuring band and background intensity using Adobe Photoshop CS5.1 software (Adobe Photoshop, RRID:SCR_014199). Four to five samples per group of vehicle versus. SB-3CT WT versus. *Fmr1* KO were run per blot. Statistical analysis was performed with unpaired *t* test using GraphPad Prism 6 software (GraphPad Prism, RRID:SCR_002798). Data represent mean $\pm$ standard error of the mean (SEM).

2.15 | Statistical analysis

All statistical analyses for cell density quantification, Western blot, behavior, and DQ gelatin assay were done using Prism (GraphPad Software). For cell density quantification, theta/gamma ratio, and behavior, two-way ANOVA was used to compare Genotype (WT $\times$ KO) versus. Treatment (SB-3CT $\times$ vehicle) followed by *post hoc* pairwise comparisons with the Bonferroni's or Tukey's correction, unless otherwise specified. For Western blot and the DQ gelatin assay, the Student's *t* test was used to assess the effects of treatment in KO or WT mice. For all analyses, 'N' = number of animals and 'n' = number of coronal sections. No power analysis was performed to calculate sample size for each experiment. The number of animals for each study was determined using previously published studies using similar techniques [EEG (Lovelace et al., 2018; Wen, Lovelace, Ethell, Binder, & Razak, 2019), PV/PNN analysis (Lovelace et al., 2019; Wen et al., 2018), behavior, and DQ gelatin (Lovelace et al., 2019)].

3 | RESULTS

In the current study, we examined whether acute pharmacological inhibition of MMP-9 activity during a developmental critical period reduces auditory hypersensitivity in developing *Fmr1* KO mice. First,

we evaluated changes in spectral power and synchronization to auditory stimuli using EEG, as well as changes in PV/PNN density. Next, we assessed anxiety-like behaviors and hyperactivity along with changes in key molecular targets associated with FXS, including PV levels and phosphorylation of TrkB and Akt.

3.1 | Developing *Fmr1* KO mice exhibit increased resting state gamma EEG power and abnormal EEG power coupling

Previously, we have shown that adult *Fmr1* KO mice exhibit increased resting state gamma power (Lovelace et al., 2018), a phenotype that is similar to that observed in humans with FXS (Ethridge et al., 2017; Wang et al., 2017). Here, we assessed whether developing *Fmr1* KO mice on C57BL/6 background (for FVB background, see [Wen et al., 2019]) also exhibit increased resting state gamma power during the P21–28 period when maturation of various neuronal circuits occurs. To do this, mice were implanted with two-channel EEG electrodes placed in the auditory (AC) and frontal cortex (FC) at P18 (Figure 1a and b), allowed to recover for 4–5 days, and baseline EEG activity was recorded at P22 prior to treatment (PRE). Resting EEG data (in the absence of auditory stimulation) were collected for 5 min and were divided into 2-s segments for spectral analysis. Resting EEG raw power ($\mu V^2/Hz$) was calculated in the AC and FC of WT and *Fmr1* KO mice by analyzing all frequency bands during the entire 5-min resting period. Examples of one-second segments of EEG traces for individual WT and *Fmr1* KO mice are shown in Figure 1c and d, respectively (AC: top; FC: bottom). The group average power spectral densities for the AC and the FC are shown in histogram (Figure 1e) and bar graph format (Figure 1f), respectively. To normalize data for each region and to determine relative changes in power across frequency bands, power values were expressed as a ratio of *Fmr1* KO ($N = 20$) to WT ($N = 22$) means (for non-normalized data, see Figure S1–1). A value of 1 indicates no mean difference in power between WT and *Fmr1* KO, whereas values above 1 indicate *Fmr1* KO > WT, and below indicate *Fmr1* KO < WT mice.

Statistical analysis was performed using a one-way MANCOVA with the percentage of time spent moving as a covariate. We compared genotype mean differences on six bands per region: Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8–13 Hz), Beta (13–30 Hz), Low Gamma (30–55 Hz), and High Gamma (65–100 Hz). Frequencies from 55 to 65 Hz were excluded in all analysis, as a 60 Hz notch filter was utilized to eliminate line noise. We confirmed assumptions of equality of covariance using Levene's test of equality of error variance and no differences between genotypes were observed in error variance (all $p > .05$). Since assumptions were not violated, we report an effect of genotype (AC: Pillai's Trace = 2.465, $p = .04$; FC: Pillai's Trace = 2.587, $p = .036$) across all six of the combined frequency variables. The low gamma band was the only frequency band significantly different between genotypes in the AC and FC (Figure 1f; AC: low gamma, $F(1,27) = 10$, $p = .003$, $\eta^2 = 0.204$; FC: low gamma, $F(1,27) = 9.012$, $p = .005$, $\eta^2 = 0.188$; WT $N = 22$, *Fmr1* KO $N = 20$;

for all frequency averages, see Table S1). These results indicate that *Fmr1* KO mice exhibit increased resting state low gamma power (30–55 Hz) early in development at P22–23.

Human EEG studies also report power abnormalities in delta, theta, alpha frequencies, and impaired coupling between low- and high-frequency oscillations in individuals with FXS and ASD (Sinclair et al., 2017; Wang et al., 2013). To determine whether there are abnormalities in power coupling in early development, a Pearson's correlation was done during the resting baseline period to assess the relationship between power across different frequencies and/or regions, using the same approach that was used in a study for human FXS (Wang et al., 2013). Results show a negative correlation in power coupling of the AC Theta/Low Gamma and AC Alpha/Low Gamma in WT mice (Figure 1g). These correlations were significantly reduced in *Fmr1* KO mice (T1G1: $p = .02$; A1G1: $p = .008$). Results also show a negative correlation in across region power coupling of the AC Theta/FC Low Gamma and AC Alpha/FC Low Gamma in WT mice (Figure 1i), and FC Theta/AC Low Gamma and FC Alpha/AC Low Gamma (Figure 1j). These correlations were also significantly reduced in *Fmr1* KO mice (T1G2: $p = .03$; A1G2: $p = .04$; T2G1: $p = .04$; A2G1: $p = .03$).

Overall, these findings indicate that abnormalities in resting gamma power and EEG power coupling, in particular changes in theta/low gamma and alpha/low gamma power coupling in the AC and cross regional impairments, are present in developing *Fmr1* KO mice as early as P22–23.

3.2 | *Fmr1* KO mice treated with MMP-9 inhibitor show no effect on resting state EEG gamma power and EEG power coupling

Next, both *Fmr1* KO and WT mice received a single i.p. injection of the MMP-2/9 inhibitor, SB-3CT (25 mg/kg), or vehicle immediately following EEG recording to assess the efficacy of acute MMP-9 inhibition to normalize resting EEG oscillation pattern (Figure 1a). One day after treatment, resting EEG baseline data were again collected for 5 min and was divided into 2-s segments for spectral analysis. The percentage of time that the mice spent moving during the recording period was not different between genotypes (Figure S1–2) or treatment groups (Figure S2–7). Figure 2 shows the average spectral power as a ratio of the corresponding control group in histogram (Figure 2a–c) and bar graph format (Figure 2d–f) for each group (AC: top; FC: bottom; for non-normalized data see Figure S2–2). To determine resting EEG in vehicle groups, vehicle-treated *Fmr1* KO mice were compared to vehicle-treated WT mice. Although resting state low (LG) and high gamma (HG) power was trending higher in the AC of vehicle-treated *Fmr1* KO ($N = 12$) compared to vehicle-treated WT ($N = 16$) mice, this was not statistically significant possibly due to large variability in vehicle-injected *Fmr1* KO mice (Figure 2d; also see Figure S2–4). Although SB-3CT-treated *Fmr1* KO ($N = 12$) mice exhibited normal low and high gamma in the AC and FC that was comparable to SB-3CT-treated WT ($N = 15$) mice (Figure 2e), comparison of SB-3CT-treated *Fmr1* KO ($N = 12$) mice to vehicle-treated *Fmr1* KO ($N = 12$) mice showed

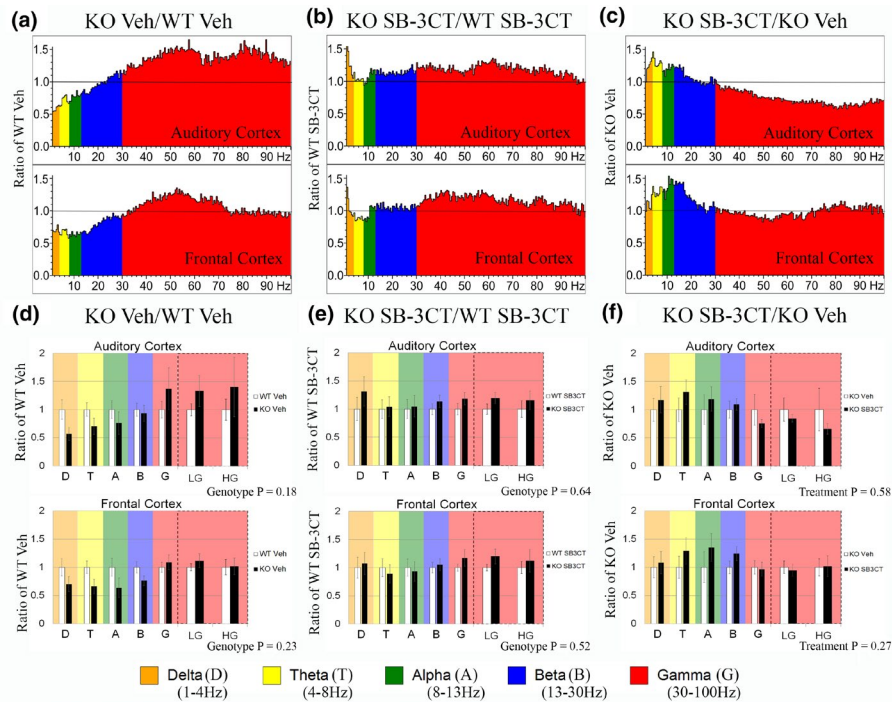


FIGURE 2 Effects of acute inhibition of MMP-9 activity on resting EEG power in *Fmr1* KO mice. Five minutes of resting EEG activity was recorded from WT and *Fmr1* KO mice post-treatment at P23-24 (from treatment groups, $N = 2$ from vehicle-treated WT and *Fmr1* KO groups, respectively, and $N = 1$ from the SB-3CT-treated *Fmr1* KO group was excluded from resting baseline EEG analysis). FFT calculated spectral power ($\mu V^2/Hz$) for each group is shown as a ratio of a corresponding control group. (a and d) Average power in the AC (top) and FC (bottom) of vehicle-treated *Fmr1* KO ($N = 14$, excluded $N = 2$) compared to vehicle-treated WT ($N = 18$, excluded $N = 2$). (b and e) Average power in the AC and FC of SB-3CT-treated *Fmr1* KO ($N = 13$, excluded $N = 1$) compared to SB-3CT-treated WT ($N = 15$, no excluded animals). (c and f) Average power of SB-3CT-treated *Fmr1* KO compared to vehicle-treated *Fmr1* KO. All graphs represent average values and the error bars indicate SEM. Delta (D), Theta (T), Alpha (A), Beta (B), Low Gamma (LG), and High Gamma (HG)

a trending reduction in low and high gamma power, but this was not statistically significant (Figure 2f; for summary of all frequency averages, see Table S2). Vehicle-treated WT mice ($N = 16$) and SB-3CT-treated WT mice ($N = 15$; for non-normalized data, see Figure S2-3) did not show any significant changes in spectral frequencies compared to WT mice before treatment (PRE) in AC or FC (Figure S2-5a and 2-5d [Vehicle-WT] and Figure S2-5b and 2-5e [SB-3CT-WT]).

To determine whether EEG power coupling abnormalities were ameliorated following treatment with SB-3CT, a Pearson's correlation was done during the resting baseline period to assess the relationship between power across different frequencies and/or regions. Results show that treatment with SB-3CT did not alter the EEG power coupling profiles in WT or *Fmr1* KO mice (Figure S2-6).

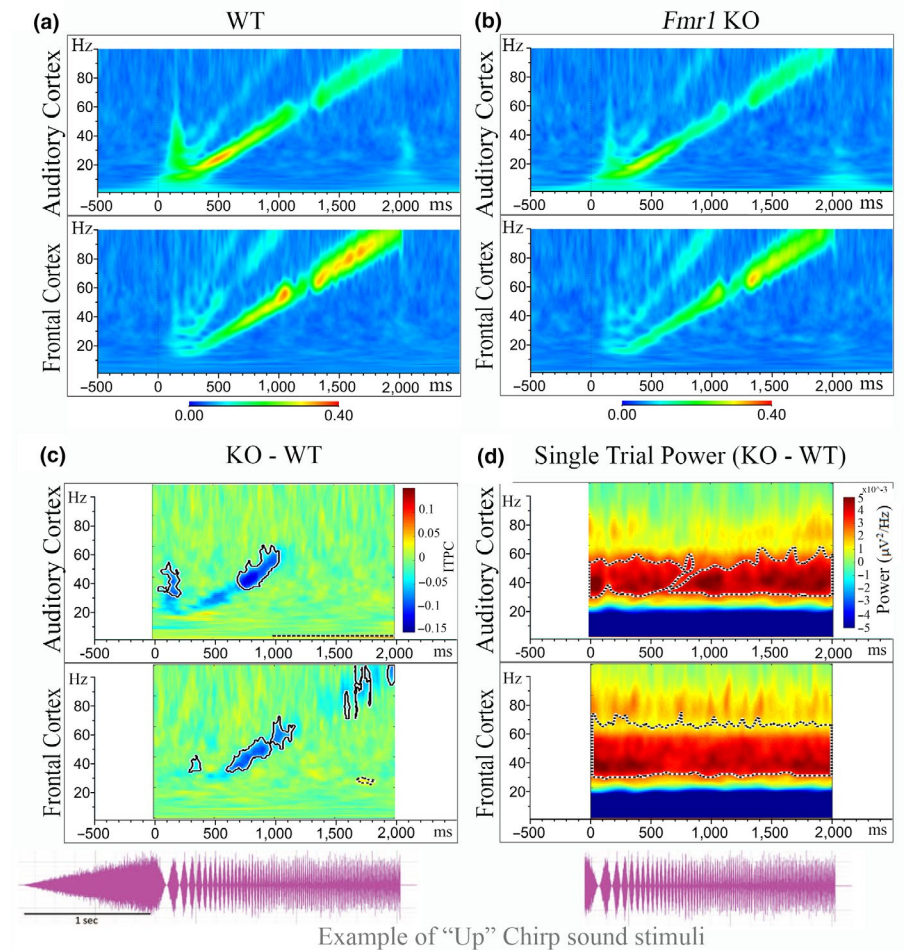
Together, these results indicate no significant effects of treatment in *Fmr1* KO mice when resting EEG power or EEG power coupling was analyzed for individual frequencies between vehicle- and SB3CT-treated groups.

3.3 | Phase locking to auditory 'up chirp' stimuli is impaired in developing *Fmr1* KO mice

To assess whether P22-23 *Fmr1* KO mice also exhibit deficits in the consistency of phase locking to time-varying stimuli as seen in adult

Fmr1 KO mice (Lovelace et al., 2018), and humans with FXS (Ethridge et al., 2017), we recorded responses to chirp stimuli. We tested both Up- and Down-chirp stimuli to ensure that the differences are specific to modulation frequencies and are not affected by the direction of frequency change in the sound (Lovelace et al., 2019). Following EEG resting state baseline recording, repeated chirp stimuli (300 trials for up, 300 trials for down) was delivered and electrocortical activity was recorded. Inter-trial phase coherence (ITPC) was calculated across trials in the time $\times$ frequency domain using Morlet Wavelet analysis (Lovelace et al., 2018). After grand average ITPC was calculated for each group, we found EEG oscillations that matched the frequency of the chirp and were seen as increased ITPC along a diagonal, from 0 to 2 s and 1-100 Hz. The means for WT PRE (Figure 3a; only 'up chirp' data are shown) were subtracted from *Fmr1* KO PRE mice (Figure 3b) to show the difference between *Fmr1* KO and WT (Figure 3c) in the AC (top) and FC (bottom). For statistical analysis, clusters of p values were calculated, and significant differences ($p < .025$) were overlaid on the chirp response to demonstrate quantitative differences between each genotype after correction for multiple comparisons (Lovelace et al., 2019). We observed a statistical difference between genotypes in one main cluster, which spans the low gamma band ITPC in both the AC (35-55 Hz) and FC (35-65 Hz); significant clusters are highlighted by bold-lined contours in Figure 3c. In the FC, there was also a statistical difference between genotypes in the high gamma band in the FC

FIGURE 3 *Fmr1* KO mice are deficient in phase locking to auditory 'UpChirp' stimuli. (a and b) Inter-Trial Phase Coherence (ITPC: ability to synchronize oscillations to stimulus frequencies) grand average for WT ($N = 23$) and *Fmr1* KO ($N = 19$) mice in the AC (top) and FC (bottom). EEG oscillations matched the frequency of the chirp and were seen as increased ITPC along a diagonal, from 0 to 2 s and 1–100 Hz. (c) ITPC means for WT PRE were subtracted from *Fmr1* KO PRE mice to show the difference between genotypes. Blue areas indicating $KO < WT$, green areas no difference, and yellow/red $KO > WT$. Significant clusters ($p < .025$) are highlighted by bold-lined contours. (d) Graph shows difference in grand average single trial power (STP) or ongoing 'background' power during auditory stimulation between WT PRE and *Fmr1* KO mice in AC (top) and FC (bottom)



(80–100 Hz). Similar patterns of statistics of ITPC were observed for both up and down chirps (down chirp data not shown).

To examine whether P22–23 *Fmr1* KO mice also exhibit an increase in background gamma power in both the AC and FC during the chirp presentation, similar to adult *Fmr1* KO mice (Lovelace et al., 2018), we assessed non-phase locked single trial power (STP) during chirp stimulation in *Fmr1* KO mice compared to WT control mice. Using the same statistical cluster analysis as for the chirp ITPC, results indicate that P22–23 *Fmr1* KO mice exhibit a significant increase in background gamma power in the AC (~30–55 Hz) and FC (~30–70 Hz) compared to WT control mice (Figure 3d).

Together, these data show that developing *Fmr1* KO mice exhibit low and high gamma synchronization deficits and increased background gamma power in both the AC and FC in a manner similar to adult *Fmr1* KO mice and humans with FXS.

3.4 | Acute MMP-9 inhibition improves phase-locking to time-varying auditory stimuli in *Fmr1* KO mice

To test whether acute treatment of SB-3CT would ameliorate the ITPC deficit to the auditory chirp stimuli, both up and down chirps were presented during EEG recording 1 day after treatment. Figure 4a and b shows the grand average ITPC values for

vehicle-treated and SB-3CT-treated *Fmr1* KO, respectively. The mean for vehicle-treated *Fmr1* KO mice was subtracted from vehicle-treated WT mice (Figure 4c) to show the difference between ITPC values in *Fmr1* KO and WT mice in both the AC and FC. Similarly, the mean for SB-3CT-treated *Fmr1* KO mice was subtracted from the mean of vehicle-treated *Fmr1* KO mice (Figure 4d). For statistical analysis, clusters of p values were calculated, and these differences were overlaid on the chirp response to demonstrate quantitative differences between each genotype after correction for multiple comparisons. Monte Carlo statistical method on cluster analysis revealed a significant reduction in average ITPC values between vehicle-treated *Fmr1* KO ($N = 12$) mice and vehicle-treated WT ($N = 17$) mice in low gamma band range (~40–50 Hz) in the AC (Figure 4c: top), consistent with the reduction observed in un-treated mice (Figure 3d). Statistical analysis of average ITPC values shows a significant enhancement in the same low gamma band range (~40–50 Hz) ITPC in the AC of SB-3CT-treated *Fmr1* KO mice ($N = 12$) to vehicle-treated *Fmr1* KO ($N = 12$) mice (Figure 4d: top). However, a significant decrease in ~30–40 Hz gamma band range ITPC band in both AC and FC was observed (time $\times$ frequency bands that are significantly different between groups are highlighted in bolded black lines). Grand average ITPC values were also calculated for vehicle-treated ($N = 17$) and SB-3CT-treated WT ($N = 14$) mice (Figure S4-1a and S4-1b,

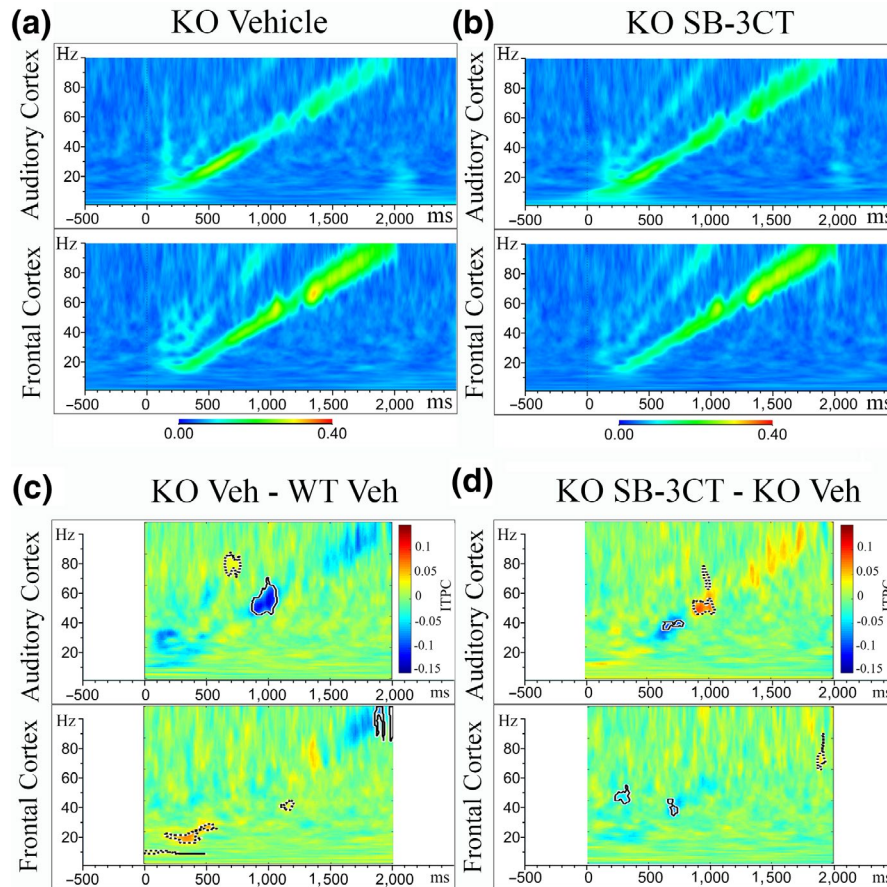


FIGURE 4 Acute SB-3CT treatment improves Inter-Trial Phase Coherence (ITPC) to auditory 'Up Chirp' stimuli in *Fmr1* KO mice. (a and b) Grand average ITPC in AC and FC of vehicle-treated *Fmr1* KO (a, $N = 12$) and SB-3CT-treated *Fmr1* KO (b, $N = 12$). Warmer colors, yellow/red, represent high ITPC values, while cooler colors, blue/green, represent low ITPC values. Significant clusters ($p < .025$) are highlighted by bold-lined contours. (c) Graph shows the difference in ITPC values between vehicle-treated *Fmr1* KO ($N = 12$) and vehicle-treated WT ($N = 17$). (d) Graph shows the difference in ITPC values between SB-3CT-treated *Fmr1* KO ($N = 12$) and vehicle-treated *Fmr1* KO ($N = 12$)

respectively). No significant differences were detected in AC or FC for average ITPC values between vehicle-treated WT ($N = 17$) mice and WT PRE ($N = 23$) mice in the low and high gamma ITPC band except for a small decrease in the FC at ~ 25 – 35 Hz (Figure S4-1c), suggesting the effects of injection on the ~ 25 – 35 Hz gamma band range. SB-3CT-treated WT ($N = 14$) mice exhibited a significant increase in high gamma band ITPC in the AC, but not FC, specifically in 70–85 Hz ITPC band compared to WT PRE (Figure S4-1d). Similar patterns and statistics of ITPC were observed for both up and down chirps. These results indicate that an acute treatment of the MMP-9 inhibitor, SB-3CT, improves fidelity of temporal responses to auditory stimuli by specifically increasing low gamma band (40–50 Hz) ITPC in developing *Fmr1* KO mouse cortex.

3.5 | Acute inhibition of MMP-9 enhances PNN formation in the developing auditory cortex of *Fmr1* KO mice

Previously, we showed evidence for delayed development of PV interneurons and PNN formation around PV interneurons in layers

2–4 of the developing AC of *Fmr1* KO mice (Wen et al., 2018). Here, we examined whether acute pharmacological inhibition of MMP-9 would ameliorate the PV/PNN deficits in P22–23 *Fmr1* KO mice. To test this, immediately after EEG recordings (1 day after treatment), mice were sacrificed, and brains were collected for PV immunostaining and PNN labeling using *Wisteria floribunda* Agglutinin (WFA; Figure 1a, PV/PNN analysis). We analyzed the density of PV-positive (Figure 5a, e, i, m), PNN-positive (Figure 5b, f, j, 5n), and PV/PNN co-labeled neurons (Figure 5c, g, k, o) in layer 4 of the AC contralateral to the EEG electrode (for layers 1, 2/3, and 5/6, see Figure S5-1). We observed a significant increase in PNN density in SB-3CT-treated *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice (Figure 5r, $p = .02$). There was also a significant increase of PNN density in SB-3CT-treated WT mice compared to vehicle-treated WT mice (Figure 5r, $p = .009$). Statistical analysis of PV/PNN co-localization revealed a significant increase in PV/PNN co-localization in SB-3CT-treated *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice (Figure 5s, $p = .008$). There was also a significant increase in PV/PNN co-localization in SB-3CT-treated WT mice compared to vehicle-treated WT mice (Figure 5s, $p = .04$; for all average values and statistics, see Tables S3 and S4).

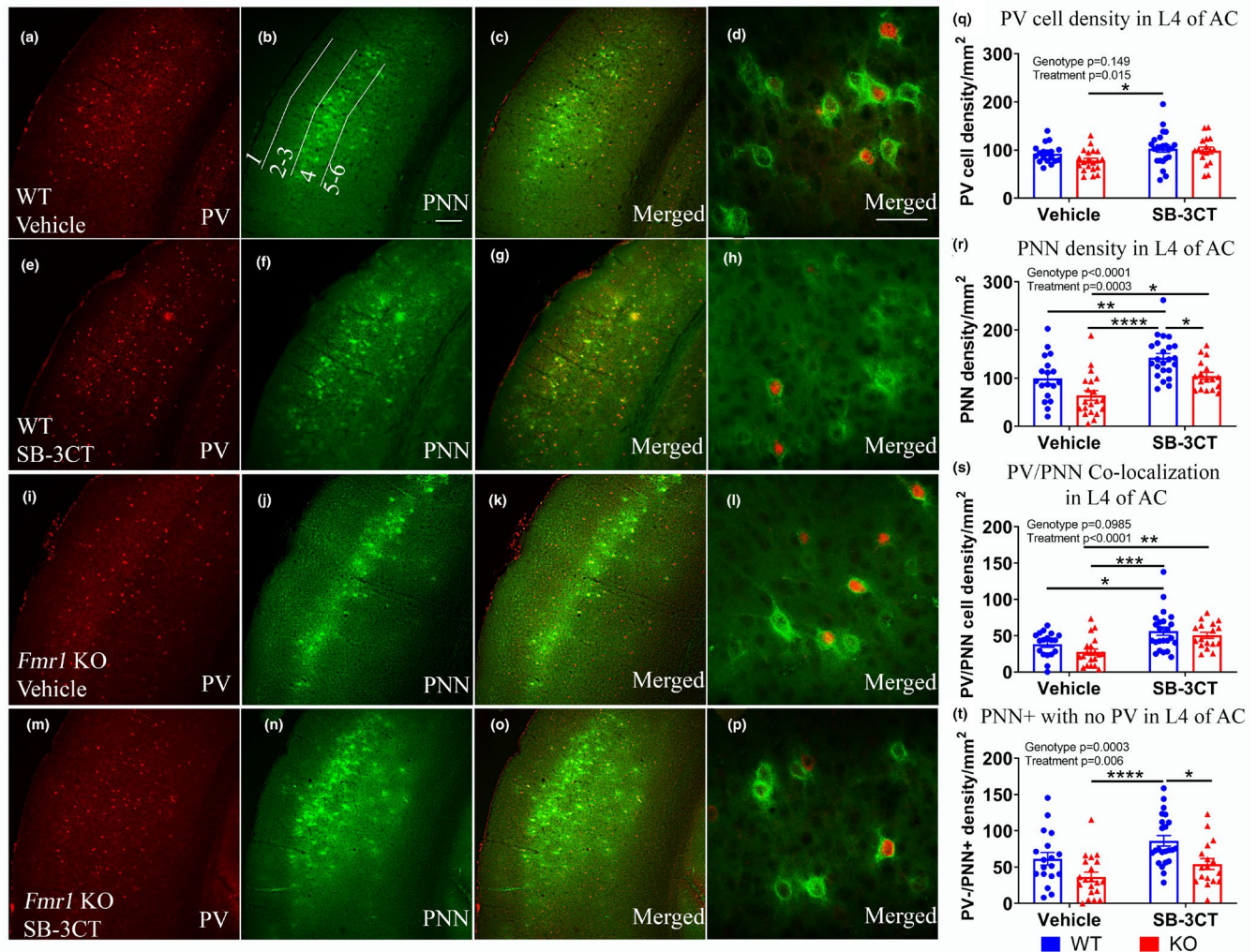


FIGURE 5 Acute SB-3CT treatment enhances PNN formation around PV cells in layer 4 auditory cortex of *Fmr1* KO mice. Confocal images of PV immunoreactivity (red; a, e, i, m), PNN labeling with WFA (green; b, f, j, n) and PV/PNN double labeling (c, g, k, o) in vehicle-treated WT (a–d), SB-3CT-treated WT (e–h), vehicle-treated *Fmr1* KO (i–l), and SB-3CT-treated *Fmr1* KO (m–p). Scale bar, 150 μ m (b). High-magnification images of PV/PNN in vehicle-treated WT (d), SB-3CT-treated WT (h), vehicle-treated *Fmr1* KO (l), and SB-3CT-treated *Fmr1* KO (p). Scale bar, 50 μ m. (q–t) Graphs show density of PV cells (q), PNN-positive cells (r), PV/PNN co-localization (s) and PV-negative cells with PNN (t). All graphs represent average values and the error bars indicate SEM ($n = 17-23$ per group, $N = 6$ per group; * $p < .05$; ** $p < .01$; *** $p < .001$; **** $p < .0001$)

Taken together, these data suggest that acute pharmacological inhibition of MMP-9 normalizes PNN development and co-localization of PNN predominantly around PV interneurons in layer 4 of the developing AC of *Fmr1* KO mice, consistent with improved phase locking at low gamma band frequencies.

3.6 | Acute inhibition of MMP-9 ameliorates anxiety and hyperactivity in *Fmr1* KO mice

Next, we assessed whether SB-3CT treatment reduces anxiety-like behaviors and hyperactivity in *Fmr1* KO mice that received an i.p. injections of SB-3CT (25 mg/kg) at P27–28 (for timeline, see Figure 6a). Anxiety-like behaviors were assessed on P28–29 mice (1 day after treatment) using the elevated plus maze (EPM)

and open field (OF). In the EPM, mice were allowed to explore the cross-shaped field for 10 min. TopScan Live software was used to evaluate anxiety-like behaviors by determining the percent of time spent in the open arms (Figure 6b). To evaluate locomotor activity and hyperactivity, the number of total entries into open and closed arms (Figure 6c), and the average speed (Figure 6d) were quantified. Results showed that vehicle-treated *Fmr1* KO mice spent less time in the open arms compared to vehicle-treated WT controls (Figure 6b). Statistical analysis using two-way ANOVA revealed significant effect of treatment, genotype, and significant interaction (for statistical comparisons, refer to Table S5). Post hoc comparisons showed that SB-3CT-treated *Fmr1* KO mice spent more time in open arms than vehicle-treated *Fmr1* KO mice (Figure 6b, $p < .0001$). Treatment with SB-3CT did not alter behavior of WT mice, suggesting specificity of the drug to the *Fmr1* KO group.

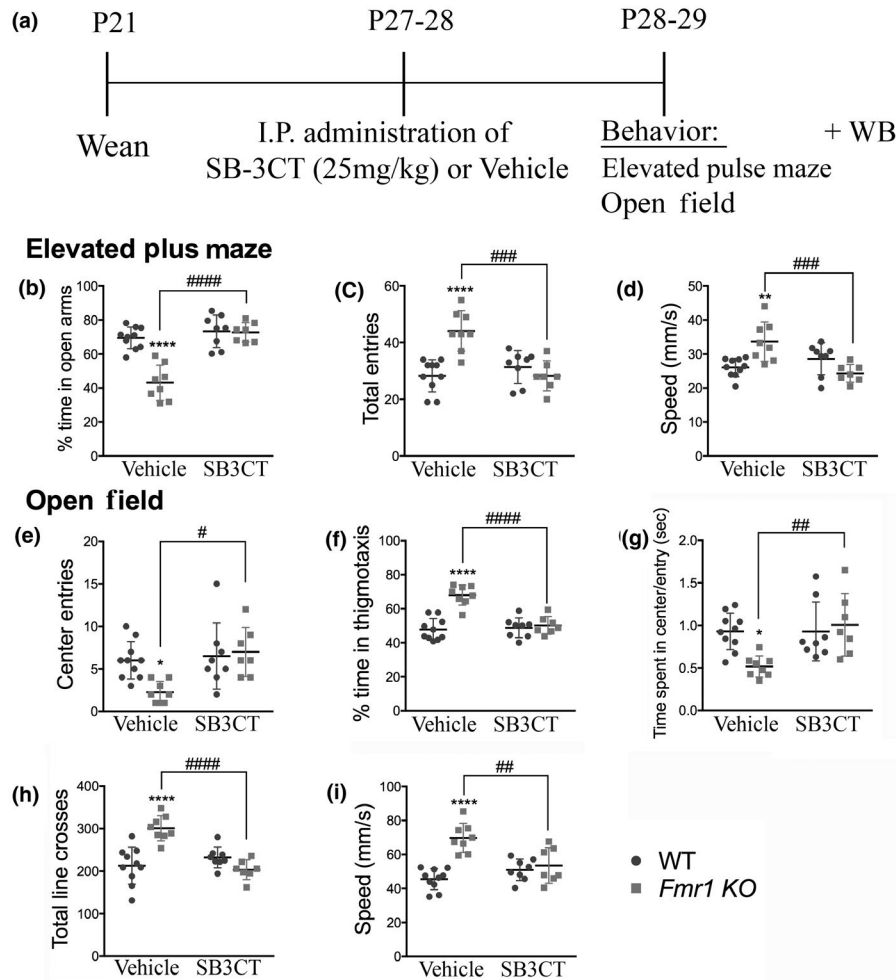


FIGURE 6 Acute SB-3CT-treatment normalizes anxiety/hyperactivity phenotype associated with *Fmr1* KO mice. (a) Experimental timeline for behavior and Western blot analysis. For this, mice were given either an intraperitoneal injection of SB-3CT (25 mg/kg) or vehicle at P27-28 and were tested for anxiety, locomotor, and hyperactivity using the open field and elevated plus maze test 1-day post-treatment. Brain samples were collected for Western blot analysis immediately after behavior. (b–d) Graphs show the percent time spent in the open arms (b), total entries into open and closed arms (c), and the average speed (d) in the elevated plus maze. (e–i) Graphs show the total number of center entries (e), the percent time spent in thigmotaxis (f), the time spent in the center per entry (g), the total number of line crosses (h), and the average speed (i) in the open-field test. All graphs represent average values and the error bars indicate SEM (WT vs. *Fmr1* KO: * $p < .05$, ** $p < .01$, **** $p < .0001$; vehicle-treated versus SB-3CT-treated *Fmr1* KO: # $p < .05$, ## $p < .01$, ### $p < .001$, #### $p < .0001$, vehicle-treated WT, $N = 10$; SB-3CT-treated WT, $N = 8$; vehicle-treated *Fmr1* KO, $N = 10$; SB-3CT-treated *Fmr1* KO, $N = 9$)

Vehicle-treated *Fmr1* KO mice also showed an increase in the number of total entries (Figure 6c, $p = .0001$) and speed (Figure 6d, $p = .0030$) compared to vehicle-treated WT controls. Treatment of *Fmr1* KO mice with SB-3CT reduced the number of total entries (Figure 6c, $p = .0001$) and speed (Figure 6d, $p = .0008$), yet it did not significantly alter the number of total entries or speed in WT controls (Figure 6c and d).

In the OF task, animals were allowed to explore an open field for 10 min. Anxiety-like behaviors were evaluated by measuring the total number of center entries (Figure 6e), the percent of time spent in thigmotaxis (Figure 6f), and the time spent in the center per entry (Figure 6g). Locomotor activity was assessed by determining the number of total line crosses (Figure 6h) and average speed (Figure 6i). All behaviors were quantified using TopScan Live software. Statistical analysis using two-way ANOVA revealed significant

main effects of treatment and interaction (for statistical comparisons, refer to Tables S5 and S6). Post hoc comparisons showed that vehicle-treated *Fmr1* KO mice made fewer center entries (Figure 6e, $p = .0316$), spent more time in thigmotaxis (Figure 6f, $p < .0001$) and spent less time in the center per entry (Figure 6g, $p = .0173$) compared to vehicle-treated WT controls. However, SB-3CT-treated *Fmr1* KO mice made more center entries (Figure 6e, $p = .0101$), spent less time in thigmotaxis (Figure 6f, $p < .0001$) and more time in the center per entry (Figure 6g, $p = .0086$) compared to vehicle-treated *Fmr1* KO mice. Statistical analysis using two-way ANOVA also revealed a significant increase in the total line crosses (Figure 6h, $p < .0001$) and average speed (Figure 6i, $p < .0001$) in vehicle-treated *Fmr1* KO mice compared to vehicle-treated WT mice. SB-3CT treatment significantly decreased the total line crosses (Figure 6h, $p < .0001$) and average speed (Figure 6i, $p = .0021$; for all average

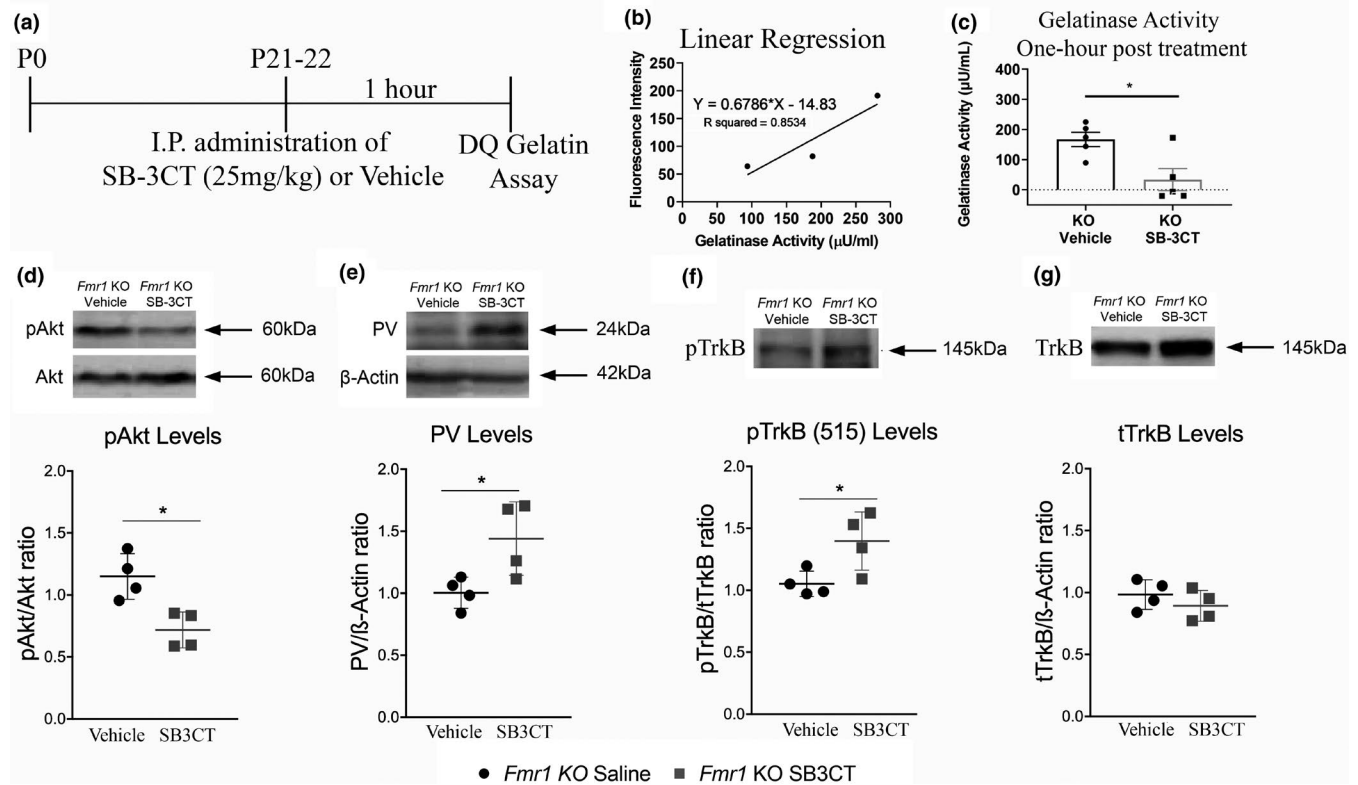


FIGURE 7 Acute MMP-9 inhibition reduces gelatinase activity and phosphorylation of Akt while increasing PV levels and phosphorylation of TrkB in the AC of *Fmr1* KO mice. To confirm that the MMP-2/9 inhibitor, SB-3CT, reduced MMP-2/9 activity following treatment, gelatinase activity was measured using a Dye-Quenched (DQ) gelatin assay. (a) Experimental timeline for DQ gelatin assay. Mice (no EEG) were given either an intraperitoneal injection of SB-3CT (25 mg/kg) or vehicle at P21–22 and brains were collected for the DQ Gelatin assay 1-hr post-injection. (b) Graph shows standard curve. (c) *Fmr1* KO mice injected with SB-3CT (25 mg/kg) show reduced gelatinase activity compared to vehicle-treated mice 1-hr post-injection ($N = 5$ per group, t test, $p = .02$). (d–g) Western blots show levels of pAkt and Akt (d), PV and actin (e), pTrkB515 (f), and TrkB (d). Graphs show pAkt/Akt (d), PV/actin (e), pTrkB515/TrkB (f), and TrkB/actin (g) ratios in the AC of vehicle-treated and SB-3CT-treated *Fmr1* KO mice ($N = 4$ per group) 24-hr post-injection. All graphs represent average values and error bars indicate SEM (* $p < .05$)

values and statistics, see Tables S5 and 6) of *Fmr1* KO mice. SB-3CT treatment did not change the behavior of WT mice in the open field.

These results suggest that acute inhibition of MMP-9 using SB-3CT alleviates anxiety/hyperactivity observed in *Fmr1* KO mice, without altering these behaviors in WT mice.

3.7 | Acute inhibition of MMP-9 with SB-3CT reduces gelatinase activity and phosphorylation of AKT while increasing PV levels and phosphorylation of TRKB

To confirm that the MMP-2/9 inhibitor, SB-3CT, reduced MMP-2/9 activity following treatment, gelatinase activity was measured using a Dye-Quenched (DQ) gelatin assay. *Fmr1* KO mice were injected with SB-3CT (25 mg/kg) or vehicle, the auditory and surrounding temporal cortex (Figure S2–1) was collected and homogenized for the DQ gelatin assay 1-hr post-injection (for timeline, see Figure 7a). A mouse recombinant MMP-9 ($\sim 1,500 \text{ pmol min}^{-1} \mu\text{g}^{-1}$) was used as a standard to calculate the approximate MMP-9 activity in the sample using a linear regression curve (Figure 7b). Results reveal that SB-3CT-treated *Fmr1* KO mice exhibit significantly decreased levels of

gelatinase activity 1-hr post-injection compared to vehicle-treated *Fmr1* KO mice (Figure 7c, t test, $N = 5$ per group, $p = .02$, Table S7). SB-3CT-treated WT mice also show significantly reduced gelatinase activity compared to vehicle-treated WT mice (Figure S7–1).

Previous studies have shown over-activation and enhanced phosphorylation of Akt/mTOR in the cortex of *Fmr1* KO mice (Lovelace et al., 2019) may underlie abnormalities in synaptic functions and hyperexcitability (Klann & Dever, 2004; Sharma et al., 2010). In addition, genetic deletion of MMP-9 restored Akt/mTOR phosphorylation to the normal levels in the hippocampus of *Fmr1* KO mice, while treatment of WT neurons with active MMP-9 enhanced Akt/mTOR phosphorylation (Sidhu et al., 2014). Therefore, to determine whether acute pharmacological MMP-9 inhibition influenced Akt/mTOR-related signaling, we analyzed Akt phosphorylation in the AC of SB-3CT and vehicle-treated *Fmr1* KO mice. *Fmr1* KO mouse brains were collected for Western blot analysis at P27–28 immediately after behavior, 1 day following treatment (for timeline see Figure 6a). Results show reduced levels of Akt phosphorylation in SB-3CT-treated *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice (Figure 7d; $N = 4$, $t(6) = 4.274$, $p = .0235$, t test). There were no significant changes in Akt phosphorylation in SB-3CT-treated compared to vehicle-treated WT mice (Figure S7–2a).

As TrkB signaling is implicated in PV cell development and survival (Nomura et al., 2017; Xenos et al., 2018), we examined the effects of acute MMP-9 inhibition on PV levels and phosphorylation of TrkB using Western blot. Results show that following SB-3CT treatment *Fmr1* KO mice exhibit increased PV levels (Figure 7e; $N = 4$, $t(6) = 2.607$, $p = .0479$, t test). We also observed an increase in TrkB phosphorylation on Tyrosine (Y) 515 in SB-3CT-treated *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice (Figure 7f; $N = 4$, $t(6) = 2.805$, $p = .0378$, t test), while there was no significant change in total TrkB levels (Figure 7g; for average values, see Table S8). Comparison of SB-3CT-treated WT versus vehicle-treated WT mice did not reveal any significant changes in PV (Figure S7-2b), pTrkB Y515 (Figure S7-2c), or total TrkB (Figure S7-2d).

4 | DISCUSSION

In this study, we show that P22–23 *Fmr1* KO mice exhibit abnormal neural oscillation patterns that parallel adult *Fmr1* KO mice and humans with FXS including increased resting state gamma power and deficits in gamma synchronization to auditory stimuli. Treatment with the MMP-9 inhibitor, SB-3CT, had no significant effect on resting EEG power or EEG power coupling in *Fmr1* KO mice. However, acute inhibition of MMP-9 activity improved fidelity of gamma frequency (~40 Hz) phase locking to time-varying auditory stimuli in SB-3CT *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice. At the molecular level, SB-3CT treatment enhanced PNN formation predominantly around PV interneurons in the developing AC of both *Fmr1* KO mice and WT mice. Assessment of anxiety/hyperactivity with EPM and OF tests demonstrated that acute inhibition of MMP-9 activity ameliorated anxiety-like behaviors and hyperactivity in *Fmr1* KO mice. Lastly, acute inhibition of MMP-9 activity reduced Akt phosphorylation while increasing PV levels and TrkB phosphorylation. Our data are consistent with previously reported benefits of minocycline treatment in humans with FXS (Schneider et al., 2013), *Fmr1* KO mice (Bilousova et al., 2009; Dansie et al., 2013; Toledo, Wen, Binder, Ethell, & Razak, 2019), and the *Drosophila* FXS model (Siller & Broadie, 2011), and further suggest that minocycline may alleviate FXS symptoms by suppressing MMP-9. These data support the notion that cellular and electrophysiological abnormalities of sensory processing are present early in development. In addition, treatment during early postnatal developmental period may be beneficial in ameliorating deficits due to higher cortical plasticity. Furthermore, reduction of MMP-9 activity may provide a suitable target to reduce auditory hypersensitivity in FXS and potentially other ASDs.

4.1 | Developing and adult *Fmr1* KO mice exhibit comparable EEG phenotypes

In this study, we show that developing *Fmr1* KO mice (P22–23) exhibit increased resting state low gamma power in both the

AC and FC (Figure 1) similar to adult *Fmr1* KO mice. Unlike adult *Fmr1* KO mice, though, developing *Fmr1* KO mice do not exhibit increased resting state delta or high gamma power (Lovelace et al., 2018). These results are consistent with data from the FVB strain of mice (Wen et al., 2019), suggesting that abnormal electrophysiological phenotypes are observed as early as P22 in developing *Fmr1* KO mice. The consistency of EEG phenotypes across age and genetic backgrounds of mice, and between humans and mice, indicates that these measures can be utilized as objective physiological biomarkers to test the effectiveness of potential drugs. Furthermore, accumulating evidence indicates that increased resting state gamma power is associated with sensory processing and communication deficits in FXS and ASD (Ethridge et al., 2017, 2019; Lovelace et al., 2018, 2019; Sinclair et al., 2017; Wang et al., 2017). Additional studies also point to abnormalities in theta and alpha power as contributing mechanisms underlying hyperexcitability (Van der Molen & Van der Molen, 2013). Theta power is linked to cortical arousal level (Barry, Clarke, Johnstone, McCarthy, & Selikowitz, 2009). Alpha power has been interpreted to reflect a neural inhibitory mechanism that regulates the processing of external, sensory information (Klimesch, 1999; Mathewson et al., 2011). In particular, GABAergic inhibitory activity is thought to contribute to this neural inhibition mediated by alpha oscillations (Jensen & Mazaheri, 2010). Together, alpha/theta synchronization has been linked to cognitive function (Klimesch, 1999) and ability to inhibit irrelevant sensory information (Van der Molen & Van der Molen, 2013). Human EEG studies report an inverted U-shaped pattern of power abnormalities showing reduced alpha frequencies, but increased delta, theta frequencies and high-frequency bands (beta, gamma) in children with ASD (Sinclair et al., 2017; Wang et al., 2013). Elevated theta and delta power and reduced alpha power is also observed in individuals with ADHD (Barry, Johnstone, & Clarke, 2003; Born et al., 2017). Our study shows a reduced EEG power correlations between Theta and Low Gamma, Alpha and Low Gamma bands within the AC as well as across regions (AC to FC and FC to AC) in *Fmr1* KO mice compared to WT mice. Overall, our findings indicate that abnormalities in EEG oscillation pattern, which are associated with hyperexcitability, cognitive performance, and ability to process sensory information, are present in developing *Fmr1* KO mice as early as P22–23.

In this study, we show that developing *Fmr1* KO mice exhibit decreased ability to produce synchronous stimulus-induced oscillations, similar to adult *Fmr1* KO mice (Lovelace et al., 2018), but the impairment is localized specifically to the low gamma frequencies (~30–55 Hz) in the AC and both low and high gamma (~65–100 Hz) frequencies in the FC. Treatment of *Fmr1* KO mice with SB-3CT leads to an improvement in phase locking to auditory chirp stimuli, in the same frequency range (~40–50 Hz) where the deficit was observed in vehicle-treated *Fmr1* KO AC (Figure 4). This result indicates that SB-3CT may be targeting specific cellular and network mechanisms in the AC resulting in improved phase locked synchronization in developing *Fmr1* KO mice.

4.2 | Molecular mechanisms influencing *Fmr1* KO EEG phenotype

It is possible that changes in PV/PNN networks affect inhibitory circuits and neural oscillation patterns. The loss of PNNs is associated with abnormal plasticity and reduced excitability of PV interneurons (Balmer, 2016; Lensjo, Christensen, Tennoe, Fyhn, & Hafting, 2017; Chu et al., 2018). This may lead to altered balance of excitatory and inhibitory circuits, and improper network synchronization and hyperexcitability (Contractor, Klyachko, & Portera-Cailliau, 2015). In fact, several studies point altered functionality of GABAergic circuits, specifically fast-spiking PV interneurons in FXS (Goel et al., 2018; Nomura et al., 2017; Selby, Zhang, & Sun, 2007). Specifically, PV cell density is significantly reduced in the AC (Kulinich et al., 2019; Wen et al., 2018) and somatosensory cortex (Selby et al., 2007) of *Fmr1* KO mice compared to WT mice. One recent study demonstrated that passive developmental sound exposure during the maturation of synaptic and intrinsic properties (P9–21) results in enhanced PV cell density and PV/PNN co-localization in the AC of *Fmr1* KO mice despite impairment of PNN formation (Kulinich et al., 2019). In contrast, genetic reduction of MMP-9 was shown to enhance the formation of PNNs around PV cells in the AC (Wen et al., 2018). Our study shows that pharmacological inhibition of MMP-9 activity alone during the critical developmental period results in enhanced PNN formation around PV interneurons. While we did not see a significant increase in PV cell density, we did observe an increase in PV levels measured by Western blot, similar to what has been observed in other ASD models (Filice, Vorkel, Sungur, Wöhr, & Schwaller, 2016). It is possible that increased PNN formation around PV cells contributed to increased PV levels, which influenced the changes in resting state EEG oscillations in SB-3CT-treated *Fmr1* KO mice (Yamada, Ohgomori, & Jinno, 2015). To more effectively target auditory hypersensitivity, a combination of sensory stimulation (Kulinich et al., 2019; Woo & Leon, 2013) with pharmacological inhibition of MMP-9 activity (Bilousova et al., 2009) may yield a synergistic effect.

Other studies have employed sound exposure or mGluR5 inhibitors during the late postnatal window (P20–30) to rescue AC plasticity deficits and found that they were only able to rescue sound exposure-induced cortical map plasticity (size of A1 and response latency) when *Fmr1* KO mice were exposed to sound between P9–20 in conjunction with daily MPEP injections (Kim, Gibboni, Kirkhart, & Bao, 2013). It is possible that inhibition of MMP-9 activity may function as a potent therapeutic for auditory hypersensitivity and anxiety/hyperactivity phenotypes during the critical developmental period. The question remains whether administration of SB-3CT during the critical developmental period has more beneficial effects in regulating resting state EEG oscillations, improving responses to sound and anxiety/hyperactivity-related behaviors in *Fmr1* KO mice versus administration of SB-3CT in adult *Fmr1* KO mice. SB-3CT has only ~45 min half-life (Gooyit et al., 2012). However, even transient inhibition of MMP-9 activity during this specific developmental window was sufficient to trigger structural and functional changes in

the auditory cortex of *Fmr1* KO mice. One potential benefit may be attributed to the metabolism of SB-3CT, which functions as an irreversible inhibitor yielding covalent modifications that inhibit the activation of MMP-2/9 (Brown et al., 2000).

In addition to the enhanced PNN formation surrounding PV interneurons in the AC, the function of PV interneurons may also be influenced by activity of TrkB receptor. TrkB is shown to play a role in gamma-band synchronization in the hippocampal network (Zheng et al., 2011) and functions as a receptor for brain-derived neurotrophic factor (BDNF). In fact, TrkB is expressed by GABAergic neurons, in particular PV-positive cells (Cellerino, Maffei, & Domenici, 1996; Lewis et al., 2005). BDNF-mediated signaling is involved in mediating cell growth and survival, neuronal differentiation, and activity-dependent regulation of inhibitory synapse development (Hong, McCord, & Greenberg, 2008) and has been linked to maturation of PV cells in the cerebral cortex (Itami, Kimura, & Nakamura, 2007). BDNF via TrkB is known to promote the development of GABAergic neurons and induces the expression of GABA-related proteins, including GAD67, GAT1, and PV (Nomura et al., 2017; Xenos et al., 2018). Notably, BDNF-TrkB signaling has been shown to be impaired in *Fmr1* KO mice (Castren & Castren, 2014). We previously reported decreased phosphorylation of TrkB in the AC of *Fmr1* KO mice compared to WT mice, which was up-regulated following developmental exposure to sound (Kulinich et al., 2019). In the current study, we showed that treatment with SB-3CT increased TrkB phosphorylation in *Fmr1* KO mice compared to vehicle-treated *Fmr1* KO mice (Figure 7). Together, inhibition of MMP-9 activity may preferentially target PV-mediated inhibitory networks by influencing the stability of PV interneurons via increased PNN formation and activation of TrkB receptors.

4.3 | Inhibition of MMP-9 activity reduces AKT phosphorylation and improves anxiety and hyperactivity phenotypes in *Fmr1* KO mice

FXS is characterized by over-activation of group I metabotropic glutamate receptors (Huber, 2002), which promote Akt/mTOR signaling leading to aberrant phosphorylation of Akt and mTOR-related proteins, and increased protein synthesis (Bear, Huber, & Warren, 2004; Sharma et al., 2010). Studies have shown that genetic reduction of p70 ribosomal S6 kinase 1 (S6K1) prevented elevated phosphorylation of translational control proteins, normalizing protein synthesis, reversing dendritic spine deficits, and improving social interactions, novel object recognition, and behavioral flexibility in *Fmr1* KO mice (Bhattacharya et al., 2012). In fact, several studies link dysregulation of mTOR signaling with dendritic and synaptic abnormalities, cognitive impairments, and behavioral performance in FXS (Bhattacharya et al., 2012; Busquets-Garcia, Maldonado, & Ozaita, 2014; Troca-Marin, Alves-Sampaio, & Montesinos, 2012). Furthermore, treatment with the antibiotic minocycline, which is known to inhibit MMP-9, also promotes dendritic spine maturation and improves behavioral performance in developing *Fmr1* KO mice

(Bilousova et al., 2009). Thus, it is possible that the reduction of Akt phosphorylation observed in *Fmr1* KO mice following treatment with SB-3CT may be linked to the changes in dendritic and synaptic profiles affecting protein synthesis, synaptic plasticity, and behavioral performance.

While *Fmr1* KO mice display multiple behavioral impairments that parallel human patients with FXS (Bernardet & Crusio, 2006), there is wide variability in behavioral results assessing anxiety, hyperactivity, social, and cognitive impairments in *Fmr1* KO mice. Several studies find these measures to be altered (Bilousova et al., 2009; Ding, Sethna, & Wang, 2014; Sinclair et al., 2017), while others show no differences (Kazdoba, Leach, Silverman, & Crawley, 2014). We show that vehicle-treated *Fmr1* KO mice spent less time in the open arms of the EPM, similar to previous studies in our laboratory (Bilousova et al., 2009), as well as increased percent time in thigmotaxis and decreased center entries in the OF, indicating higher anxiety. *Fmr1* KO mice also displayed increased hyperactivity indicated by increased speed in both EPM and OF tests. Interestingly, treatment with SB-3CT reduced both anxiety and hyperactivity measures in *Fmr1* KO mice. These results suggest that SB-3CT effectively ameliorates behavioral impairments observed in developing *Fmr1* KO mice when MMP-9 activity is inhibited during the specific developmental period.

In conclusion, we have shown that acute inhibition of MMP-9 activity during a specific critical developmental period (P22–P28) may serve to modulate PV neuron-mediated inhibitory circuits, neural oscillatory patterns, and behavioral impairments associated with FXS. These findings implicate MMP-9 as a potent candidate contributing to the FXS phenotype and indicate that targeted inhibition of MMP-9 activity may be a promising therapeutic agent.

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All experiments were conducted in compliance with the ARRIVE guidelines.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

ORCID

Patricia S. Pribhoy  <https://orcid.org/0000-0001-8733-7447>

Jonathan W. Lovelace  <https://orcid.org/0000-0001-5734-6801>

Iryna M. Ethell  <https://orcid.org/0000-0002-1324-6611>

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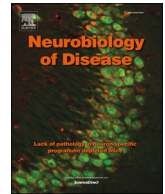


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SUPPORTING INFORMATION

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Multielectrode array analysis of EEG biomarkers in a mouse model of Fragile X Syndrome

Carrie R. Jonak^a, Jonathan W. Lovelace^c, Iryna M. Ethell^{a,b}, Khaleel A. Razak^{b,c}, Devin K. Binder^{a,b,*}

^a Division of Biomedical Sciences, School of Medicine, University of California, Riverside, United States of America

^b Neuroscience Graduate Program, University of California, Riverside, United States of America

^c Department of Psychology, University of California, Riverside, United States of America

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ABSTRACT

Fragile X Syndrome (FXS) is a leading known genetic cause of intellectual disability with symptoms that include increased anxiety and social and sensory processing deficits. Recent EEG studies in humans with FXS have identified neural oscillation deficits that include increased resting state gamma power, increased amplitude of auditory evoked potentials, and reduced inter-trial phase coherence of sound-evoked gamma oscillations. Identification of comparable EEG biomarkers in mouse models of FXS could facilitate the pre-clinical to clinical therapeutic pipeline. However, while human EEG studies have involved 128-channel scalp EEG acquisition, no mouse studies have been performed with more than three EEG channels. In the current study, we employed a recently developed 30-channel mouse multielectrode array (MEA) system to record and analyze resting and stimulus-evoked EEG signals in WT vs. *Fmr1* KO mice. Using this system, we now report robust MEA-derived phenotypes including higher resting EEG power, altered event-related potentials (ERPs) and reduced inter-trial phase coherence to auditory chirp stimuli in *Fmr1* KO mice that are remarkably similar to those reported in humans with FXS. We propose that the MEA system can be used for: (i) derivation of higher-level EEG parameters; (ii) EEG biomarkers for drug testing; and (ii) mechanistic studies of FXS pathophysiology.

1. Introduction

Fragile X Syndrome (FXS) is the most common genetic cause of intellectual disability with symptoms that overlap with autism spectrum disorders (ASD) (Crawford et al., 2001). FXS is caused by a mutation in the *Fragile X Mental Retardation 1* (*Fmr1*) gene and a loss of Fragile X Mental Retardation Protein (FMRP) (Yu et al., 1991). FMRP is an RNA-binding protein that regulates synaptic function through regulation of protein translation (Darnell et al., 2011). Symptoms associated with FXS include increased anxiety, repetitive behaviors, social communication deficits, delayed language development and abnormal sensory processing (Abbeduto and Hagerman, 1997; Berry-Kravis, 2002; Hagerman et al., 2009; Miller et al., 1999; Musumeci et al., 1999; Roberts et al., 2001; Sabaratnam et al., 2001; Sinclair et al., 2017c; Van der Molen et al., 2010; Wisniewski et al., 1991). Abnormal sensory processing in FXS includes hypersensitivity and reduced habituation to repeated sensory stimuli (Castrén et al., 2003; Schneider et al., 2013).

Auditory processing deficits are common in both humans with FXS (Castrén et al., 2003; Ethridge et al., 2016; Schneider et al., 2013; Van

der Molen and Van der Molen, 2013a) and *Fmr1* knockout (KO) mice (Lovelace et al., 2016, 2018; Rotschafer and Razak, 2013, 2014; Wen et al., 2018), a mouse model of FXS (Bernardet and Crusio, 2006). Recent EEG recordings from humans showed altered cortical oscillatory activity that may contribute to sensory hypersensitivity and social communication deficits in FXS (Ethridge et al., 2017; Wang et al., 2017). Gamma frequency power was enhanced in humans with FXS compared to healthy controls (Wang et al., 2017). When neural oscillations were induced with auditory “chirp” stimuli, inter-trial phase coherence (phase-locking) was reduced in humans with FXS, particularly at gamma frequencies (Ethridge et al., 2017). The non-phase locked single-trial power was, however, enhanced in FXS. These results suggest that enhanced background gamma oscillations (“gamma noise”) may contribute to hypersensitivity and interfere with stimulus-evoked synchronization in FXS. Importantly, these phenotypes were correlated with parent reports of social communication deficits and hypersensitive sensory responses suggesting clinical relevance of the EEG measures (Ethridge et al., 2017).

Identification of comparable biomarkers in humans and validated

* Corresponding author at: Division of Biomedical Sciences, University of California, 900 University Avenue, Riverside, CA 92521, United States of America.
E-mail address: dbinder@ucr.edu (D.K. Binder).

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animal models is a critical step in facilitating pre-clinical to clinical therapeutic pipelines to treat neurodevelopmental disorders (Berry-Kravis et al., 2018). In an initial set of studies, we recorded EEGs in awake, freely moving *Fmr1* KO mice using similar stimuli as in the human studies. We found remarkably similar phenotypes in frontal and auditory cortex of the *Fmr1* KO mice including enhanced resting state gamma power and reduced inter-trial phase coherence (ITPC) to auditory “chirp” stimuli (Lovelace et al., 2018). However, these initial studies were done with three epidural screw electrodes (frontal, auditory, and occipital). Thus, a potentially important technical difference between the human studies and our mouse studies is that we have used different methods to collect and analyze EEG data between species. The data collected from humans with FXS utilized large EEG arrays with 128 leads, which allows spatial mapping of oscillations, examination of the spread of neural excitation across cortex, and powerful principal component analysis of multiple channels. Our recordings with 3 cortical electrodes used a common ground in the occipital lobe, and recording electrodes in the frontal and auditory cortices, resulting in 2 channels of derived data. Because of this electrode limitation in mice in our initial studies, the spatial representation of oscillations was limited, which could cause us to miss important alterations in oscillations that were revealed in human data, especially amplitude and frequency coupling across regions. For these reasons, it is desirable to align mouse electrophysiology data acquisition methods to more closely match those used in human studies.

To address these issues, we have developed and applied multi-electrode array (MEA) analysis in mice (Jonak et al., 2018). Our system involves stable chronic *in vivo* implantation of a planar multielectrode array (MEA) on the surface of the mouse skull and enables low-noise 30-channel simultaneous EEG, which can then be used for resting and stimulus-evoked EEG acquisition in awake, freely moving mice (Jonak et al., 2018). For auditory stimulation, we used paradigms analogous to those used in human studies (Ethridge et al., 2017) and identical to those previously reported in the 2-channel data (Lovelace et al., 2018). Using this system, we now report MEA-derived EEG phenotypes in *Fmr1* KO mice that are similar to those observed in humans and provide novel insights into abnormal sensory processing in FXS. In particular, we demonstrate robust MEA-derived phenotypes of altered resting EEG power, event-related potentials (ERPs), single-trial and train-related EEG power, and inter-trial phase coherence (ITPC) to auditory chirp stimuli in *Fmr1* KO mice.

2. Methods

2.1. Animals

Fmr1 KO (B6.129P2-*Fmr1*^{tm1Cgr}/J, stock #003025) (Bakker et al., 1994) and C57BL/6J WT (stock #000664) mice were obtained from Jackson Laboratories at 8 weeks of age and were housed in our animal facility before and after surgical procedures. Animals were maintained under a 12-hour light/dark cycle and were provided irradiated rodent diet (PicoLab, 5053) and water *ad libitum*. All experiments were performed with approval from the University of California Animal Care and Use Committee and in accordance with the National Institutes of Health Animal Care and Use Guidelines. EEG recordings were obtained from 11 *Fmr1* KO and 10 WT mice. Males between 2 and 3 months of age were used for all EEG recordings.

2.2. MEA implantation

Mice were anesthetized with isoflurane inhalation (0.2–0.5%) and given an i.p. injection cocktail of ketamine 80 mg/kg (Zoetis, 10004027) and xylazine 10 mg/kg (Bimeda, 1XYL003). Mice were aseptically prepared for surgery and secured in a stereotaxic apparatus. Artificial tear ointment (Henry Schein, 01169568321) was applied to the eyes to prevent drying. Toe pinch reflex was used to

measure anesthetic depth throughout the surgery, and supplemental doses of K/X were administered as needed. Once the mouse was anesthetized, a midline sagittal incision was made along the scalp to expose the skull. A cotton-tip applicator was used to remove the periosteum from the skull and to clean skull with saline. A surgical marker was used to mark bregma and positions of three screws. A dental drill was used to drill 1 mm diameter holes in the skull overlying the left frontal cortex, left cerebellum and right cerebellum. Screws (PlasticsOne, 00-96 X 1/16) were advanced into drilled holes until secure; special care was taken not to advance the screws beyond the point of contact with the dura. The probe grounding wire was placed in the nuchal musculature and the probe was placed on the skull surface carefully aligning the “+” in the center of the probe with bregma. Saline was added to the top of the probe to aid in adherence to the skull surface and allowed to dry. A 4-0 silk tie was used to secure the probe ribbon between the two cerebellum screws and Teflon/plastic wrap was placed on top of the probe. Dental cement (Kuraray, 3382KA) was applied around the screws, on the base of the cotton-tip applicator post, and the Teflon/plastic wrap covering the probe. Waterproof medical tape was used to secure the cotton-tip applicator to the probe connector. Triple antibiotic was applied along the edges of the dental cement followed by a subcutaneous injection of buprenorphine 0.1 mg/kg (Reckitt & Colman, 5053624). Mice were placed on a heating pad to aid in recovery from anesthesia and additional doses of buprenorphine were administered every 6–8 h for continuous analgesia during the first 48 h after surgery. Mice were then individually housed with nesting material (Ancare, NES3600) for environmental enrichment, returned to the vivarium and monitored daily until the day of EEG recordings. Animals were recorded between 2 and 4 days after surgery.

2.3. Acoustic stimulation

All experiments were conducted in a sound-attenuated chamber lined with anechoic foam (Gretch-Ken Industries, Oregon). Acoustic stimuli were generated using RVPDSEX software and RZ6 hardware (Tucker Davis Technologies, FL) and presented through a free-field speaker (MF1 Multi-Field Magnetic Speaker; Tucker-Davis Technologies, FL) located 12 in. directly above the cage. Sound pressure level (SPL) was modified using programmable attenuators in the RZ6 system. The speaker output was ~70 dB SPL at the floor of the recording chamber with fluctuation of ± 3 dB for frequencies between 5 and 35 kHz as measured with a ¼ inch Bruel & Kjaer microphone.

After 5 min of EEG recording without any sound (resting EEG), we first presented broadband noise to record auditory event-related potentials (ERPs) to identify various components (P1, N1, P2) for comparison to previous animal (Lovelace et al., 2018; Lovelace et al., 2016; Wen et al., 2019) and human (Castrén et al., 2003; Van der Molen et al., 2012) studies. ERPs were recorded in response to trains of broadband noise with each train consisting of 10 repetitions (1 Hz repetition rate). Each noise stimulus was 100 ms in duration, with a 5 ms rise/fall time and was presented at 70 dB SPL. The inter-train interval was 8 s. The total duration of broadband stimuli was 30 min.

Second, we used an acoustic stimulation paradigm that has been used in humans with FXS to enhance translational relevance. Ethridge et al. (2017) used a chirp-modulated tone (henceforth, ‘chirp’) to induce synchronized oscillations in their EEG recordings (Ethridge et al., 2017). The chirp stimulus used here was broadband noise whose amplitude was modulated by a sinusoid with linearly increasing frequencies from 1 to 100 Hz (Artieda et al., 2004; Pérez-Alcázar et al., 2008; Purcell et al., 2004). Each stimulus was 2 s in duration, and the depth of modulation was 100%. To avoid onset responses contaminating phase locking to the amplitude modulation of the chirp, the stimulus was ramped in sound level from 0 to 100% over 1 s (rise time) which then smoothly transitioned into chirp modulation of the noise. Chirp trains were presented 300 times each with a random inter-chirp interval between 1 and 1.5 s. The total duration of chirp stimuli was

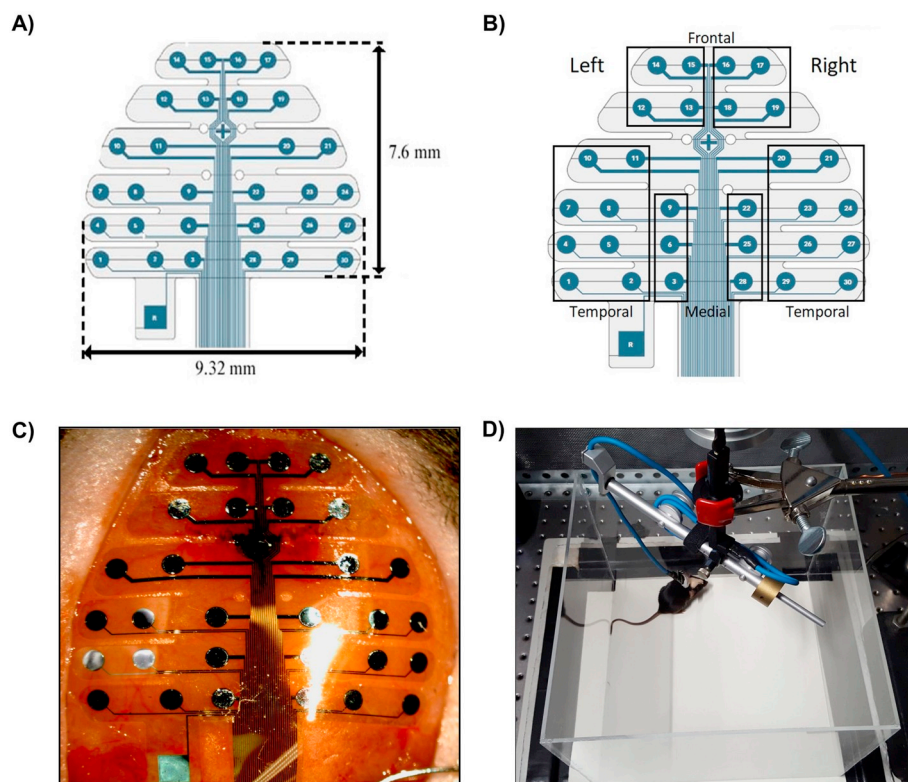


Fig. 1. A. Schematic of multielectrode array probe. B. Division into six regions (left and right frontal, temporal, and medial). C. MEA probe on mouse skull surface *in situ*. Note that the “+” is situated over bregma. D. Freely moving mouse connected to MEA recording apparatus.

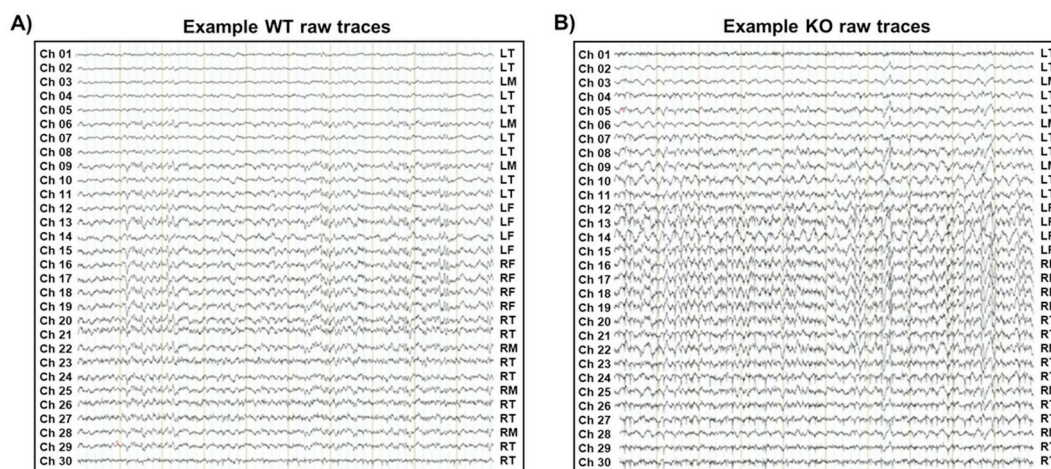


Fig. 2. Example of resting MEA EEG obtained from WT (A) and *Fmr1* KO (B) mice. Note higher amplitude and frequency in the *Fmr1* KO EEG traces. LT = left temporal; LM = left medial; LF = left frontal; RT = right temporal; RM = right medial; RT = right temporal.

25 min. The chirp facilitates a rapid measurement of transient oscillatory entrainment (delta to gamma frequency range) to auditory stimuli of varying frequencies and can be used to compare oscillatory responses in different groups in clinical and pre-clinical settings (Purcell et al., 2004). Inter-trial phase coherence analysis (phase locking factor) (Tallon-Baudry et al., 1996) can then be used to determine the ability of neural generators to synchronize oscillations to the frequencies present in the repeated stimulus.

2.4. Electrophysiology

Resting and auditory ERP recordings were obtained using the SmartBox (NeuroNexus) from awake and freely moving mice (Jonak

et al., 2018). A headstage and tether was connected to the probe post of the mouse skull (implanted during surgery) under brief isoflurane anesthesia. The mouse was then placed inside a grounded Faraday cage after recovery from isoflurane. This tether was then connected to a commutator located directly above the cage. Mice were then allowed to habituate to being connected to the tether for 20 min before EEG recordings were obtained.

The SmartBox acquisition system was connected to the commutator to which the animal was attached. Acquisition hardware was set to lower (0.5 Hz) and upper (500 Hz) filters and data were sampled at a rate of 1250 Hz. Sound delivery was synchronized with EEG recordings using a TTL pulse to mark the onset of each sound in a train. Five minutes of resting EEG was recorded in which no auditory stimuli were

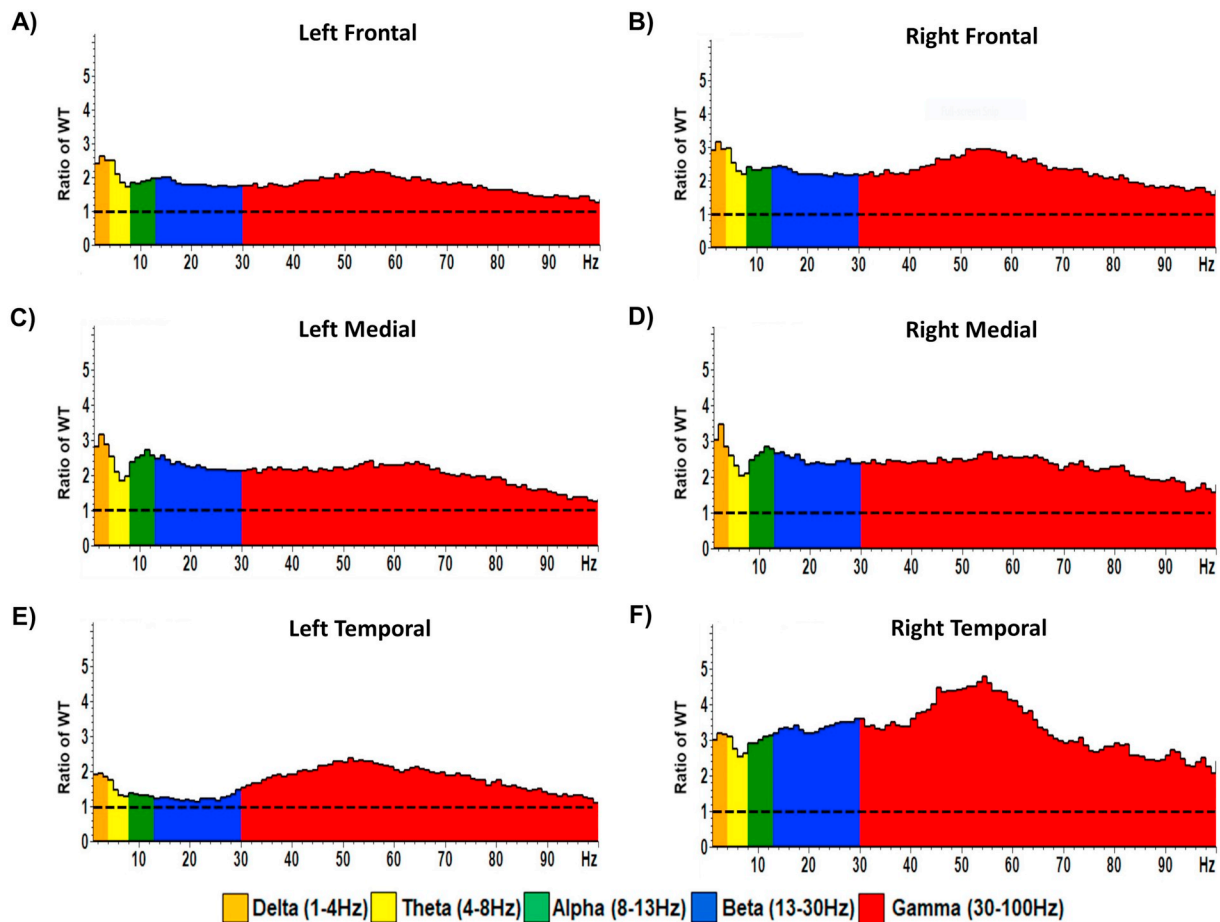


Fig. 3. Ratio of *Fmr1* KO to WT EEG power across frequency bands for distinct brain regions (A–F). Five minutes of resting EEG data from various indicated brain regions was recorded and FFT analysis was done to determine spectral power. Values above 1 indicate higher EEG power in *Fmr1* KO compared with WT mice.

presented. This was followed by ERP recordings in response to trains of broadband noise and chirp stimuli. After these experiments were completed, mice were euthanized.

2.5. Data analyses

Data analyses were done using a combination of Analyzer 2.1 (Brain Vision Inc.), MATLAB, and SPSS. Data were extracted from the Smartbox files and saved in a file format compatible with Analyzer 2.1 software. Data were first down sampled to 625 Hz and a 60 Hz notch filter was used. EEG artifacts were removed using a semi-automatic procedure in Analyzer 2.1 for all recordings. Less than 20% of data were rejected due to artifacts from any single mouse.

Resting (no auditory stimulus) EEG data were divided into 1 second segments and Fast Fourier Transforms (FFT) was run on each segment using 0.5 Hz bins and then average power ($\mu\text{V}/\text{Hz}^2$) was calculated for each mouse from 1 to 100 Hz. Power was then further binned into standard frequency bands: Delta (1–4 Hz), Theta (4–8 Hz), Alpha (8–13 Hz), Beta (13–30 Hz), and Gamma was divided into “Low Gamma” (30–55 Hz), and “High Gamma” (65–100 Hz).

Single ERP peak analysis was done by obtaining average traces across the first response of all BBN trains (total of 200 trials). Peaks were determined by pre-defined time windows after stimulus onset: P1 (10–30 ms), N1 (30–75 ms), and P2 (75–150 ms).

Two different measures of power were used to analyze single ERP responses as well as responses throughout trains of BBN. Event-related power of single ERP responses was calculated by taking the average trace from the first response (window is ± 500 ms from sound onset) in each of the 1 Hz trains (a total of 200 repetitions) and performing

Morlet Wavelet deconvolution using power density ($\mu\text{V}^2/\text{Hz}$) on the average trace. This same procedure was also used to determine event-related power during entire 1 Hz trains of BBN as well, with windows adjusted to encompass the entire train.

Chirp trains were analyzed using Morlet wavelet analysis. The trains were segmented into 2-second windows during the chirp stimulation. EEG traces were processed with Morlet wavelets from 1 to 100 Hz using complex number output (voltage density, $\mu\text{V}/\text{Hz}$) for inter-trial phase coherence (ITPC) calculations, and power density ($\mu\text{V}^2/\text{Hz}$) for phase locked power (PL power) calculations and baseline corrected non-phase locked single-trial power (induced power). Wavelets were run with a Morlet parameter of 10 as this gave the best frequency/power discrimination. This parameter was chosen since studies in humans found most robust difference around 40 Hz, where this parameter is centered (Ethridge et al., 2017).

To measure phase synchronization at each frequency across trials, inter-trial phase coherence (ITPC) was calculated. The equation used to calculate ITPC is:

$$ITPC(f, t) = \frac{1}{n} \sum_{k=1}^n \frac{F_k(f, t)}{|F_k(f, t)|}$$

where f is the frequency, t is the time point, and k is trial number. Thus, $F_k(f, t)$ refers to the complex wavelet coefficient at a given frequency and time for the k th trial. There were no less than 275 chirp trials (out of 300) for any given mouse after segments containing artifacts were rejected.

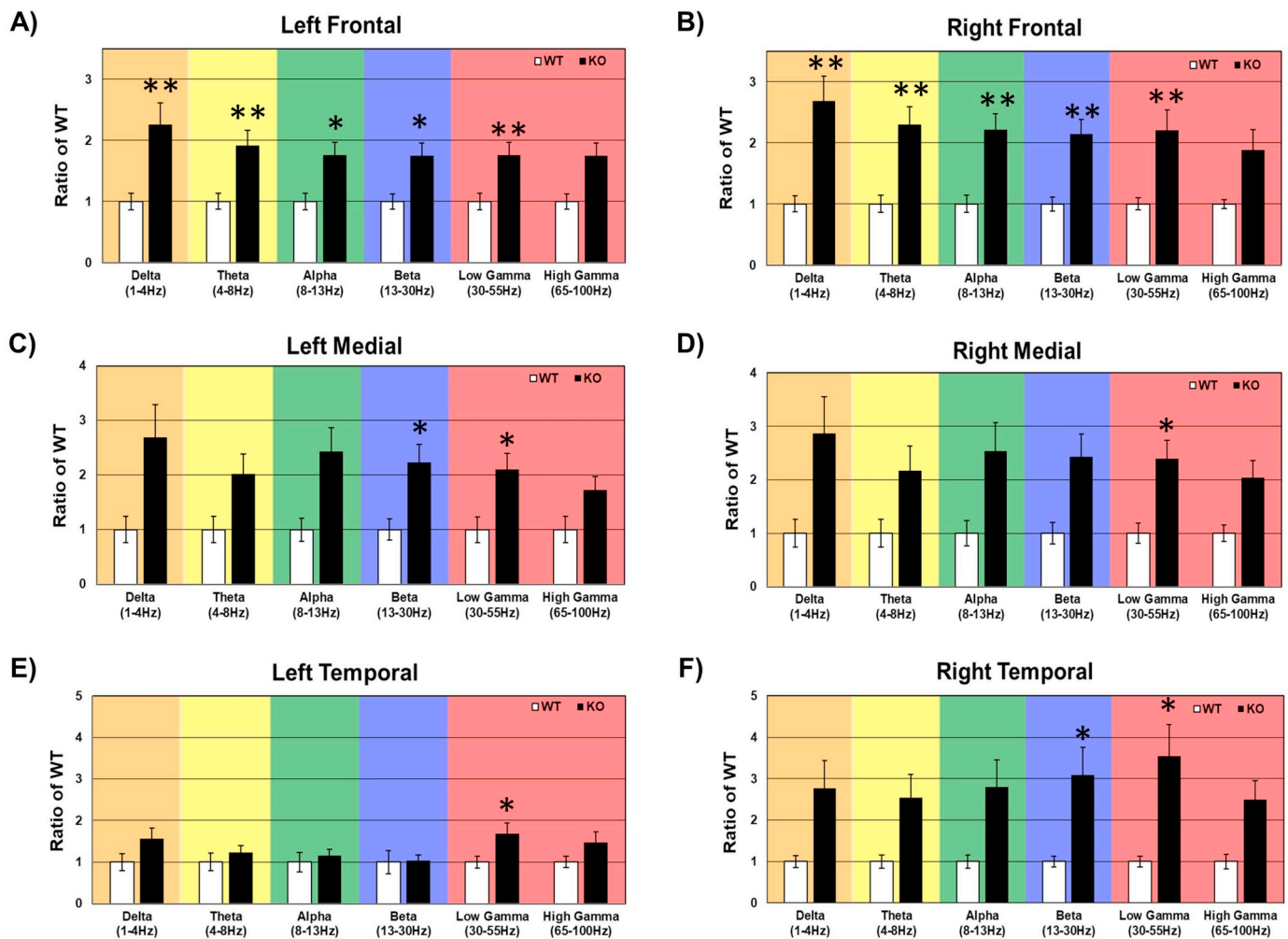


Fig. 4. Quantitation of ratio of *Fmr1* KO to WT EEG power across frequency bands for distinct brain regions (A–F). Values above 1 indicate higher EEG power in *Fmr1* KO compared with WT mice. * $p < .05$; ** $p < .01$. $n = 11$ *Fmr1* KO, $n = 10$ WT mice.

2.6. Statistical analyses

The term ‘resting’ is used to indicate EEGs recorded in these mice without any specific auditory stimuli. The data were analyzed for 2 factors: Genotype (WT, *Fmr1* KO) and Frequency (delta to gamma) for the cortical regions (left frontal, right frontal, left medial, right medial, left temporal and right temporal). We analyzed the raw data using 2-way ANOVA. Data were expressed as ratio of WT values to gauge relative differences in various factors using the same scale.

Statistical group comparisons of broadband noise trains and chirp responses (ITPC) were quantified by wavelet analysis. Statistical analysis was conducted by binning time into 625 parts and frequency into 100 parts, resulting in a 100×625 matrix. Non-parametric cluster analysis was used to determine contiguous regions in the matrix that were significantly different from a distribution of 1000 randomized Monte Carlo permutations based on previously published methods (Maris and Oostenveld, 2007). Briefly, if the cluster sizes of the real genotype assignments (both positive and negative direction, resulting in a two-tailed alpha of $p = .025$) were larger than 97.25% of the random group assignments, those clusters were considered significantly different between genotypes. This method avoids statistical assumptions about the data and corrects for multiple comparisons.

In all cases where genotype means are reported, SEM was used. In all cases, p values $< .05$ were considered significant for ANOVA and Student's t -tests. Where t -tests were performed, r was calculated as an effect size. When interactions were found and multiple comparisons for

ANOVA were made, data were analyzed on each factor for simple effects and corrected for using Bonferroni adjustments. If assumptions of sphericity were violated for repeated measures ANOVA, the Greenhouse-Geiser correction was used.

3. Results

3.1. Multielectrode array analysis of resting EEG

We developed a method for stable chronic *in vivo* implantation of a planar MEA on the surface of the mouse skull (Fig. 1) (Jonak et al., 2018). This can then be used for baseline and stimulus-evoked EEG acquisition in awake, freely moving mice. Features of our protocol include: (1) standardized implantation procedure; (2) reproducible placement of probe over the skull surface with bregma as reference; (3) Teflon/plastic wrap protective layer for the MEA probe to enable reusability; (4) secure implantation with dental cement and screw fixation; (5) fixation of the headstage with an anchoring “post”; (6) use of commutator to allow free movement of the mouse and cables without restriction; (7) reproducible artifact-free 30-channel EEG; and (8) reusability of the MEA probes. With this method, we reliably obtain 30-channel low-noise EEG from awake mice. Resting and stimulus-evoked EEG recordings can be readily obtained and analyzed. In the current study, we applied this MEA technology to analyze resting and stimulus-evoked EEG in the Fragile X mouse model, *Fmr1* KO mice. An example of resting 30-channel MEA EEG obtained from WT vs. *Fmr1* KO mice is

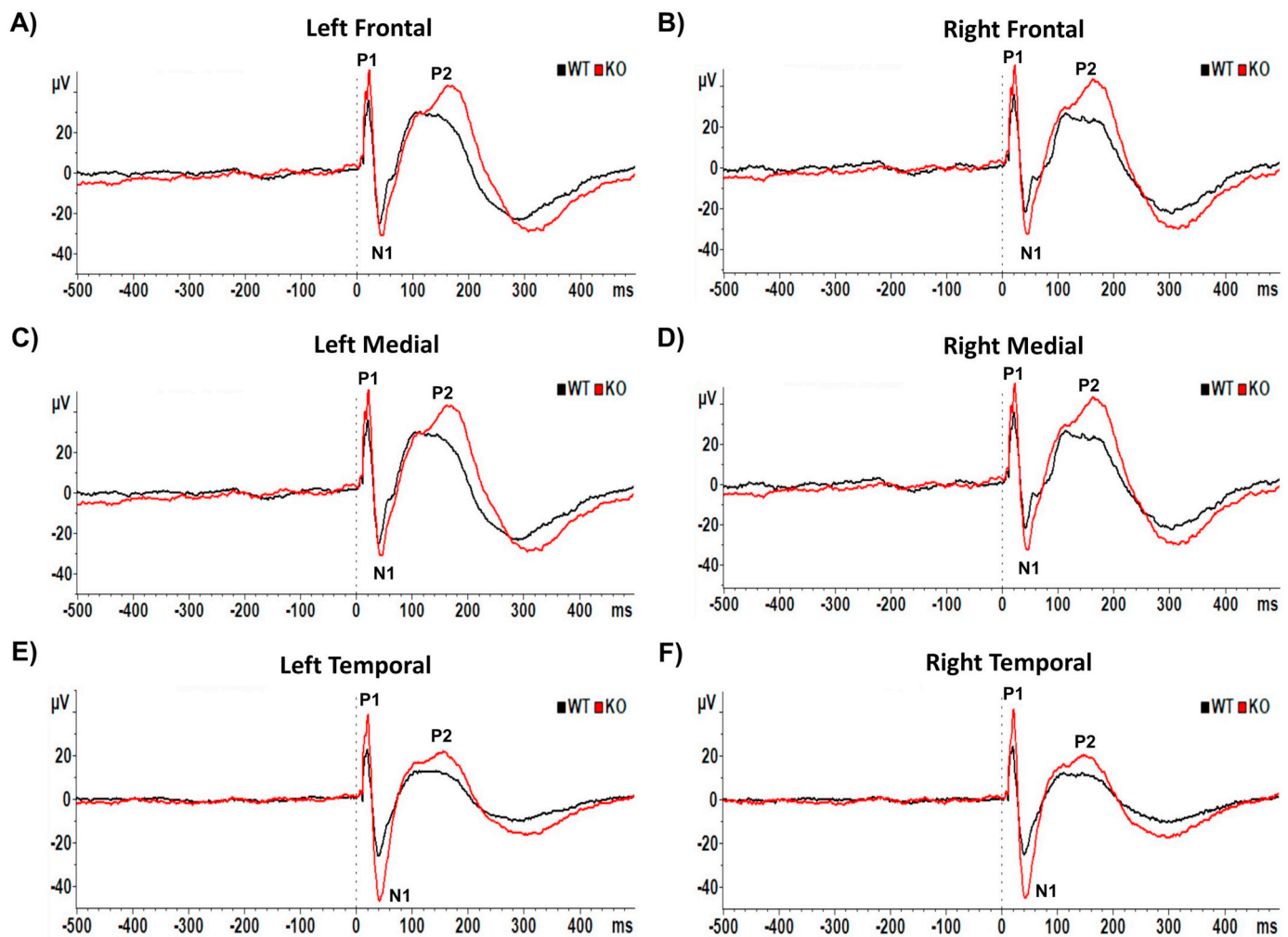


Fig. 5. Multielectrode array analysis of auditory event-related potentials (ERPs) in WT vs. *Fmr1* KO mice. Single ERP peak analysis was done by obtaining average traces across the first response of all BBN trains (total of 200 trials). Peaks were determined by pre-defined time windows after stimulus onset: P1 (10–30 ms), N1 (30–75 ms), and P2 (75–150 ms). WT and *Fmr1* KO ERP morphologies are shown by brain region.

shown in Fig. 2. This demonstrates that low-noise resting MEA EEG can be obtained, and qualitative visual comparison suggests a difference in amplitude and frequency characteristics of the resting EEG between WT and *Fmr1* KO mice. To further evaluate spectral characteristics of baseline MEA EEG in WT vs. *Fmr1* KO mice, we performed power spectral density analysis across brain regions (left frontal, right frontal, left medial, right medial, left temporal, right temporal). These six regions (Fig. 1B) were empirically defined electrode clusters by region to allow group comparisons, although of course any combination of the 30 electrodes could be used or compared if desired. *Fmr1* KO mice were found to have higher resting EEG power across frequency bands (Fig. 3). Interestingly, there was a trend toward higher resting EEG power in the right hemisphere compared with the left hemisphere in *Fmr1* KO mice, especially in the temporal region (Fig. 3F vs. Fig. 3E).

Resting EEG data were then analyzed with two-way ANOVA for 2 factors: Genotype (WT, *Fmr1* KO) and Frequency (delta to gamma) for the cortical regions (left frontal, right frontal, left medial, right medial, left temporal and right temporal) (Fig. 4). In the left frontal region, significant increases in EEG power in *Fmr1* KO mice were found in delta, theta, alpha, beta, and low gamma frequency bands (Fig. 4A). In the right frontal region, increases in delta, theta, alpha, beta, and low gamma power were found as well (Fig. 4B). In the left medial region, significant increases in EEG power in *Fmr1* KO mice were found in beta and low gamma bands (Fig. 4C). In the right medial region, a significant increase was found just in low gamma power (Fig. 4D). In the left

temporal region, a significant increase was found just in low gamma power (Fig. 4E). In the right temporal region, significant increases in beta and low gamma power was observed (Fig. 4F). These data are based on MEA analyses of $n = 11$ *Fmr1* KO and $n = 10$ WT mice. These data show significant increases in EEG power in distinct frequency bands and different cortical areas in awake and freely moving *Fmr1* KO mice compared to WT mice.

3.2. Increased amplitude of auditory ERPs in *Fmr1* KO mice

Next, we aimed to detect and analyze auditory event-related potentials (ERPs) using our *in vivo* MEA system. Indeed, we were able to record auditory ERPs from 1 Hz broadband noise with normal morphologies, as well as identify and quantify P1, N1, and P2 components (Fig. 5). We subsequently analyzed ERP component amplitudes and latencies by brain region in the same WT and *Fmr1* KO mice that were subjected to resting EEG analysis ($n = 11$ *Fmr1* KO, $n = 10$ WT). In *Fmr1* KO mice, significant increases were found in left frontal P1 amplitude (Fig. 6A), left frontal P2 latency (Fig. 6B), left and right medial N1 amplitudes (Fig. 6C), left temporal P1, N1, and P2 amplitudes and right temporal N1 and P2 amplitudes (Fig. 6E). Therefore, we did find increased ERP N1 amplitudes described previously in other studies (Lovelace et al., 2018) but interestingly these were region-specific, with significantly increased N1 amplitudes in the medial and temporal regions, but not the frontal region.

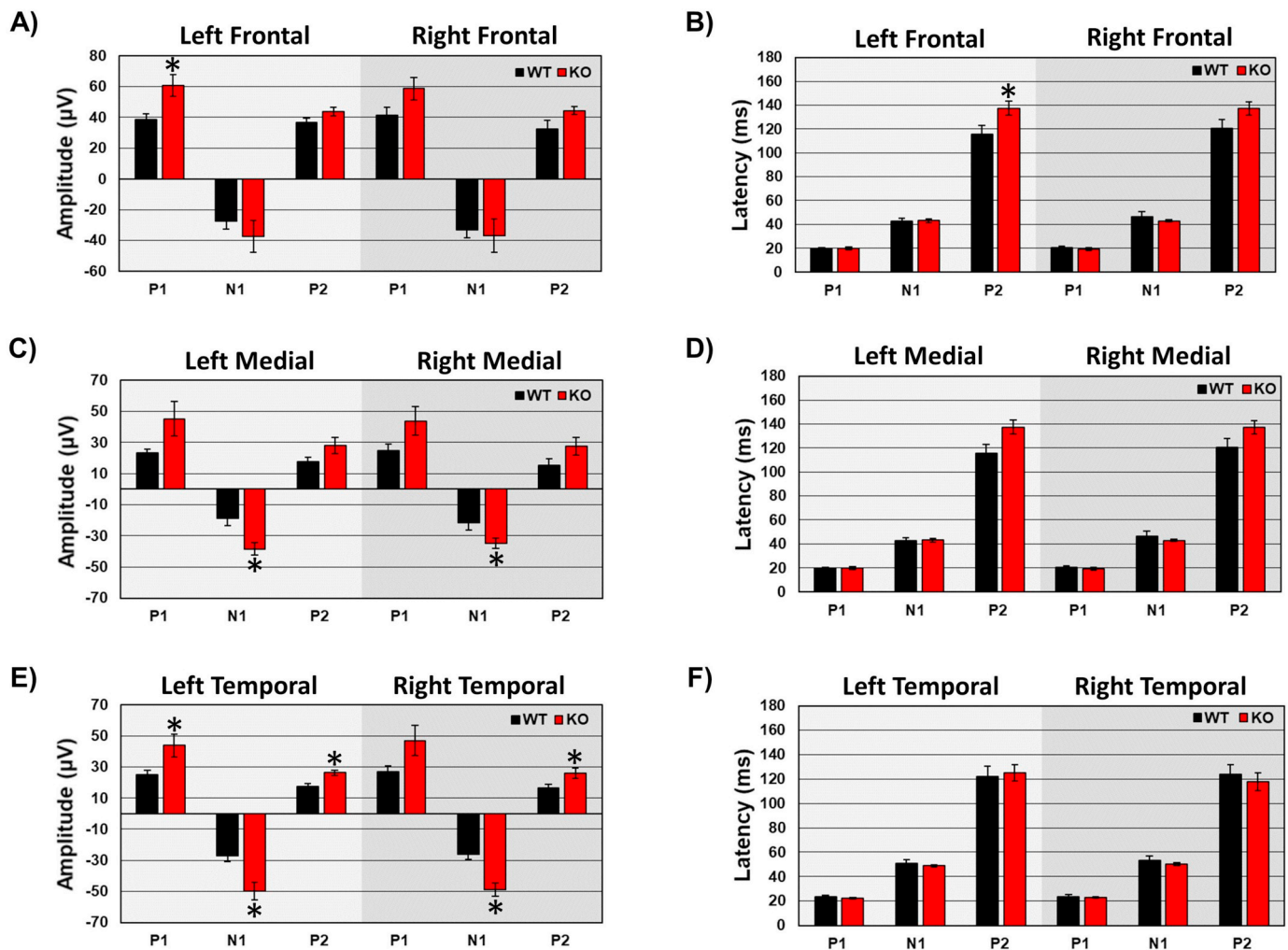


Fig. 6. Quantitation of auditory event-related potential (ERP) amplitudes and latencies in WT vs. *Fmr1* KO mice. In *Fmr1* KO mice, significant increases were found in left frontal P1 amplitude (A), left frontal P2 latency (B), left and right medial N1 amplitudes (C), left temporal P1, N1, and P2 amplitudes and right temporal N1 and P2 amplitudes (E). * $p < .05$. $n = 11$ *Fmr1* KO, $n = 10$ WT mice.

3.3. Increased event-related power following auditory stimulation in *Fmr1* KO mice

Previous studies found increases in event-related power in *Fmr1* KO mice following auditory stimulation (Lovelace et al., 2019), but potential region-specific differences are not known. Therefore, we analyzed event-related power from the MEA data following auditory stimulation (both single and train stimulation). Event-related power following the first auditory stimulation of each train revealed significant differences between WT and *Fmr1* KO mice. *Fmr1* KO mice demonstrated increased event-related power in all brain regions (Fig. 7A, C, D, E, F) except right frontal (Fig. 7B), indicating that the increased power following a sound stimulus is essentially a brain-wide phenomenon providing important insight into neural correlates of sensory hypersensitivity.

We also analyzed event-related power during entire 1 Hz trains of BBN auditory stimuli. Each BBN stimulus in the train led to a measurable stimulus-related EEG power response (Fig. 8). Significant differences in 1 Hz train event-related power were noted between WT and *Fmr1* KO mice. *Fmr1* KO mice demonstrated increased 1 Hz train event-related power in all brain regions (Fig. 8A–F).

3.4. Reduced phase locking in *Fmr1* KO mice to temporally modulated stimuli

Inter-trial phase coherence (ITPC) (also called phase locking factor) measures the reliability of synchronization of neural responses to repetitions of the auditory chirp stimuli, with the EEG response entrained to the chirp modulation frequency (Fig. 9). In WT mice, there was a robust response to the chirp stimulus throughout the brain regions studied (Fig. 9A–F, left panels). In *Fmr1* KO mice, ITPC following chirp stimulation was significantly decreased in all brain regions (Fig. 9A–F, middle and right panels). In particular, ITPC in *Fmr1* KO mice appeared to be decreased in a specific frequency band ranging from approximately 40–60 Hz (low gamma range) (Fig. 9A–F, right panels). Thus, there was a marked deficit in phase locking to the frequency modulated auditory stimulus in *Fmr1* KO mice throughout the brain, particularly in the low gamma range.

4. Discussion

In this paper, we used 30-channel mouse skull surface MEA in *Fmr1* KO mice vs. WT mice for the first time and generated several novel findings. First, *Fmr1* KO mice were found to have higher resting EEG power across multiple frequency bands. Second, the specific frequency bands (delta, theta, alpha, beta, and low gamma) that demonstrated

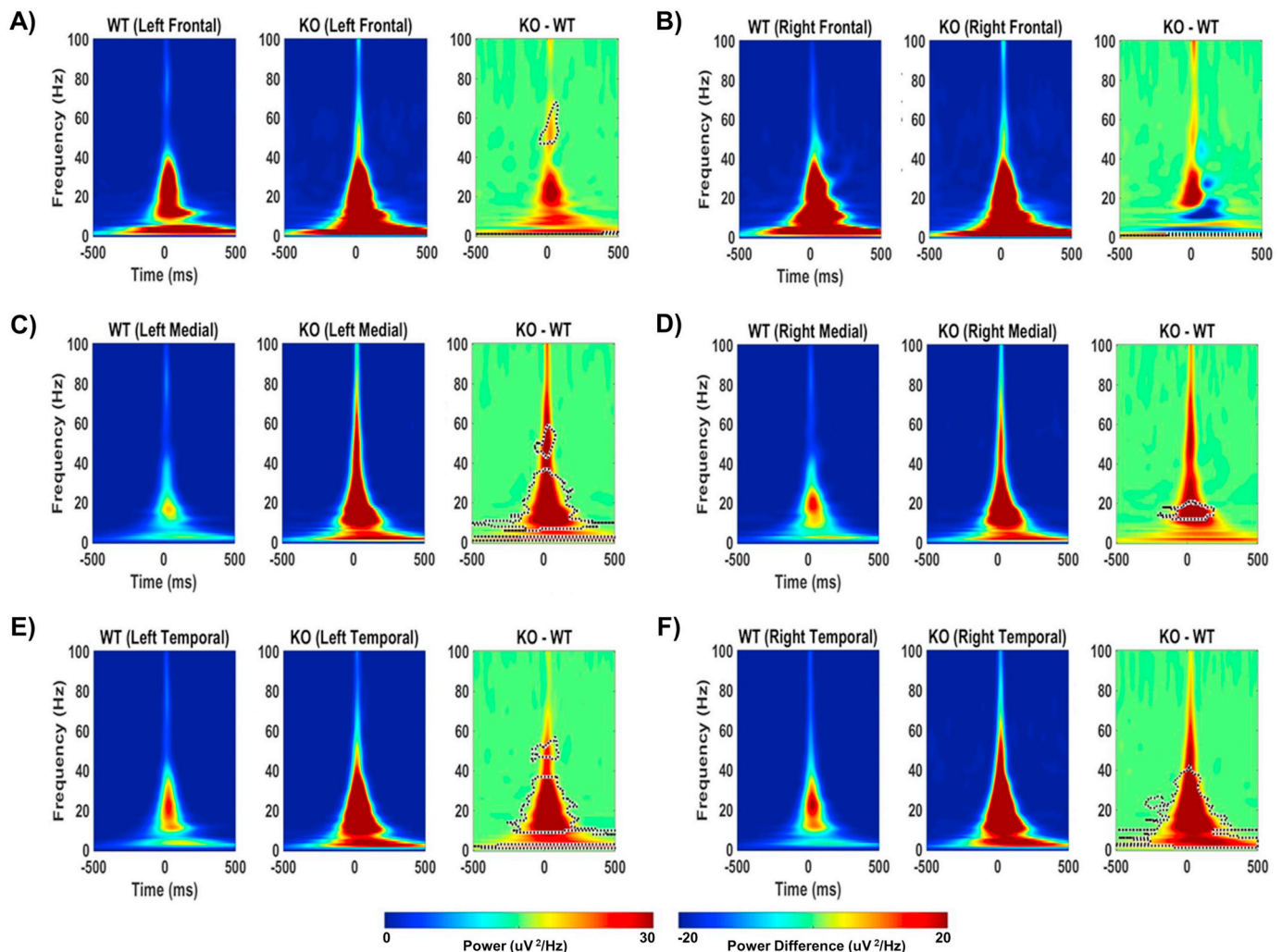


Fig. 7. Single-trial event-related power following auditory stimulation in WT vs. *Fmr1* KO mice. For each brain region (A–F), the left panel shows the averaged WT event-related power, the middle panel shows the averaged *Fmr1* KO event-related power and the right panel shows KO–WT (subtraction). Scales at the bottom show power and power difference in $\mu\text{V}^2/\text{Hz}$. Significant increases in event-related power in *Fmr1* KO compared to WT mice are outlined in black-dotted areas.

increased resting EEG power in *Fmr1* KO mice were region-specific. Third, significant region-specific alterations in ERP component amplitudes were observed in *Fmr1* KO mice. Fourth, *Fmr1* KO mice demonstrated increased sound-induced power in all brain regions. Fifth, inter-trial phase coherence to auditory “chirp” stimuli was significantly decreased in all brain regions in *Fmr1* KO mice. These findings provide a robust set of EEG phenotypes that are likely neural correlates of sensory hypersensitivity in FXS, and indicate remarkably similar EEG phenotypes in *Fmr1* KO mice and humans with FXS.

4.1. Genotype differences in resting EEG power spectral density

Our previous studies demonstrated increased resting state gamma (30–100 Hz) power in auditory and frontal cortex of *Fmr1* KO mice (Lovelace et al., 2018, 2019). Enhanced resting gamma power is consistent with a previous study of *Fmr1* KO mice (Sinclair et al., 2017a). Our current findings demonstrate widespread increases in resting EEG power across multiple frequency bands in *Fmr1* KO mice (Fig. 3). This indicates that the increase in resting EEG power is a global brain-wide phenomenon in *Fmr1* KO mice. Interestingly, changes in EEG power were not limited to the gamma frequency. It is possible that the 30-channel MEA provides much greater sensitivity to the EEG changes at lower frequencies than prior studies (Lovelace et al., 2018; Sinclair et al., 2017a). In the frontal region, for example, increases in EEG

power in *Fmr1* KO mice were observed in delta, theta, alpha, beta, and low gamma bands (Fig. 4A, B). Furthermore, these increases were region-specific, with medial and temporal regions exhibiting significant increases in low gamma (Fig. 4E, F). In addition, increases in EEG power in *Fmr1* KO mice appeared more pronounced in right temporal vs. left temporal regions (Fig. 3F vs. Fig. 3E). In this initial report with the MEA technique, we elected to group 30-channel data into these 6 “regions” to make these comparisons, but clearly it is feasible to individually analyze each of the 30 single channels if desired, other anatomic groupings and/or hemispheric lateralization (Fig. 1B). Future studies will also be able to test whether these resting EEG changes are normalized with pharmacological treatment.

4.2. Genotype differences in ERPs and event-related power

Studies of humans with FXS have demonstrated alterations in ERPs including increased N1 amplitude (Castrén et al., 2003; Van der Molen et al., 2012). Our previous 2-channel study in the *Fmr1* KO mice demonstrated increased N1 amplitude and increased event-related power (Wen et al., 2019). Interestingly, in the current MEA study we found significantly increased N1 amplitudes in the medial and temporal regions bilaterally but not the frontal region (Figs. 5 and 6). ERP latencies were not found to be different except for left frontal P2 (Fig. 6B). In contrast, event-related power was significantly higher in all brain

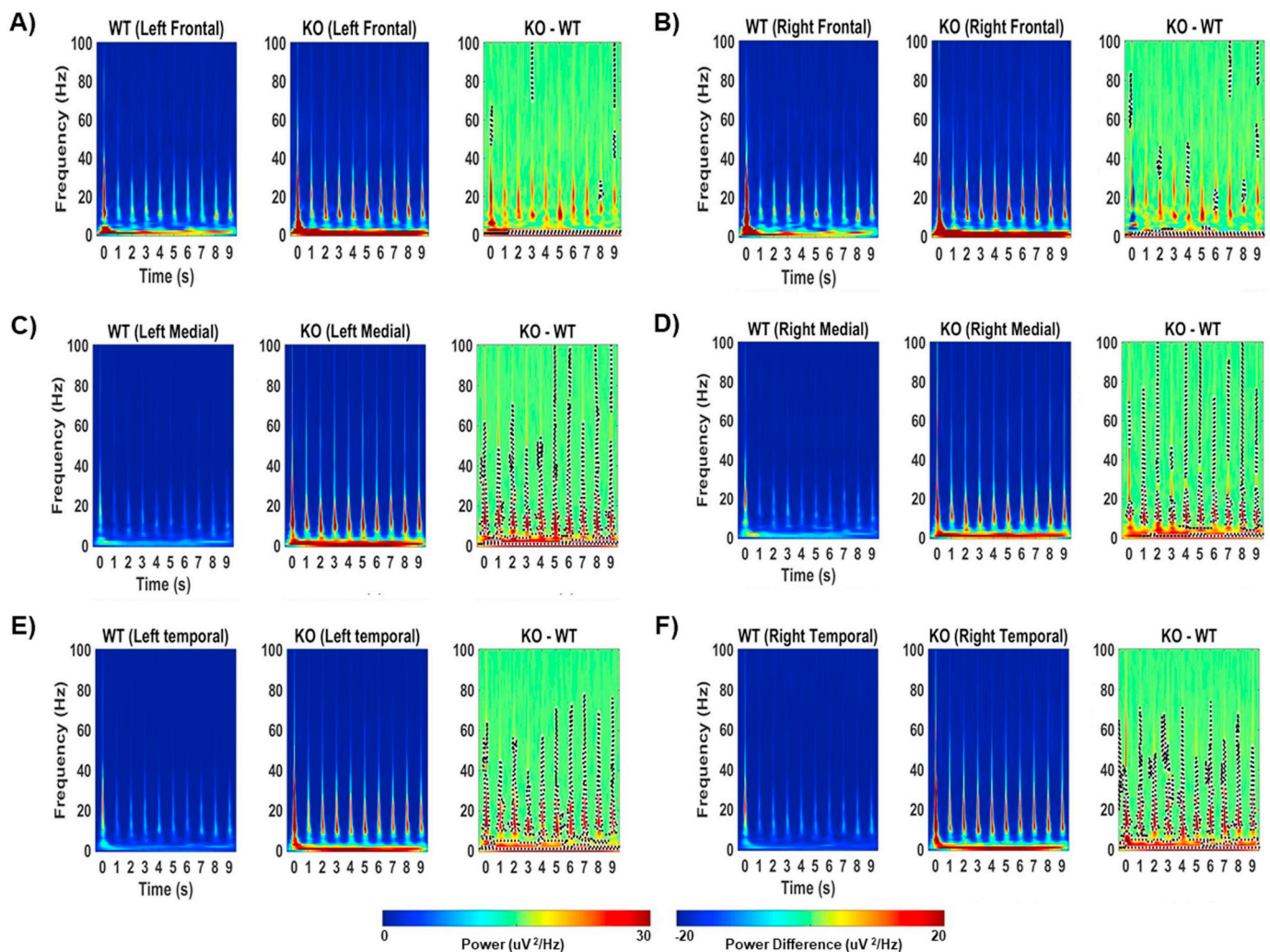


Fig. 8. Event-related power following 1 Hz train of auditory stimulation in WT vs. *Fmr1* KO mice. For each brain region (A–F), the left panel shows the averaged WT event-related power, the middle panel shows the averaged *Fmr1* KO event-related power and the right panel shows KO–WT. Scales at the bottom show power and power difference in $\mu\text{V}^2/\text{Hz}$. Significant increases in event-related power in *Fmr1* KO compared to WT mice are outlined in black-dotted areas.

regions except right frontal when assessed as single-trial power (Fig. 7) and in *all* brain regions in response to 1 Hz stimulus trains (Fig. 8). Thus, event-related power (either single stimulus or train) may be a more reliable EEG biomarker than ERP amplitudes and latencies to distinguish WT and *Fmr1* KO mice. Furthermore, we note that event-related power data are not dependent on ERP morphology characteristics but rather show the overall “power” response of the brain to the auditory stimulus. As such, frequency characteristics can also be distinguished which provide more information than ERP amplitude and latency.

Enhancement of ERP N1 amplitude and event-related power is likely to be related to underlying neurophysiological response magnitudes to a given auditory stimulus. Previous studies in the adult *Fmr1* KO mice auditory cortex showed enhanced responses to tones and broader frequency tuning of single neurons (Rotschafer and Razak, 2013). In addition, there is reduced habituation of ERPs to repeated sounds in *Fmr1* KO mice as compared to WT mice (Lovelace et al., 2016). Together, these data indicate that for any sound, more neurons will respond with a greater response magnitude over a sustained period. The observations of enhanced ERP amplitude as well as single-trial and 1 Hz train-related power are consistent with this interpretation. Overall, these and our previous results (Lovelace et al., 2018, 2019) in auditory cortex are similar to other findings of sensory hyperexcitability due to circuit changes in *Fmr1* KO mice. For example, there is an abnormally large

size of whisker-evoked somatosensory cortical maps in adult *Fmr1* KO mice (He et al., 2019). Various circuit alterations including single cell response magnitudes, developmental changes involving inhibitory circuitry and overall circuit synchrony may account for these changes (Berzhanskaya et al., 2017; Contractor et al., 2015; Goncalves et al., 2013; Sinclair et al., 2017b; Talbot et al., 2018; Wen et al., 2018).

4.3. Genotype differences in evoked neural synchronization

Recent EEG studies of humans with FXS have reported enhanced resting gamma power, reduced chirp-evoked phase locking in gamma frequencies and enhanced single-trial power (Ethridge et al., 2017; Wang et al., 2017). These EEG abnormalities were correlated with clinically relevant measures including heightened sensory sensitivity and autism-associated social impairment (Social Communication Questionnaire), indicating translational relevance (Ethridge et al., 2017). Given the robust recent human data indicating reduced chirp-evoked phase locking in gamma frequencies, we aimed to determine whether this could be reliably observed in the *Fmr1* KO mice with MEA analysis. Indeed, we observed a marked impairment in ITPC or “phase-locking” to chirp stimuli in *Fmr1* KO mice (Fig. 9) indicating reduced ability to synchronize to the frequency-modulated stimulus. ITPC following auditory chirp stimulation was significantly decreased in *all* brain regions. Interestingly, this appeared to be frequency-specific, with

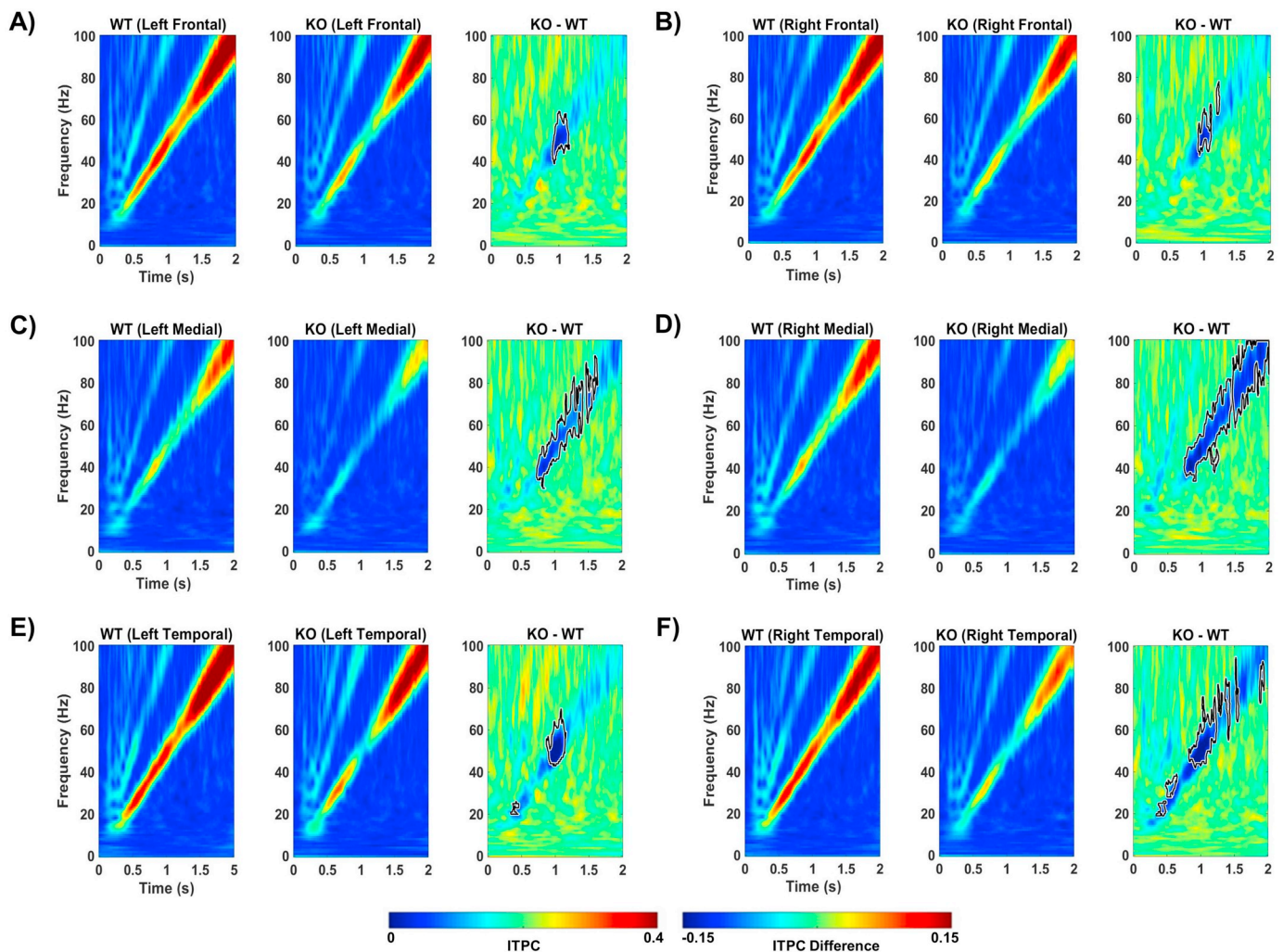


Fig. 9. Multielectrode array analysis of auditory chirp stimulation in WT vs. *Fmr1* KO mice. For each brain region (A–F), the left panel shows the averaged WT inter-trial phase coherence (ITPC or phase-locking factor), the middle panel shows the averaged *Fmr1* KO ITPC and the right panel shows KO–WT. Scales at the bottom show ITPC and ITPC difference in $\mu\text{V}^2/\text{Hz}$. Significant decreases in ITPC in *Fmr1* KO compared to WT mice are shown in black-outlined areas. Blue areas in the right panels (KO–WT) are negative ITPC differences since ITPC values in KO are less than WT mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the greatest deficit in phase locking in the 40–60 Hz range (Fig. 9). Thus, ITPC following auditory chirp stimulation may be a reliable EEG biomarker to distinguish stimulus-induced brain responses in WT vs. *Fmr1* KO mice and in human studies (Ethridge et al., 2017).

4.4. Parallel EEG abnormalities in *Fmr1* KO mice and FXS humans

Several parallel EEG abnormalities have been observed in both humans with FXS and *Fmr1* KO mice: (1) enhanced resting gamma power; (2) enhanced single-trial power; and (3) reduced phase-locking to chirp stimuli. We hypothesize that this combination of factors may be associated with both sensory hypersensitivity and also reduced auditory stimulus discrimination more generally. Previous studies have found deficits in auditory information processing in FXS patients, such as impaired auditory stimulus discrimination (Van der Molen et al., 2012). Our model would predict that enhanced resting gamma (in the unstimulated brain) constitutes background “gamma noise” that would impair synchronized response to a stimulus, particularly in the gamma range. Hence, the ITPC deficit appears particularly in the low gamma range, where we observed significant increase in resting EEG power. Increased single-trial power, which we also observe in parallel with the human data, may relate to auditory cortical hyperexcitability; however,

an exaggerated cortical response to individual stimuli together with background “gamma noise” would presumably be maladaptive in generating temporally synchronized responses to rapid auditory stimuli. This hypothesis could be tested by determining whether pharmacological interventions that ameliorate the above EEG abnormalities also improve auditory stimulus discrimination. Proof-of-principle for this comes from a study in which racemic baclofen, a GABA_B agonist, was able to improve working memory and anxiety-related behavior in conjunction with reduction in auditory-evoked gamma oscillations (Sinclair et al., 2017a). Alternatively, selective manipulation of inhibition could be employed as has been done recently to restore parvalbumin neuron function in *Fmr1* KO mouse visual cortex resulting in improvement in visual discrimination (Goel et al., 2018).

The similarity in EEG measures between humans and mice indicate that EEG/ERP recordings can serve as objective, physiological probes that serve as surrogate biomarkers to develop therapeutics to treat symptoms of FXS (Schneider et al., 2013; Sinclair et al., 2017a). More broadly, increased high-frequency EEG power has been found in autism spectrum disorders (Orekhova et al., 2007; Sinclair et al., 2017b; Wang et al., 2013). Low-frequency EEG abnormalities have also been found in humans with FXS with enhanced theta band power relative to healthy controls (Van der Molen and Van der Molen, 2013b; Wang et al., 2017).

These similarities in EEG measures between humans and mice suggest the importance of studying basic sensory processing using analogous experimental design and approach in generating translation-relevant biomarkers (Sinclair et al., 2017b). These measures may be useful outcome measures in the preclinical to clinical drug development pipeline and can be also employed in stratification of patient population for appropriate treatment strategies using a combination of EEGs and pharmacology. In particular, the finding that a particular drug candidate has robust effects on normalizing EEG parameters such as resting gamma power and phase-locked synchronization in both humans and mice would enable targeting and correlation of those drugs with clinical parameters (Berry-Kravis et al., 2018).

We envision MEA EEG analysis in particular to be useful in several distinct contexts in mouse models of neurodevelopmental disorders (NDD) (Ewen et al., 2019; Vasa et al., 2016). (1) *Further delineation of EEG biomarkers*. Simply having 30 channels to compare as opposed to 2 channels in our previous studies has enabled more region-specific analysis. Distinct stimulus parameters could also be used with the same MEA system, such as visual evoked potentials (VEPs) (Land et al., 2019). Furthermore, more complex EEG parameters can be derived from the 30-channel MEA data. In addition to resting EEG power, ERP amplitudes and latencies, single-trial power and ITPC as reported in this study, the availability of 30 channels with a well-defined spatial orientation (Fig. 1) enables spatial analysis of EEG signal propagation across the cortical surface as well as analysis of phase synchronization (Jonak et al., 2018; Wang et al., 2013), cross-frequency amplitude coupling, and phase-amplitude coupling (Munia and Aviyente, 2019). Altered theta-gamma coupling and increased phase synchronization were observed in FXS subjects (Wang et al., 2017), parameters which can now be derived with mouse MEA analysis. Altered cross-frequency coupling has been correlated to cognition in several other contexts (Munia and Aviyente, 2019; Rodriguez-Larios and Alaerts, 2019) and is feasible with high-density mouse MEA data. Derivation of such measures in *Fmr1* KO mice or other animal models of FXS (Dahlhaus, 2018) may uncover additional EEG biomarkers specific to FXS pathophysiology. In addition, application of MEA to other models of NDD may similarly enable identification of translational EEG biomarkers. For example, in Rett syndrome (RTT), specific changes in EEG spectral power have been correlated with lower cognitive assessment scores (Roche et al., 2019); thus, use of MEA EEG in animal models of RTT may enable identification of parallel MEA EEG biomarkers in RTT animal models, enabling use as therapeutic targets. Another example is Angelman syndrome, in which delta rhythmicity (Sidorov et al., 2017) and gamma coherence during sleep (den Bakker et al., 2018) have been associated with the disease state. (2) *Pharmacological studies*. We have demonstrated feasibility in affixing the 30-channel MEA to the skull surface of awake, behaving mice for several weeks (Jonak et al., 2018) allowing both acute and chronic studies of drug effects on EEG parameters/biomarkers in *Fmr1* KO mice (or for any other mouse model). Each candidate drug could then be assessed for its efficacy in normalizing MEA EEG parameters with both acute and chronic administration. (3) *Mechanistic studies*. Combined use of the MEA EEG system with genetic mouse models and/or DREADDs could enable dissection of cell type-specific contributions to EEG abnormalities in FXS and in NDD more generally. For example, selective deletion of *Fmr1* from forebrain excitatory neurons has recently been found to increase resting EEG gamma power (Lovelace et al., 2019); MEA analysis with parameters described above could assess the effects of multiple distinct cell-type-specific deletions on specific MEA EEG parameters.

In summary, we have employed MEA EEG analysis in *Fmr1* KO vs. WT mice for the first time. These studies have revealed specific EEG biomarkers in this FXS mouse model. We believe that the MEA system will serve as a robust method for further definition of EEG biomarkers in diverse disorders as well as an enabling technology for mechanistic and therapeutic studies.

Declaration of Competing Interest

None.

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RESEARCH ARTICLE | Nervous System Pathophysiology

Abnormal development of auditory responses in the inferior colliculus of a mouse model of Fragile X Syndrome

Anna O. Nguyen,¹ Devin K. Binder,^{2,3} Iryna M. Ethell,^{2,3} and Khaleel A. Razak^{2,4}

¹Bioengineering Program, University of California, Riverside, California; ²Graduate Neuroscience Program, University of California, Riverside, California; ³Division of Biomedical Sciences, University of California, Riverside, California; and ⁴Psychology Department, University of California, Riverside, California

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Nguyen AO, Binder DK, Ethell IM, Razak KA. Abnormal development of auditory responses in the inferior colliculus of a mouse model of Fragile X Syndrome. *J Neurophysiol* 123: 2101–2121, 2020. First published April 22, 2020; doi:10.1152/jn.00706.2019.—Sensory processing abnormalities are frequently associated with autism spectrum disorders, but the underlying mechanisms are unclear. Here we studied auditory processing in a mouse model of Fragile X Syndrome (FXS), a leading known genetic cause of autism and intellectual disability. Both humans with FXS and the *Fragile X mental retardation gene* (*Fmr1*) knockout (KO) mouse model show auditory hypersensitivity, with the latter showing a strong propensity for audiogenic seizures (AGS) early in development. Because midbrain abnormalities cause AGS, we investigated whether the inferior colliculus (IC) of the *Fmr1* KO mice shows abnormal auditory processing compared with wild-type (WT) controls at specific developmental time points. Using antibodies against neural activity marker c-Fos, we found increased density of c-Fos+ neurons in the IC, but not auditory cortex, of *Fmr1* KO mice at P21 and P34 following sound presentation. In vivo single-unit recordings showed that IC neurons of *Fmr1* KO mice are hyperresponsive to tone bursts and amplitude-modulated tones during development and show broader frequency tuning curves. There were no differences in rate-level responses or phase locking to amplitude-modulated tones in IC neurons between genotypes. Taken together, these data provide evidence for the development of auditory hyperresponsiveness in the IC of *Fmr1* KO mice. Although most human and mouse work in autism and sensory processing has centered on the forebrain, our new findings, along with recent work on the lower brainstem, suggest that abnormal subcortical responses may underlie auditory hypersensitivity in autism spectrum disorders.

NEW & NOTEWORTHY Autism spectrum disorders (ASD) are commonly associated with sensory sensitivity issues, but the underlying mechanisms are unclear. This study presents novel evidence for neural correlates of auditory hypersensitivity in the developing inferior colliculus (IC) in *Fmr1* knockout (KO) mouse, a mouse model of Fragile X Syndrome (FXS), a leading genetic cause of ASD. Responses begin to show genotype differences between postnatal days 14 and 21, suggesting an early developmental treatment window.

autism; development; Fragile X Syndrome; inferior colliculus; seizures; sensory hypersensitivity

INTRODUCTION

Fragile X Syndrome (FXS) is a leading genetic cause of intellectual disability and autism that affects ~1 in 4,000 males and 1 in 8,000 females. An expansion of CGG repeats in the 5' untranslated region of the *Fragile X mental retardation 1* (*Fmr1*) gene causes its silencing and a loss of Fragile X Mental Retardation Protein (FMRP). FMRP is an RNA binding protein involved in translation regulation. The resulting abnormal protein synthesis in the brain during development leads to symptoms of FXS that include cognitive, anxiety and social deficits, hyperactivity, language impairments, increased susceptibility to seizures, and sensory impairments (Smith et al. 2012). A consistent and debilitating symptom of FXS is abnormal sensory reactivity (Rais et al. 2018), particularly hypersensitivity to sensory stimuli (Hitoglou et al. 2010; Rogers et al. 2003). Hypersensitivity manifests strongly in the auditory domain, but the underlying mechanisms are only beginning to be elucidated (Ethridge et al. 2016; Garcia-Pino et al. 2017; Wang et al. 2017).

An animal model of FXS, the *Fmr1* knockout (KO) mouse, displays several core FXS-like phenotypes including hyperactivity, social abnormalities, electrophysiological, and dendritic spine deficits (Bakker and Oostra 2003; Kazdoba et al. 2014). Importantly, the *Fmr1* KO mouse also shows auditory hypersensitivity, including enhanced cortical responses to sounds, increased propensity for AGS, and abnormal sensorimotor gating (Chen and Toth 2001; Frankland et al. 2004; Kokash et al. 2019; Rotschafer and Razak 2013; Wen et al. 2018). Electroencephalographic (EEG) recordings demonstrate remarkably similar phenotypes in the *Fmr1* KO mouse and humans with FXS (Castrén et al. 2003; Ethridge et al. 2017; Lovelace et al. 2016, 2018; Schneider et al. 2013). These include increased amplitude and reduced habituation of sound-evoked responses, increased resting gamma power, and reduced consistency in phase locking to amplitude-modulated sounds. In vivo single unit and EEG recordings from the auditory cortex of *Fmr1* KO mice show abnormally increased responses to sounds during development and in adults (Kulinich et al. 2020; Rotschafer and Razak 2013; Wen et al. 2018, 2019).

FMRP is expressed at multiple levels of the auditory system, and deficits in auditory processing are reported in the lower brainstem of the *Fmr1* KO mice (Beebe et al. 2014; Garcia-

Correspondence: K. A. Razak (Khaleel@ucr.edu).

Pino et al. 2017; Rotschafer and Cramer 2017; Rotschafer et al. 2015; Wang et al. 2014). A recent study showed that selective deletion of *Fmr1* from forebrain excitatory neurons only partially recapitulates cortical EEG phenotypes and showed that increased resting state low (30–60 Hz)- but not high (70–100 Hz)-gamma power reported in global *Fmr1* KO is present in the forebrain-specific knockout (Lovelace et al. 2020). These studies suggest that at least some of the abnormal responses recorded in the cortex are generated subcortically.

Audiogenic seizure (AGS) is a robust and consistently reported phenotype in the *Fmr1* KO mice, particularly between postnatal day (P)20 and P30 (Dölen et al. 2007; Michalon et al. 2012; Musumeci et al. 2007; Pacey et al. 2009; Yan et al. 2005). Multiple studies have suggested that abnormalities of the midbrain inferior and superior colliculi (IC, SC) underlie specific patterns of sensitivity and behaviors associated with AGS (Faingold and Randall, 1999; Faingold 2002; Millan et al. 1986). Indeed, Gonzalez et al. (2019) showed that AGSs are induced by *Fmr1* deletion in VGlut2-expressing excitatory neurons in the inferior colliculus (IC). We recently found elevated levels of matrix metalloproteinase-9 in the developing IC of *Fmr1* KO mice compared with wild-type (WT) mice and deficits in prepulse inhibition of acoustic startle in *Fmr1* KO mice (Kokash et al. 2019), further suggesting early developmental deficits in IC. These studies suggest that responses IC neurons of *Fmr1* KO mice are abnormal during development and may underlie auditory hypersensitivity. The main goal of this study was to test this hypothesis and to identify developmental changes in IC responses to sound in *Fmr1* KO mice.

We used two different methods to quantify IC auditory responses in WT and *Fmr1* KO mice. The first aim was to use immunostaining for c-Fos to determine the number of activated cells in the IC, auditory thalamus, and auditory cortex at P21 and P34. We found a significantly higher number of activated cells in the IC of the *Fmr1* KO compared with WT mice. Second, we performed *in vivo* single unit recordings from IC at P14, P21, and P34 to determine minimum thresholds, response magnitudes, frequency tuning, and responses to amplitude-modulated tones. We report significant genotype differences of IC responses that correlate with the development of auditory hyperresponsiveness in *Fmr1* KO mice.

METHODS

Animals. All animal procedures were approved by the University of California, Riverside Institution Animal Care and Use Committee. Breeding pairs of FVB.129P2-Fmr1tm1Cgr/J (*Fmr1* KO) and their congenic controls (WT) mice were obtained from Jackson Laboratories and bred in-house. All mice were housed in a 12:12-h light-dark cycle and given standard laboratory chow and water *ad libitum*. Immunostaining against c-Fos was done on the brain slices of P21–22 WT ($n = 16$), P34–39 WT ($n = 23$), P21–22 *Fmr1* KO ($n = 15$), and P34–39 *Fmr1* KO ($n = 23$) mice. Single-unit electrophysiological recordings were obtained from the IC of P14–15 WT ($n = 9$), P21–22 WT ($n = 11$), P34–39 WT ($n = 12$), P14–15 *Fmr1* KO ($n = 10$), P21–22 *Fmr1* KO ($n = 10$), and P34–39 *Fmr1* KO ($n = 9$) mice. These groups will be referred to as P14, P21, and P34 mice below. Male mice were used for all experiments.

Sound exposure paradigm. Our goal was to examine the levels of neural activity marker c-Fos in the auditory pathway of *Fmr1* KO mice in response to relatively loud sounds but without any overt motor behaviors associated with AGS. AGS behaviors include wild running and jumping and tonic seizure episodes that may lead to death (Dansie

et al. 2013; Gonzalez et al. 2019). *Fmr1* KO mice, but not WT mice, are prone to AGS. Therefore, if the sound causes seizures, the associated motor responses involved would only be present in the KO mice and render the two groups incomparable in terms of sensory responses. Therefore, we performed pilot tests to identify the highest sound level that does not cause any motor responses associated with AGSs. These pilot data showed that the AGS threshold for P34 *Fmr1* KO mice was >90 dB SPL so we used 80 or 90 dB for 15 min (5- to 50-kHz bandwidth, 500-ms upward modulated frequency sweep followed by 500-ms downward modulated frequency sweep). However, 90-dB sounds induced AGSs in the P21 group, so we used 85 dB SPL in this age group with 1,000 ms of quiet in between each 1,000 ms of sound. Based on offline video analysis, none of the mice used showed any motor behaviors associated with AGSs. Therefore, there was no exclusion of mice to account for motor behaviors. To perform c-Fos immunostaining, up to four male mice in the P21 or P34 group were placed in a standard mouse cage with no food or water. Mice used for immunohistochemistry of c-Fos were habituated for 3 h in a sound attenuated booth (Gretch-Ken, Inc.) before stimulus presentation. This would facilitate isolation of c-Fos expression to the stimulus and minimize background c-Fos expression. In addition, these mice remained in the sound attenuation booth for 45 min after offset of the sound stimulus and before transcardial perfusion. Control groups underwent the same procedure except no sounds were presented. Auditory stimuli were generated using custom software (BATLAB, Dr. Don Gans, Kent State University or Sparkle, Portfors Laboratory, Washington State University) and delivered through a programmable attenuator (PA5, TDT) and a speaker (FT17H, Fostex International) placed face down on top of the cage lid. Sound levels were measured with a sound level meter (735, B&K Precision) at a distance from the speaker to the cage bottom. A lamp was used to provide light for a video camera to record behaviors during 5 min of baseline with no sound presentation and 15 min of sound presentation.

Immunohistochemistry. We examined the IC, the auditory thalamus, and core auditory cortex to determine if there are regional differences in c-Fos expression across genotypes. Two age groups of mice were used for the analysis of c-Fos+ cell density: P21 and P34. Mice were euthanized with sodium pentobarbital (Fatal-Plus, 125 mg/kg ip) for perfusion. Transcardial perfusion was done with cold 0.1 M PBS (pH 7.4) followed by cold 4% PFA (pH 7.6). The brain tissues were extracted and postfixed overnight in 4% PFA before storage in 0.1 M PBS at 4°C until further tissue processing in the future. The brains were cryoprotected in 30% sucrose for 2 days before being sectioned (CM 1860, Leica Biosystems) in the coronal plane at 40- μ m thickness. All immunohistochemistry steps were done on a shaker at room temperature unless otherwise noted. For each mouse, two slices for each region of interest (IC, medial geniculate body, and auditory cortex) were used for c-Fos immunohistochemistry. Slices were chosen at ~50% in the rostrocaudal extent of the IC and MGB based on the Allen mouse brain atlas. This allowed for consistency in slice locations across mice. The shape of these nuclei also varies across the rostrocaudal extent, facilitating selection of comparable sections across mice. Consistency across mice in selecting the auditory cortex sections was based on the location of the dentate gyrus of the hippocampus. We have previously validated this method in Martin del Campo et al. (2012). Slices were washed three times for 5 min in 0.1 M PBS followed by blocking with 5% normal goat serum (NGS) for 1 h. Slices were then washed with PBS for 10 min followed by 0.5% triton X-100 for 10 min. Next, the slices were incubated overnight in 4°C in primary rabbit anti-c-Fos antibody [1:100; SC-52, Santa Cruz, Research Resource Identifier (RRID):AB_2106783] in 1% NGS and 0.1% Tween-20 in 0.1 M PBS. This antibody is commonly used in mouse studies (e.g., Howorth et al. 2009; Numa et al. 2019), including in studies of the central auditory system (Fulop et al. 2019). On the next day, the slices were washed three times for 5 min with PBS and incubated in secondary antibody (1:500; donkey anti-rabbit Alexa 594) with 1% NGS and 0.1% Tween 20 in PBS for

2 h. Then, the slices were washed in PBS three times for 5 min and mounted on a glass slide with a mounting medium (Vectashield H-1200, Vector Laboratories) and the edges were sealed (Cytoseal 60, Richard-Allan Scientific). The slides were stored in the dark at 4°C until imaging was done. Stained sections were imaged using a confocal microscope (SP5, Leica Microsystems) with $\times 10$ objective and a stack of 20 optical images was collected with $1,024 \times 1,024$ resolutions at 2- μm z-steps. Image analysis was performed using ImageJ Software (NIH). Because the MGB is composed of multiple divisions with different functions, we evaluated c-Fos-positive cell density in each division separately using Allen Brain Atlas. The dimensions of the windows used for the cell counting in the different divisions of the MGB are provided in Table 1. The sizes of these windows were selected based on sufficient coverage of the divisions of interest across all photomicrographs. A 400- μm wide window that was at 45° angle to the midline of a coronal section was used as the counting window for the IC (Fig. 1, A and B). Large images were stitched as needed using the “stitch” plugin (Preibisch et al. 2009) for ImageJ to obtain high resolution images for counting. A rolling ball background subtraction was done for all images (rolling ball radius = 6.6 μm) facilitating a removal of smooth continuous backgrounds. c-Fos+ cell counts were based on intensity autothresholding of the pixels (Geometric Triangle Function) and size ($>13.2 \mu\text{m}^2$) in ImageJ for all images. The Geometric Triangle Function is an autothresholding feature of ImageJ. This allows consistent thresholding parameters to be applied to all images, thus ensuring uniform thresholding across slices. The triangle algorithm draws a line “b” from the maximum peak to the lowest value in an image’s histogram. An orthogonal line “d” is computed from line “b” to the maximal distance of the histogram. A bin is formed from line “d” to the maximum pixel intensity value. Then, all pixels in the image are converted to binary values (pixels within the bin as determined by the triangle algorithm and pixels not within the bin). Clusters of pixels that is $>20 \text{ px}^2$ ($13.2 \mu\text{m}^2$) are counted as c-Fos+ cells.

In vivo electrophysiology recordings from IC. In vivo extracellular single unit recordings were conducted in urethane (1 g/kg)- and xylazine (20 mg/kg) (intraperitoneal injection)-anesthetized mice. Supplemental doses of anesthesia were given during recording sessions, as needed. A craniotomy was performed using a micro drill (Foredom Electric Co.) with coordinates based on skull landmarks. The IC was identified based on the transverse sinus vein, auditory responses, tonotopy, and post hoc histology from Fluoro-Ruby dye injected in the recording site. A negative feedback rectal thermometer was used to maintain the temperature of the mice at $38 \pm 1^\circ\text{C}$ throughout the recording session. A calibrated speaker was placed contralateral to the recorded IC at a 45° angle and 6 cm away from the ear. A glass electrode (1 M NaCl, 2–10 M Ω impedance) was advanced using a micromanipulator (Kopf 2660) to depths between 200–2000 μm in the IC. Sound stimulation and data acquisition were driven by SPARKLE software (Sparkle Data Acquisition, Portfors Lab). Single units were isolated and identified based on amplitude and constancy of spikes. Unless otherwise noted, each stimulus was repeated 20 times with a 2-Hz repetition rate. The stimulus duration was 50 ms including a 2-ms rise/fall time. The recording window used was 250 ms from stimulus onset except for the sinusoidal amplitude modulated (SAM) tones, in which the recording window was 1,000 ms. Poststimulus time histogram data were analyzed offline. The number of neurons

recorded from each group were as follows: $n = 78$ from P14–15 WT, $n = 77$ from P21–22 WT and $n = 102$ from P34–39 WT; $n = 81$ from P14–15 *Fmr1* KO, $n = 84$ from P21–22 *Fmr1* KO, and $n = 83$ from P34–39 *Fmr1* KO mice. Upon isolation of a neuron, spontaneous activity and response selectivity were quantified as described below.

Spontaneous activity and frequency response area. Spontaneous activity was recorded within the 250-ms recording window in the absence of any stimuli. The number of action potentials in the recording window was sampled over 20 repetitions (with no sound, 2-Hz repetition rate). Frequency tuning curves were constructed by measuring responses to tones with frequencies between 4 and 48 kHz in 4-kHz steps, and sound levels between 10- and 90-dB SPL in 10-dB steps. Each frequency/sound level combination was presented 20 times with a 2-Hz repetition rate. Characteristic frequency (CF) was defined as the frequency to which the neuron responded at the lowest sound level tested. Bandwidth (BW)10, BW20, and BW30 was the range of frequencies to which the neuron responded at 10 dB, 20 dB, and 30 dB above minimum threshold, respectively.

Rate-level functions. We determined the rate-level function of each neuron to quantify the changes in response magnitude with increasing sound levels. The CF tone was presented at levels between 10- and 90-dB SPL in a pseudorandom manner. The number of action potentials over 20 repetitions of each sound level was counted to plot the rate-level function. Percent turnover (%TO) and dynamic range were calculated from the rate-level functions as defined by (Phillips and Kelly 1989). %TO was taken as

$$\%TO = \frac{\text{Max Response} - \text{Response to 90 dB SPL}}{\text{Max Response}} \times 100.$$

The higher the value of %TO, the more nonmonotonic is the relationship between sound level and response magnitude and indicates that the response increases and then decreases as sound levels increase. A low %TO indicates either that the response increases continuously with sound level or that it saturates. We hypothesized that the *Fmr1* KO IC neurons would show reduced %TO compared with WT IC neurons. The dynamic range is the range of sound levels over which the response increases from 10% to 90% of maximum response. Across the population, the dynamic range is indicative of how rapidly the IC gets activated with increasing sound levels. We hypothesized that the *Fmr1* KO IC neurons would show narrower dynamic range compared with WT IC neurons.

Response magnitude and first spike latency. The average number of spikes per stimulus and the median first spike latency was calculated from the response of neurons to 20 repetitions (2-Hz rate) of CF tones presented at 15 dB and 30 dB above minimum threshold.

Selectivity for sinusoidal amplitude modulated sounds. Sinusoidal amplitude modulated (SAM) tones were used to determine temporal properties of IC neurons. The carrier frequency was at CF presented 15 dB above minimum threshold (500-ms duration and 2-ms rise/fall time). The recording window was 1,000 ms in duration. The carrier tone was 100% depth modulated at the following frequencies: 5, 10, 20, 50, 100, and 200 Hz. Rate modulation transfer function (rMTF) was defined as the number of spikes per stimulus presentation for the duration of the stimulus presentation (500 ms). Temporal modulation transfer function (tMTF) was quantified as the vector strength (VS; Goldberg and Brown 1969). Each spike time was correlated to the period phase (0–360°). The VS was determined at each modulation frequency (5, 10, 20, 50, 100, and 200 Hz). In the tMTF period analysis, the first 100 ms of the recording duration was not included to omit the onset response to the sound stimulus.

Statistical analysis. For the c-Fos analysis, the average of cell counts from two slices per brain region per animal was used with animal number as sample size. Mice were separated into quiet and sound-exposed groups. The P21 mice received one sound level (85 dB), and the P34 groups received one of two sound levels (80 or 90

Table 1. Window sizes for c-Fos+ cell analysis in the MGB subdivisions

Medial Geniculate Body (MGB) Subnuclei	Cell Count Window Size, μm
Supragenulate nucleus (SGN)	100 $\times$ 175
Dorsal division of the MGB (MGd)	250 $\times$ 200
Ventral division of the MGB (MGv)	250 $\times$ 350
Medial division of the MGB (MGm)	200 $\times$ 250
Peripeduncular nucleus (PP)	530 $\times$ 200

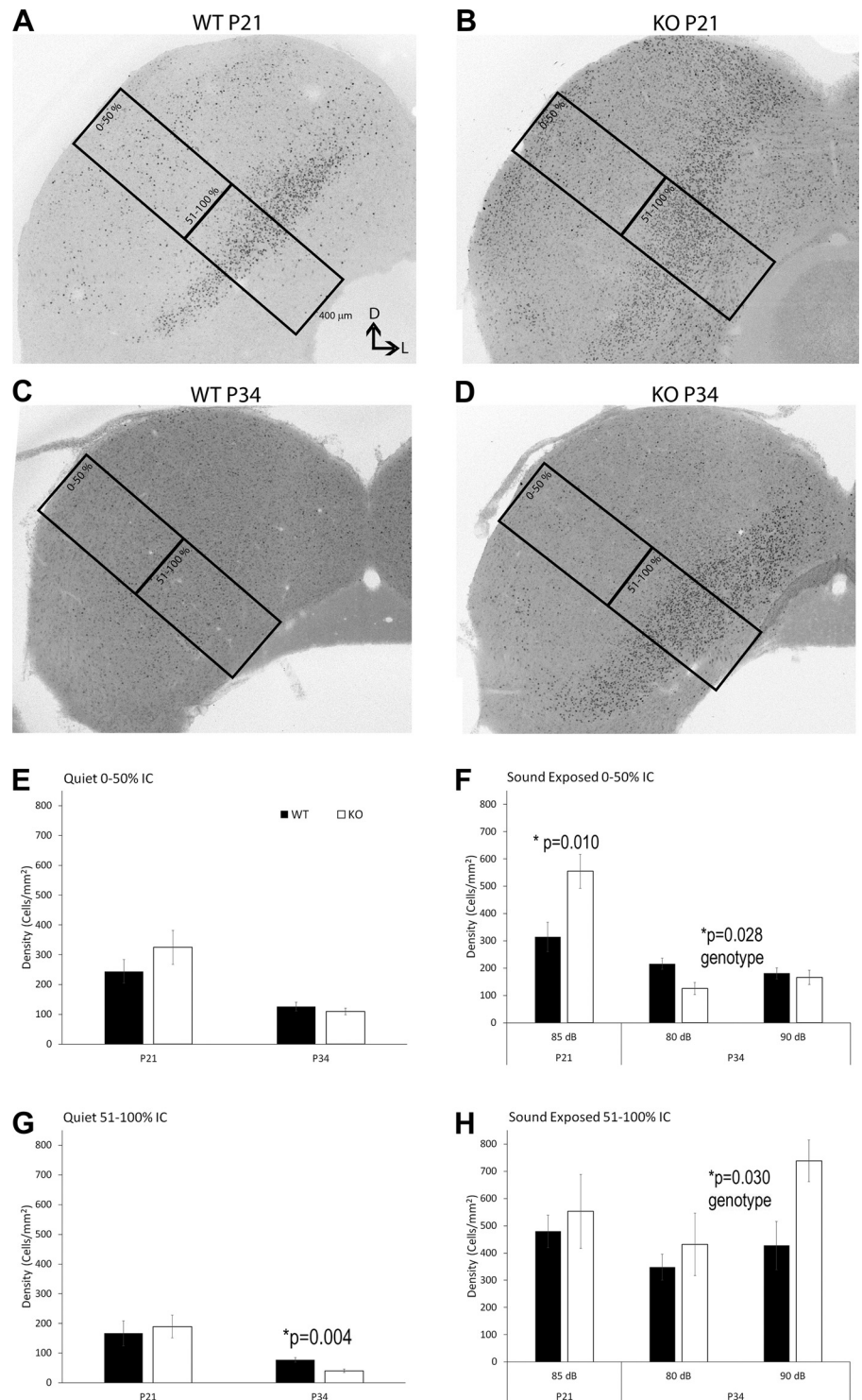


Fig. 1. c-Fos+ cell density is increased in the inferior colliculus (IC) of *Fmr1* knockout (KO) mice. *A* and *B*: example photomicrographs of c-Fos immunoreactivity in the IC obtained from postnatal day (P)21 wild-type (WT) (*A*) and P21 *Fmr1* KO mice (*B*) following sound exposure (85 dB). *C* and *D*: example photomicrographs of c-Fos immunoreactivity in the IC at P34 following 90 dB sound exposure of WT (*C*) and *Fmr1* KO mice (*D*). Rectangular boxes in *A–D* show counting windows (400- μ m width) that span the IC in a dorsolateral to medioventral direction. *E*: for the 0–50% counting window (dorsolateral half), there were no significant genotype differences in the density of c-Fos+ cells at both ages in the quiet group. *F*: in the sound-exposed group, for the 0–50% window, there was a significant increase in c-Fos+ cell density at P21 in *Fmr1* KO mice (* P = 0.010) and a genotype effect at P34 (P = 0.028). *G*: in the 51–100% window (medioventral half), there was a significant decrease in c-Fos+ cell density in the *Fmr1* KO mice at P34 under the quiet condition (* P = 0.004). *H*: for the sound-exposed group, there was no genotype difference at P21 in 51–100% window of the IC. At P34, there was significant increase in c-Fos+ cell density in *Fmr1* KO compared with WT (P = 0.030) mice. Error bars show SE.

dB). If the counts of c-Fos+ cells within a brain region showed normal distribution, then a Student's *t* test was used for genotype comparisons. If any data set within a brain region was not normally distributed, then all genotype comparisons within that brain region used the nonparametric Mann-Whitney *U* test. Genotype differences were analyzed by comparing the means of *Fmr1* KO versus WT separately for P21 and P34 groups. For the electrophysiology data, a two-way ANOVA with age and genotype as factors was performed to test main effects and interactions. The number of neurons was used as sample size for electrophysiology data. Unless otherwise noted, P <

0.05 was considered significant for ANOVA, Student's *t* test, and Mann-Whitney *U* test.

RESULTS

Density of c-Fos+ cells was higher in the IC of Fmr1 KO compared with WT mice at P21 and P34. The first major aim of the study was to determine potential genotype- and age-dependent differences in the number of c-Fos+ -activated cells

in the IC, MGB, and auditory cortex. The density of c-Fos+ cells was counted in the quiet and sound-exposed conditions in P21 and P34 mice of both genotypes, corresponding to high and low AGS susceptible ages, respectively (Musumeci et al. 2000). After habituation for 3 h in a sound-attenuated booth, a siren-like sound (alternating 5- to 50-kHz upswEEP for 500 ms and 50- to 5-kHz downswEEP for 500 ms) was played for 15 min. Following perfusion, images of c-Fos-immunoreactive labeling were collected and a 400- μ m-wide window, drawn diagonally in the dorsolateral to ventromedial direction of the IC, was selected for cell counting (Fig. 1, A–D). This window was further subdivided into two halves for analysis covering 0–50% and 51–100% of the IC in the dorsolateral to ventromedial direction.

In the quiet condition, a Student's *t* test showed no significant genotype difference in the 0–50% region of the IC at P21 [$t(11) = -1.186$, $P = 0.261$] or P34 [Fig. 1E; $t(12) = 0.841$, $P = 0.417$]. In the 51–100% region (Fig. 1G), there was also no significant genotype difference at P21. However, at P34 the density of c-Fos+ cells was significantly lower in the 51–100% region of the IC *Fmr1* KO mice compared with WT mice [$t(12) = 3.618$, $P = 0.004$]. Thus, for ambient sound levels, the density of c-Fos+ cells in the IC was not higher in the *Fmr1* KO mice compared with WT mice at either P21 or P34.

However, when sounds were presented, the *Fmr1* KO mouse IC showed higher c-Fos+ cell density than WT group at both P21 and P34. Only a single sound level (85 dB) was tested for the P21 group so the data were analyzed using a Student's *t* test. At P34, we tested mice at either 80- or 90-dB SPL and used a two-way ANOVA (sound level and genotype as factors) for the c-Fos+ cell density analysis. At P21, there was a significant increase in the sound-evoked c-Fos+ cell density in the 0–50% IC window of *Fmr1* KO mice compared with WT mice [Fig. 1F, $t(16) = -2.907$, $P = 0.010$]. Interestingly, for the P34 group, there was a significant decrease in the density of c-Fos+ cells in the 0–50% IC window of *Fmr1* KO mice compared with the WT mice [$F(1,27) = 5.415$, $P = 0.028$].

In the more ventromedial (51–100%) half of the IC (Fig. 1H), there was no significant difference at P21 between WT and *Fmr1* KO mice [$t(16) = -1.444$, $P = 0.168$]. At P34, c-Fos+ cell density was significantly higher in *Fmr1* KO mice compared with WT mice [$F(1,27) = 5.216$, $P = 0.030$]. There was also a main effect of sound level with significantly higher c-Fos+ cell density at 90 dB compared with an 80-dB sound level [$F(1,27) = 4.998$, $P = 0.034$]. There was no genotype $\times$ sound interaction [$F(1,27) = 1.734$, $P = 0.199$] at P34. Thus, when exposed to sound, c-Fos+ cell density was higher in the IC of the *Fmr1* KO than WT mice, suggesting auditory hypersensitivity in the IC at both P21 and P34. The region of the IC showing increased c-Fos+ cell density in the *Fmr1* KO mice shifts with age (P21→P34) from more dorsolateral IC to more ventromedial locations.

Division-specific genotype differences in c-Fos+ cell density in the medial geniculate body. The MGB comprises multiple divisions, including the medial (MGm), the ventral (MGv), the dorsal (MGd) divisions, and the supragenicular nucleus (SGN) (Fig. 2). The adjacent peripeduncular nucleus (PP) may also be involved in auditory processing through reciprocal connections with the IC (Arnault and Roger 1987). For the quiet condition data, a Mann-Whitney *U* test was used for genotype comparison in each MGB division. At P21, there was

a significant increase in c-Fos+ cell density in *Fmr1* KO mice compared with WT mice in the MGm ($U = 5$, $P = 0.014$). There was no significant difference in other divisions of the MGB at P21 [PP ($U = 20$, $P = 0.604$), MGv ($U = 3.5$, $P = 0.543$), MGd ($U = 18$, $P = 0.420$), and SGN ($U = 14.5$, $P = 0.217$)]. At P34, there was a significant increase in c-Fos+ cell density in *Fmr1* KO, compared with WT, mice in the MGv ($U = 8$, $P = 0.01$) but not in the other divisions [PP ($U = 26$, $P = 0.574$), MGd ($U = 15.5$, $P = 0.083$), MGm ($U = 17$, $P = 0.106$), and SGN ($U = 28.5$, $P = 0.712$)].

In the sound-exposure condition, at P21, there was a significant increase in c-Fos+ cell density in the SGN ($U = 7.5$, $P = 0.003$) and PP ($U = 19$, $P = 0.05$) of the *Fmr1* KO mice compared with the WT. All other divisions showed no significant differences [MGv ($U = 38.5$, $P = 0.858$), MGd ($U = 22$, $P = 0.102$), and MGm ($U = 20$, $P = 0.069$)]. At P34, no genotype differences were present in any MGB division for sounds presented at 80 dB SPL: PP ($U = 17.5$, $P = 0.370$), MGv ($U = 17.5$, $P = 0.368$), MGd ($U = 13.5$, $P = 0.158$), MGm ($U = 24$, $P = 0.949$), and SGN ($U = 20$, $P = 0.565$). When sound was at 90 dB, only the PP ($U = 29$, $P = 0.05$) showed a genotype difference. There were no differences in the other divisions [MGv ($U = 33$, $P = 0.083$), MGd ($U = 43$, $P = 0.283$), MGm ($U = 30.5$, $P = 0.061$), and SGN ($U = 54$, $P = 0.760$)]. Thus, for ambient sound conditions, *Fmr1* KO mice show increased number of c-Fos+-activated cells in the MGm and MGv. When sound was presented, the SGN and PP showed higher number of c-Fos+ cells in *Fmr1* KO mice.

Auditory cortex does not show increased c-Fos+ cell density in *Fmr1* KO mice. Single unit recordings from auditory cortex (Wen et al. 2018) showed higher response magnitude in P21 *Fmr1* KO mice compared with WT mice, suggesting hyperactivity of individual neurons in the auditory cortex. Here, to investigate whether more neurons were activated in the auditory cortex of *Fmr1* KO mice, we quantified the density of c-Fos+ cells (Fig. 3). A 400- μ m-wide rectangular window that spanned the length of cortical layers I–VI was used to quantify c-Fos+ cell density (Fig. 3, A and B). In the quiet condition (Fig. 3C), there were no genotype differences in the number of c-Fos+ cells in the auditory cortex of WT and *Fmr1* KO mice at P21 ($U = 16$, $P = 0.302$) or P34 ($U = 26$, $P = 0.529$; Fig. 3C, left). In the sound exposure condition (Fig. 3D), there was no significant genotype difference in c-Fos+ cell density at P21 ($U = 25$, $P = 0.321$). At P34, Mann-Whitney tests showed a significant genotype difference when mice were exposed to 80 dB ($U = 6$, $P = 0.018$), but the number of c-Fos+ cells was lower in the *Fmr1* KO mouse cortex. There was no difference for the 90-dB sound level ($U = 33$, $P = 0.364$).

Electrophysiology. Extracellular single unit recordings were obtained from the IC in both genotypes at three different developmental ages: P14, P21, and P30. Spontaneous activity, rate-level functions, frequency tuning curves, and responses to amplitude-modulated tones were compared across age and genotype.

Spontaneous activity of IC neurons shows CF-specific genotype differences. Spontaneous activity was measured by counting the number of spikes over 20 repetitions of the recording window (250 ms) with no sound stimulus. The average spontaneous activity across all recorded neurons for each genotype and age was then used in a two-way ANOVA (age and genotype as factors) to identify statistical differences. The overall spontaneous activity in the IC was low, likely due

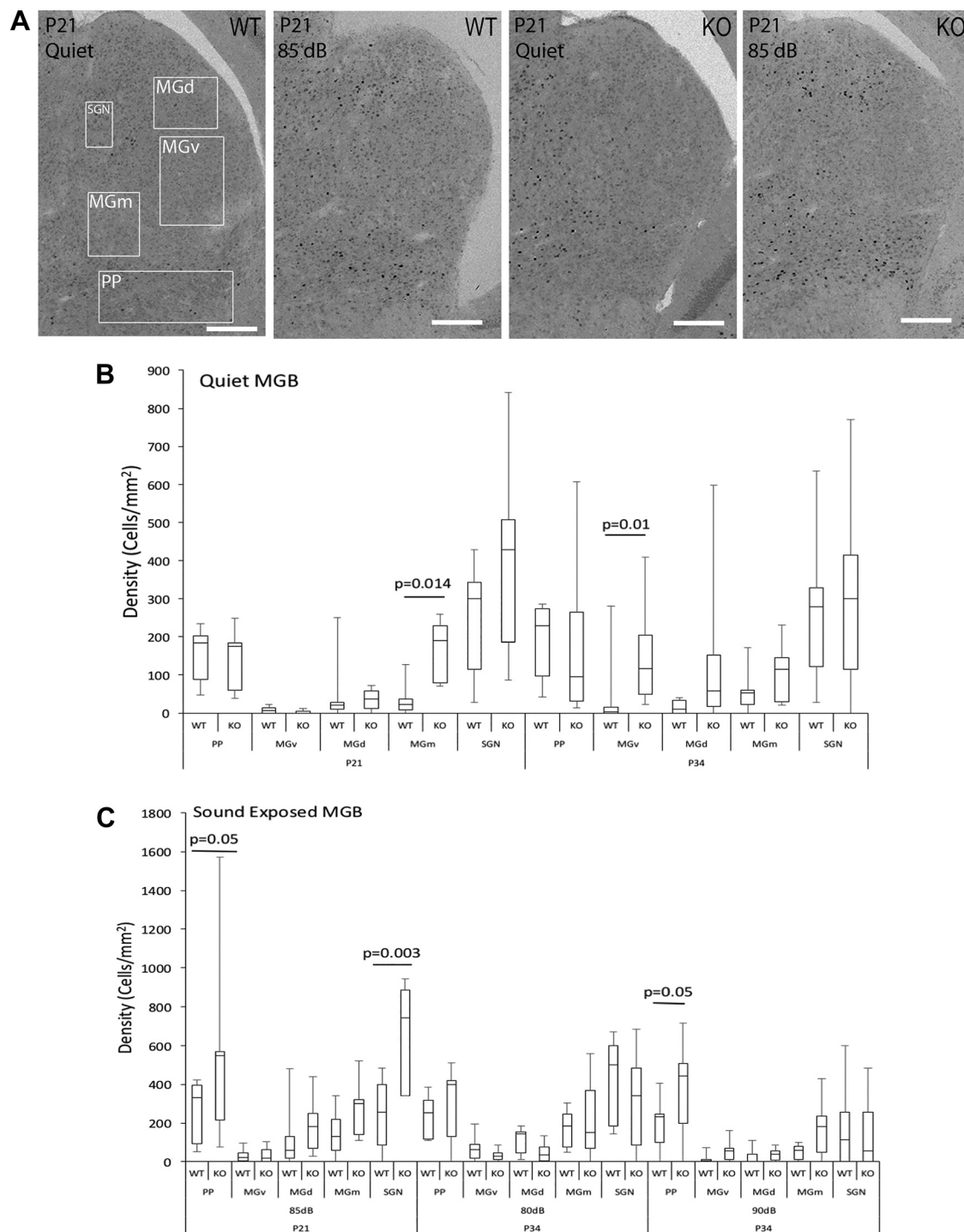


Fig. 2. Subdivision-specific genotype differences in c-Fos+ cell density in the medial geniculate body (MGB). *A*: example photomicrographs of c-Fos immunoreactivity in the MGB of wild-type (WT) and *Fmr1* knockout (KO) mice obtained at postnatal day (P)21. *B*: box and whisker plot of c-Fos+ cell density in the quiet groups at P21 and P34. *C*: box and whisker plot of c-Fos+ cell density in the sound-exposed groups at P21 and P34. Scale bar = 200 μ m. SGN, supragenulate nucleus; PP, peripeduncular nucleus; MGd, dorsal division of the MGB; MGv, ventral division of the MGB; MGm, medial division of the MGB.

to the anesthesia (Fig. 4A). However, it was possible to detect a significant main effect of age [$F(2,499) = 11.153$, $P = 0.000018$] with Bonferroni post hoc comparison showing a reduction in spontaneous activity with age (P14 vs. P21, $P = 0.024$; P14 vs. P34, $P = 0.000007$; and P21 vs. P34, $P = 0.124$). There was no main effect of genotype [$F(1, 499) = 2.333$, $P = 0.127$] or significant genotype $\times$ age

interaction [$F(2,499) = 1.186$, $P = 0.306$]. Thus, when all the neurons were considered together, spontaneous activity decreased during development in the IC, with no genotype differences.

Because there were regional genotype differences in the c-Fos+ cell density in the IC, we analyzed the electrophysiology data by classifying neurons according to CF, with low and

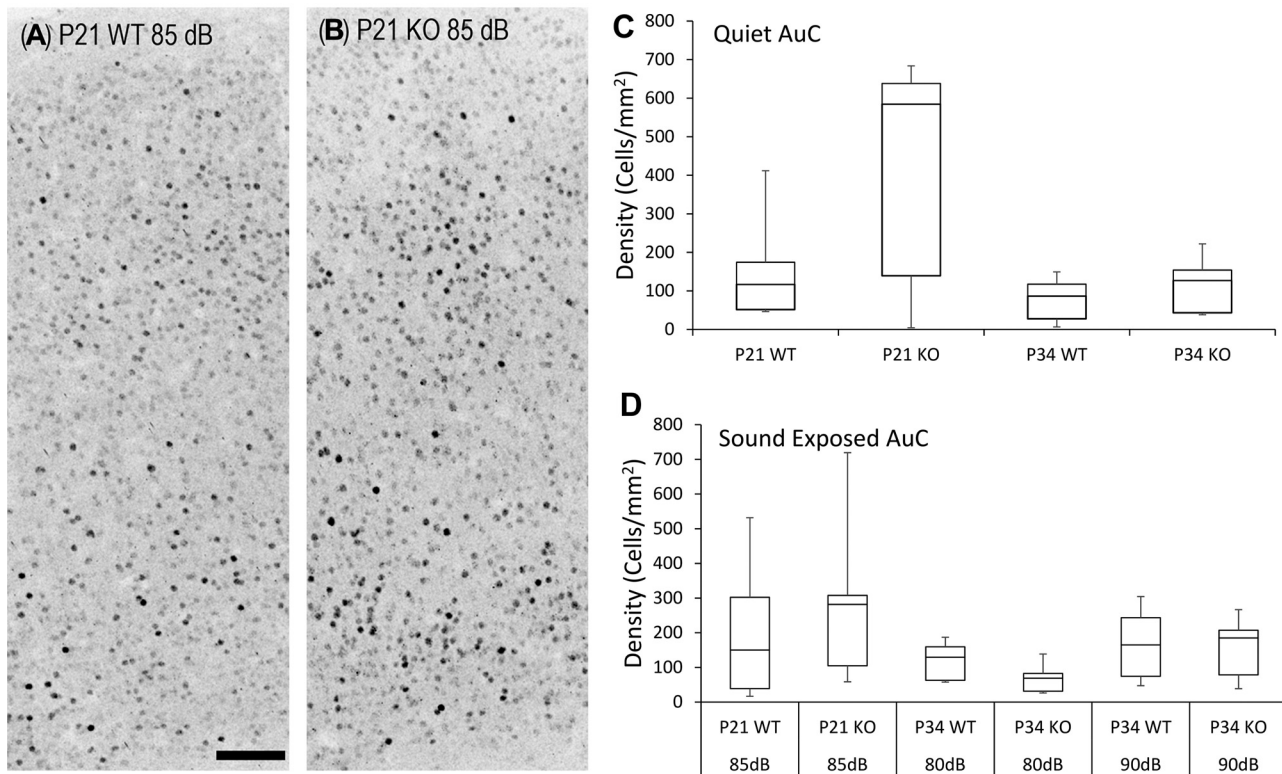


Fig. 3. Genotype comparisons of c-Fos+ cell density in the auditory cortex. *A* and *B*: example photomicrographs of sound driven c-Fos immunoreactivity in auditory cortex (AuC) of postnatal day (P)21 wild-type (WT; *A*) and *Fmr1* knockout (KO; *B*) mice. All 6 cortical layers are shown in *A* and *B* with a pial surface at *top*. Scale bar = 100 μm. *C*: box and whisker plot of c-Fos+ cell density in the quiet condition. *D*: box and whisker plot of c-Fos+ cell density in the sound-exposed condition.

high CF groups separated with a 20-kHz cut-off range. We chose the 20-kHz cut-off frequency because the IC tonotopic map splits approximately into two halves at this CF (Felix and Portfors 2007). The full statistics for the CF-classified data are provided in Table 2. *Fmr1* KO neurons with CF < 20 kHz produced more spontaneous spikes than their WT counterparts. There was an interaction between age and genotype, but no main effect of age. For neurons with CF ≥ 20 kHz, we observed no main effect of genotype or significant genotype × age interaction. A significant main effect of age was present, with Bonferroni post hoc comparison showing a decrease in spontaneous activity with age (P14 vs. P21, $P = 0.000182$; P14 vs. P34, $P < 0.0001$; P21 vs. P34, $P = 0.015$) (Fig. 4B). In terms of spontaneous activity in the IC, these data indicate a larger effect of genotype in neurons with CF < 20 kHz and an effect of age in neurons with CF ≥ 20 kHz.

Minimum thresholds of IC neurons show CF-specific genotype differences. We next compared minimum threshold (MT), defined as the lowest sound level that evoked a response to tones. When all neurons were considered regardless of CF, there was no significant main effect of genotype [$F(1,501) = 1.834$, $P = 0.176$] (Fig. 4C). Analysis demonstrated a significant genotype × age interaction [$F(2,501) = 3.818$, $P = 0.023$] and a main effect of age [$F(2,501) = 11.586$, $P = 0.000012$] with Bonferroni post hoc (P14 vs. P21, $P = 0.000115$; P14 vs. P34, $P = 1.00$; P21 vs. P34, $P = 0.000037$), showing the lowest average MT at P21 compared with P14 and P34.

When separated by CF (Table 2), neurons with CF < 20 kHz showed no main effect of genotype or genotype × age interaction for MT. There was a significant main effect of age with

Bonferroni post hoc analysis showing a difference in MT between P14 versus P21 ($P = 0.001$) and P21 versus P34 ($P < 0.0001$) but not for P14 versus P34 ($P = 0.261$) (Fig. 4D). For neurons with CF ≥ 20 kHz, a significant main effect of genotype was seen with a lower MT in *Fmr1* KO compared with WT IC. There was no genotype × age interaction. A main effect of age ($P < 0.0001$) was also observed for MT with Bonferroni post hoc comparison showing a difference between P14 versus P21 ($P = 0.000005$) and P14 versus P34 ($P = 0.007$) but not P21 versus P34 ($P = 0.105$) (Fig. 4D). These data suggest that reduced MT in high CF neurons may underlie auditory hyperresponsiveness in *Fmr1* KO mice.

IC neurons in *Fmr1* KO mice are hyperresponsive to tones. Response magnitude to 20 repetitions of the CF tone was measured and compared across age and genotype. For all neurons combined (Fig. 5A), the average response magnitude at MT + 15 dB (15 dB above threshold) showed no significant genotype × age interaction [$F(2,481) = 1.495$, $P = 0.225$]. There was a significant main effect of genotype with more spikes/stimulus in *Fmr1* KO mice compared with WT mice [$F(1,481) = 5.249$, $P = 0.022$]. There was also a main effect of age [$F(2,481) = 6.257$, $P = 0.002$] with Bonferroni post hoc comparisons being significant for P21 versus P34 ($P = 0.004$) and P14 versus P34 ($P = 0.005$) but not for P14 versus P21 ($P = 1.00$) (Fig. 5A). Sound-evoked responses were reduced with age in both WT and KO IC. For CF tone responses at MT + 30 dB (Fig. 5B), there was a significant genotype × age interaction [$F(2,477) = 4.303$, $P = 0.014$] and a significant main effect of genotype [$F(1,477) = 4.772$, $P = 0.029$], with *Fmr1* KO mouse neurons responding with more spikes than

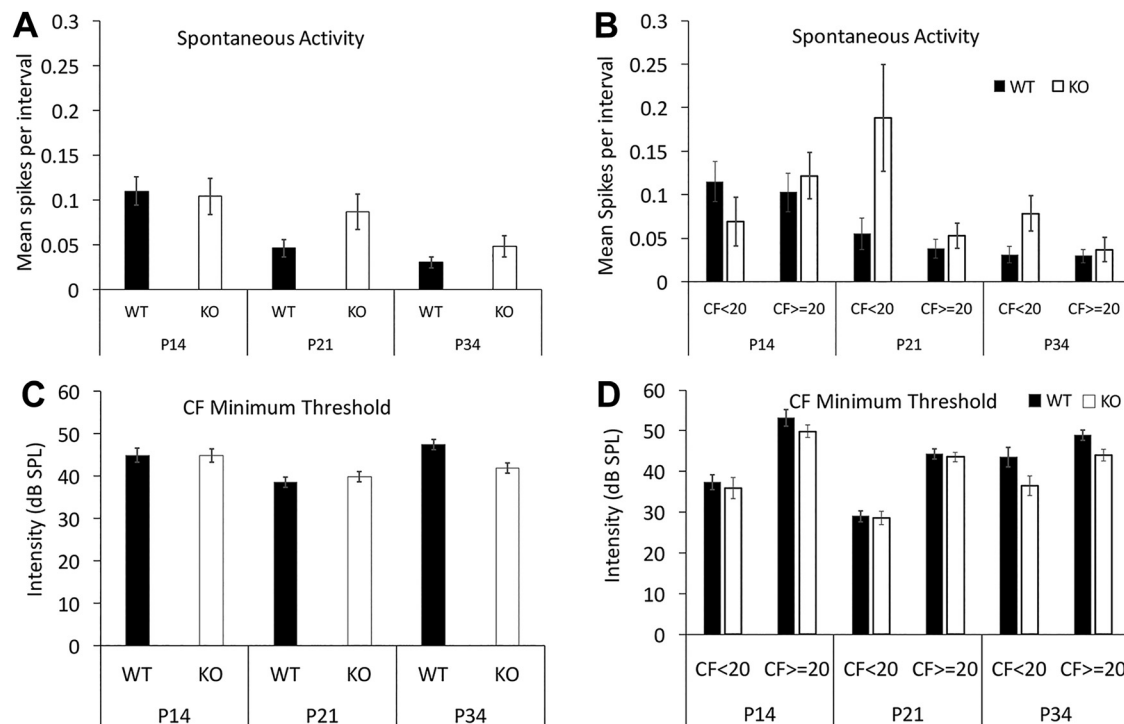


Fig. 4. Spontaneous activity and minimum threshold of *Fmr1* knockout (KO) and wild-type (WT) inferior colliculus (IC) neurons at postnatal day (P)14, P21, and P34. The graphs in *A* and *B* show the average number of spikes per acquisition window (250 ms, 20 repetitions) in the absence of any stimulus. *A*: two-way ANOVA (age $\times$ genotype) shows a main effect of age ($P = 0.000018$), with spontaneous firing rate decreasing across development. *B*: for neurons with characteristic frequency (CF) <20 kHz, a two-way ANOVA revealed significant interaction between genotype $\times$ age ($P = 0.008$) and significant main effects of genotype but no significant main effect of age (genotype: $P = 0.04$; age: $P = 0.08$). For neurons with CF ≥ 20 kHz, there was no significant genotype $\times$ age interaction or main effects of genotype ($P = 0.949$, $P = 0.335$, respectively). There was a significant main effect of age ($P = 0.000004$). *C*: the average minimum threshold at CF for neurons in IC showed no main effects of genotype ($P = 0.176$). There was a significant interaction between genotype $\times$ age and a main effect of age ($P = 0.023$, $P = 0.000012$, respectively). *D*: for neurons with CF <20 kHz, there was no significant genotype $\times$ age interaction or main effect of genotype ($P = 0.282$, $P = 0.09$, respectively). There was a significant main effect of age ($P = 0.000004$). For neurons with CF ≥ 20 kHz, there was no significant interaction between genotype $\times$ age ($P = 0.299$). There was a significant main effect for both genotype and age ($P = 0.000005$). Error bars show SE.

WT neurons. There was a significant main effect of age [$F(2,477) = 6.313$, $P = 0.002$] with Bonferroni post hoc tests revealing no significant differences in P14 versus P21 ($P = 1.00$) but a significant reduction with age in P21 versus P34 ($P = 0.001$) and P14 versus P34 ($P = 0.019$) comparisons.

For neurons with CF <20 kHz (Table 2), responses to tones at MT + 15 dB showed a significant main effect of genotype with *Fmr1* KO neurons responding more than WT neurons (Fig. 5C). There was no significant main effect of age or genotype $\times$ age interactions. Similarly, for tones presented at

Table 2. Statistical analysis of data classified according to CF (<20 kHz vs. ≥ 20 kHz) for spontaneous activity, minimum threshold, and sound-driven activity at MT + 15 dB and MT + 30 dB

	CF <20 kHz	CF ≥ 20 kHz
Spontaneous activity		
Genotype	$F(1,167) = 4.274$, $P = 0.04$	$F(1,333) = 0.933$, $P = 0.335$
Age	$F(2,167) = 2.565$, $P = 0.08$	$F(2,333) = 12.938$, $P < 0.0001$
Genotype-age interactions	$F(2,167) = 4.928$, $P = 0.008$	$F(2,333) = 0.052$, $P = 0.949$
Minimum threshold		
Genotype	$F(1,164) = 2.915$, $P = 0.09$	$F(1,331) = 6.436$, $P = 0.012$
Age	$F(2,164) = 13.385$, $P < 0.0001$	$F(2,331) = 12.583$, $P < 0.0001$
Genotype-age interactions	$F(2,164) = 1.274$, $P = 0.282$	$F(2,331) = 1.211$, $P = 0.299$
Response magnitude MT + 15 dB		
Genotype	$F(1,157) = 14.878$, $P = 0.00016$	$F(1,316) = 0.002$, $P = 0.968$
Age	$F(2,157) = 1.256$, $P = 0.288$	$F(2,316) = 6.132$, $P = 0.002$
Genotype-age interactions	$F(2,157) = 0.277$, $P = 0.759$	$F(2,316) = 1.534$, $P = 0.217$
Response magnitude MT + 30 dB		
Genotype	$F(1,155) = 18.529$, $P = 0.000030$	$F(1,314) = 0.041$, $P = 0.840$
Age	$F(2,155) = 1.625$, $P = 0.2$	$F(2,314) = 5.390$, $P = 0.005$
Genotype-age interactions	$F(2,155) = 2.117$, $P = 0.124$	$F(2,314) = 5.174$, $P = 0.006$

MT, minimum threshold; CF, characteristic frequency. Underscore indicates significant values.

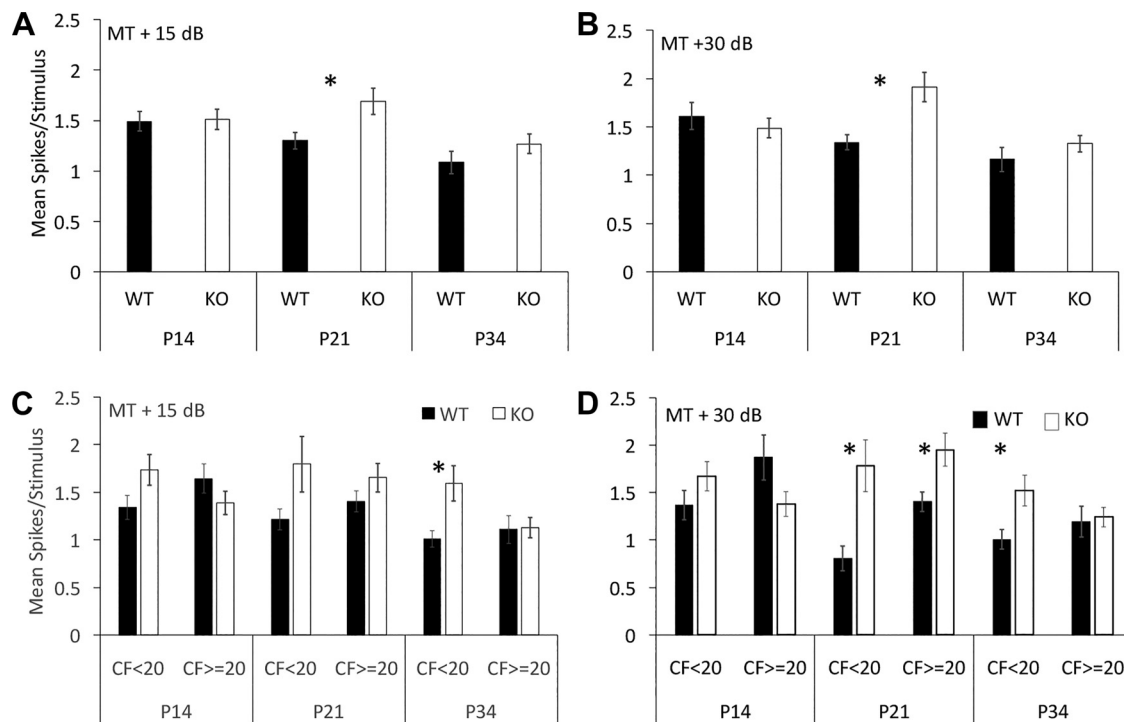


Fig. 5. *Fmr1* knockout (KO) inferior colliculus (IC) neurons show increased response magnitude to characteristic frequency (CF) tones than wild-type (WT) neurons. **A:** average response magnitude when tested with CF tones at 15 dB above minimum threshold (MT + 15 dB). There was a significant main effect of genotype and age ($P = 0.022$, $P = 0.002$, respectively) but no significant interaction between genotype $\times$ age ($P = 0.225$). **B:** average response magnitude when tested with CF tones at 30 dB above minimum threshold (MT + 30 dB). There was a significant interaction between genotype $\times$ age ($P = 0.014$), main effect of genotype ($P = 0.029$), and main effect of age ($P = 0.002$). **C:** response magnitude at MT + 15 dB for neurons separated by CF (CF <20 kHz vs. CF $\geq$ 20 kHz). For neurons with CF <20 kHz, there was no significant genotype $\times$ age interaction ($P = 0.759$) or main effect of age ($P = 0.288$). A significant main effect of genotype ($P = 0.00016$) was present. For neurons with CF $\geq$ 20 kHz, there was a significant main effect of age ($P = 0.002$) but no significant genotype $\times$ age interaction ($P = 0.217$) or main effect of genotype ($P = 0.968$). **D:** response magnitude at MT + 30 dB for neurons separated by CF. For neurons with CF <20 kHz, there was no significant interaction between genotype $\times$ age ($P = 0.124$) or main effect of age ($P = 0.2$). There was a significant main effect of genotype ($P = 0.000030$). For neurons with CF $\geq$ 20 kHz, there was a significant interaction between genotype $\times$ age ($P = 0.006$) and main effect of age ($P = 0.005$), but there was no significant main effect of genotype ($P = 0.840$). Error bars show SE. * $P < 0.05$.

MT + 30 dB (Fig. 5D), there was a significant main effect of genotype (Fig. 5D) but no main effect of age or genotype $\times$ age interactions.

For neurons with CF $\geq$ 20 kHz, response magnitude for tones presented at MT + 15 dB, (Fig. 5C) showed a main effect of age. There were no significant differences in Bonferroni post hoc comparison of P14 versus P21 ($P = 1.00$), but a significant difference was present for the P14 versus P34 ($P = 0.022$) and P21 versus P34 ($P = 0.003$) comparisons. There was no main effect of genotype or genotype $\times$ age interactions. At MT + 30 dB (Fig. 5D), there was no main effect of genotype. There was a main effect of age with no significant difference using Bonferroni post hoc comparisons in P14 versus P21 ($P = 1.000$) and P14 versus P34 ($P = 0.092$) but a significant difference in P21 versus P34 comparisons ($P = 0.003$). A significant genotype $\times$ age interaction was also present.

Overall, the number of action potentials elicited by the CF tone was higher in IC of *Fmr1* KO mice for neurons with CF <20 kHz. IC neurons with CF $\geq$ 20 kHz showed a reduction in response magnitude with age but no genotype differences. These results were similar to those seen with spontaneous activity.

Rate-level functions of IC neurons are not different between genotypes. Higher response magnitude at lower sound levels may predispose the *Fmr1* KO mice to AGS at high sound

levels (Faingold et al. 1991). To quantify the relationship between increasing sound levels and response magnitude, rate-level functions were plotted for CF tone responses measured with sound levels between 10- and 90-dB SPL (e.g., Fig. 6A). Percent turnover (%TO) is a measure of the non-monotonicity of rate-level functions and indicates the extent to which responses are reduced after reaching a peak with increasing sound levels. We hypothesized that %TO is lower in *Fmr1* KO mice compared with WT mice. The dynamic range (DR) is the range of sound levels over which the response increases from 10% to 90% of maximum. We tested the hypothesis that DR was narrower in the *Fmr1* KO mouse IC than in WT mice. This would cause the *Fmr1* KO mouse IC to be activated to near maximal levels even with small increases in sound level.

When all neurons were combined across CF (Fig. 6B), there was no main effect of genotype in the %TO [$F(1,482) = 0.559$, $P = 0.455$] or genotype $\times$ age interactions [$F(2,482) = 0.716$, $P = 0.489$]. There was a significant effect of age [$F(2,482) = 6.422$, $P = 0.002$] with Bonferroni post hoc significant differences at P14 versus P21 ($P = 0.011$) and P14 versus P34 ($P = 0.004$) but not at P21 versus P34 ($P = 1.00$). Percent TO decreased with age, indicating that neurons become more monotonic with age. For DR as well (Fig. 6C), there was no main effect of genotype [$F(1,356) = 0.601$, $P = 0.439$] or genotype $\times$ age interactions [$F(2,356) = 1.935$, $P = 0.146$].

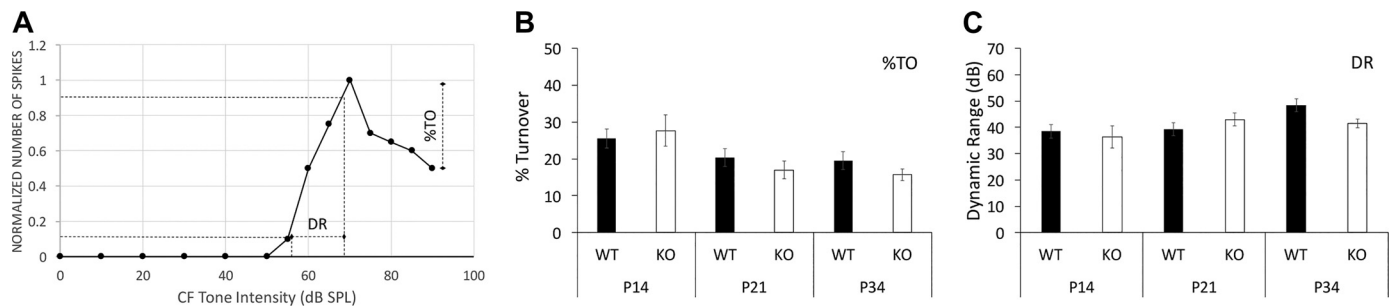


Fig. 6. Rate-level responses of inferior colliculus (IC) neurons were mostly unaffected by genotype at all ages tested. **A**: an example rate-level response function in a wild-type (WT) IC neuron [postnatal day (P)21]. Vertical dashed lines from left to right indicate sound level for 10% of maximum response and 90% of maximum response. Dynamic range (DR) was the range of sound levels over which responses increased from 10% to 90% of the maximum response. Percent turnover (%TO) is the degree of nonmonotonicity of the rate-level function and measures the extent to which response at the highest sound level tested is reduced compared with the maximum response. In this example, the %TO was ~50%. **B**: for %TO, there was no significant genotype $\times$ age interaction ($P = 0.489$) or main effect of genotype ($P = 0.455$). There was a main effect of age ($P = 0.002$), with neurons showing reduced %TO (less nonmonotonic) with age. **C**: dynamic range of neurons showed a main effect of age ($P = 0.031$) but no significant genotype $\times$ age interaction ($P = 0.146$) or main effect of genotype ($P = 0.439$). Error bars show SE.

There was a main effect of age [$F(2,356) = 3.522$, $P = 0.031$]. Bonferroni post hoc comparisons did not show any specific pairwise differences [P14 vs. P21, $P = 0.189$; P14 vs. P34, $P = 0.129$; and P21 vs. P34, $P = 1.00$].

When the data were split by CF (Table 3), the statistical trends were similar to those observed for the full data set. For %TO, neurons with CF <20 kHz showed no main effects of genotype or genotype $\times$ age interaction. There was a main effect of age with Bonferroni post hoc comparisons showing no significant pairwise differences (P14 vs. P21, $P = 0.061$; P14 vs. P34, $P = 0.138$; and P21 vs. P34, $P = 1.00$). For neurons with CF $\geq$ 20 kHz, there was a decrease in %TO in *Fmr1* KO mice compared with WT mice. This indicates neurons were more monotonic in WT mice, in a disagreement with our original hypothesis. There was no significant genotype $\times$ age interaction or main effect of age. For the dynamic range measurement, neurons with CF <20 kHz showed no main effect of genotype or genotype $\times$ age interaction. There was a significant main effect of age with Bonferroni post hoc comparisons showing differences between P14 and P21 ($P = 0.004$) and P14 and P34 ($P = 0.013$) but not between P21 and P34 ($P = 1.00$). For neurons with CF $\geq$ 20 kHz, there were no main effects or interactions. Taken together, these data suggest

that rate-level relationships in IC neurons were relatively normal in the *Fmr1* KO mice and may not contribute to auditory hypersensitivity in FXS.

Low-frequency IC neurons show longer response latency in Fmr1 KO mice. The median first spike latency of IC neuron responses to CF tone was measured at MT + 15 dB and MT + 30 dB (Fig. 7). For MT + 15 dB data, a two-way ANOVA showed a significant main effect of age for latency [$F(2, 477) = 35.43$, $P < 0.0001$, Fig. 7A]. There was no significant main effect of genotype [$F(1,477) = 0.613$, $P = 0.434$] or genotype $\times$ age interactions [$F(2,477) = 1.262$, $P = 0.284$]. Bonferroni post hoc analysis revealed a significant difference in the P14 versus P21 ($P < 0.0001$), P14 versus P34 ($P < 0.0001$), and P21 versus P34 ($P = 0.00082$) comparisons, with latencies decreasing with age. Likewise, for CF tones presented at MT + 30 dB (Fig. 7B), there was a significant main effect of age [$F(2,475) = 114.773$, $P < 0.0001$] with Bonferroni post hoc comparison at P14 versus P21 ($P < 0.0001$), P14 versus P34 ($P < 0.0001$), and P21 versus P34 ($P < 0.0001$) pairs, showing decreasing latency with age. There was no main effect of genotype [$F(1,475) = 0.904$, $P = 0.342$] or genotype $\times$ age interactions [$F(2, 475) = 0.087$, $P = 0.917$].

Table 3. Statistical analysis of data classified according to CF (<20 kHz vs. $\geq$ 20 kHz) for percent turnover, dynamic range, and response latency to CF tones presented at MT + 15 dB and MT + 30 dB

	CF <20 kHz	CF $\geq$ 20 kHz
Percent Turnover		
Genotype	$F(1,161) = 0.516$, $P = 0.473$	$F(1,330) = 4.368$, $P = 0.037$
Age	$F(2,161) = 3.624$, $P = 0.029$	$F(2,330) = 0.729$, $P = 0.483$
Genotype-age interactions	$F(2,161) = 0.405$, $P = 0.668$	$F(2,330) = 2.704$, $P = 0.068$
Dynamic range		
Genotype	$F(1,127) = 0.003$, $P = 0.959$	$F(1,223) = 0.011$, $P = 0.915$
Age	$F(2,127) = 8.188$, $P = 0.0004$	$F(2,223) = 0.053$, $P = 0.948$
Genotype-age interactions	$F(2,127) = 2.614$, $P = 0.077$	$F(2,223) = 1.719$, $P = 0.182$
Response latency at MT + 15 dB		
Genotype	$F(1,163) = 4.878$, $P = 0.029$	$F(1,315) = 3.430$, $P = 0.065$
Age	$F(2,163) = 10.682$, $P = 0.000044$	$F(2,315) = 34.024$, $P < 0.0001$
Genotype-age interactions	$F(2,163) = 0.297$, $P = 0.743$	$F(2,315) = 0.130$, $P = 0.878$
Response latency at MT + 30 dB		
Genotype	$F(1,143) = 4.128$, $P = 0.044$	$F(1,312) = 0.253$, $P = 0.615$
Age	$F(2,143) = 44.573$, $P < 0.0001$	$F(2,312) = 65.186$, $P < 0.0001$
Genotype-age interactions	$F(2,143) = 1.405$, $P = 0.249$	$F(2,312) = 0.045$, $P = 0.956$

MT, minimum threshold; CF, characteristic frequency.

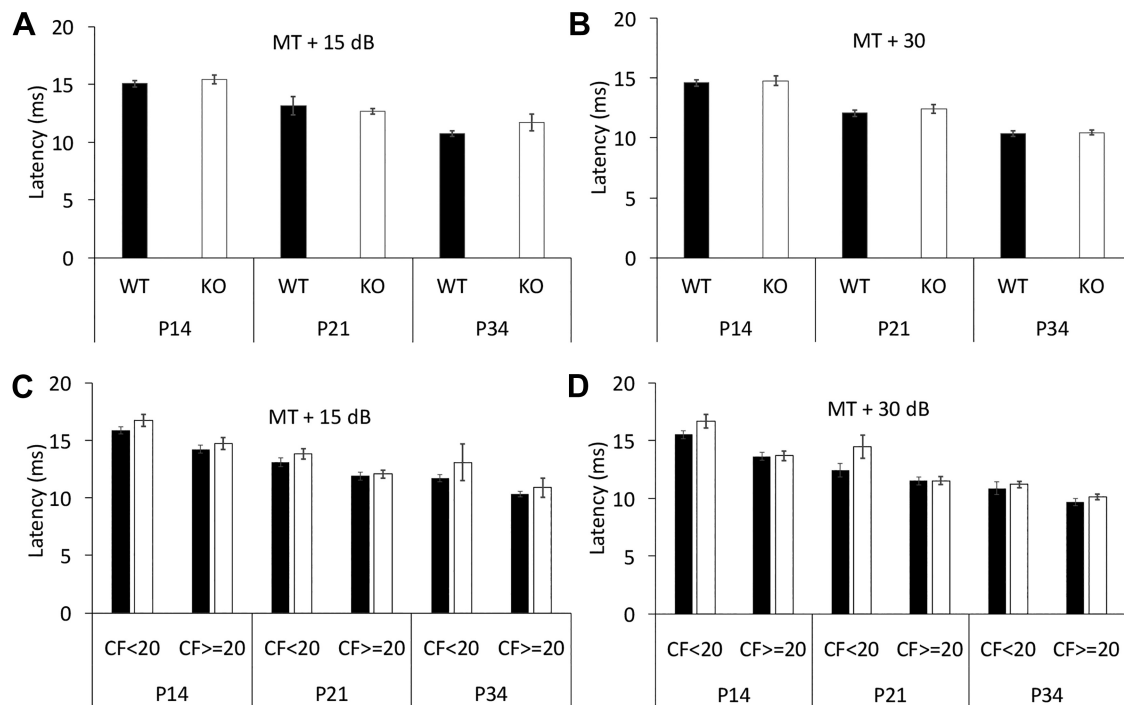


Fig. 7. No genotype differences were observed in median first spike latencies of neuronal response to characteristic frequency (CF) tones. *A*: the median first spike latency of responses to CF tone presented at minimum threshold (MT) + 15 dB showed no significant interaction between genotype $\times$ age ($P = 0.284$) or main effect of genotype ($P = 0.434$). There was a significant main effect of age ($P < 0.0001$), with latency decreasing with age. *B*: for latencies in response to CF tones at MT + 30 dB, there was no significant genotype $\times$ age interaction ($P = 0.917$) or main effect of genotype ($P = 0.342$). There was a significant main effect of age ($P < 0.0001$), with latencies decreasing with age. *C*: for neurons with CF < 20 kHz, there was no significant interaction between genotype $\times$ age ($P = 0.743$) for latencies measured with tones presented at MT + 15 dB. However, there was a significant main effect of genotype ($P = 0.029$) and main effect of age ($P < 0.0001$). For neurons with CF ≥ 20 kHz, there was a significant main effect of age ($P < 0.001$) but no significance in genotype $\times$ age interaction or main effect of genotype ($P = 0.878$, $P = 0.065$, respectively). *D*: when tested with tones presented at MT + 30 dB, the median first spike latency for neurons with CF < 20 kHz showed significant main effect of genotype and age ($P = 0.044$, $P < 0.0001$, respectively) but no significant genotype $\times$ age interactions ($P = 0.249$). For neurons with CF ≥ 20 kHz, there was a significant main effect of age ($P < 0.0001$) but no significant main effect of genotype ($P = 0.615$) or genotype $\times$ age interaction ($P = 0.956$). Error bars show SE.

When neurons were classified by CF (Table 3), neurons with CF < 20 kHz showed a significant main effect of genotype with longer latency in the KO compared with WT for tones with sound level of MT + 15 dB (Fig. 7C). There was also a main effect of age with Bonferroni post hoc comparison of P14 versus P21 ($P = 0.0042$), P14 versus P34 ($P = 0.00018$), and P21 versus P34 ($P = 1.00$) pairs, showing decreasing latency with age. There was no significant difference in genotype $\times$ age interaction. For neurons with CF ≥ 20 kHz, there was no significant main effect of genotype or genotype $\times$ age interactions. There was a significant main effect of age with Bonferroni post hoc analysis showing differences for P14 versus P21 ($P < 0.0001$), P14 versus P34 ($P < 0.0001$), and P21 versus P34 ($P = 0.0053$) pairs, with latency decreasing with age.

Similarly, at MT + 30 dB (Fig. 7D), neurons with CF < 20 kHz showed a main effect of genotype and a main effect of age ($P < 0.0001$) with Bonferroni post hoc comparisons showing differences for the P14 versus P21 ($P = 0.0023$), P14 versus P34 ($P < 0.0001$), and P21 versus P34 ($P = 0.000055$) pairs, with latency decreasing with age. There was no significant genotype $\times$ age interaction. For neurons with CF ≥ 20 kHz, there was a significant main effect of age with Bonferroni post hoc comparisons showing differences for the P14 versus P21 ($P < 0.0001$), P14 versus P34 ($P < 0.0001$), and P21 versus P34 ($P < 0.0001$) pairs, with latencies decreasing with age. There was no significant

main effect of genotype or genotype $\times$ age interactions. Thus the median first spike latency in response to CF tones decreased during development across all CFs, with genotype differences (slower latency in *Fmr1* KO neurons) seen for neurons with CF < 20 kHz.

Low-frequency IC neurons in the Fmr1 KO mice show broader frequency tuning. To determine if frequency selectivity is affected in the IC of *Fmr1* KO mice, we performed a genotype and age comparison of frequency response area bandwidths (BW) at three different sound levels: 10, 20, and 30 dB above MT (BW10, BW20, and BW30, respectively). Frequency response areas (e.g., Fig. 8A) were plotted by measuring the number of action potentials to 20 repetitions of each frequency/sound level combination ranging from 4 to 48 kHz (4-Hz steps) and 10–90 dB SPL (10-dB steps). The bandwidth of the frequency response area at 10 (BW10), 20 (BW20), and 30 (BW30) dB above the neuron's MT was measured to quantify frequency selectivity.

For BW10, there was a significant genotype $\times$ age interaction [$F(2,307) = 4.971$, $P = 0.008$; Fig. 8B] but no main effect of genotype [$F(1,307) = 0.679$, $P = 0.410$] or age [$F(2,307) = 2.556$, $P = 0.079$]. A main effect of genotype was seen for BW20 (Fig. 8B), with broader tuning curves in *Fmr1* KO mice compared with WT mice [$F(1,307) = 3.927$, $P = 0.048$]. There was also a significant main effect of age [$F(2,307) = 6.096$, $P = 0.003$] with Bonferroni post hoc test showing a significant difference in P14 versus P21 ($P =$

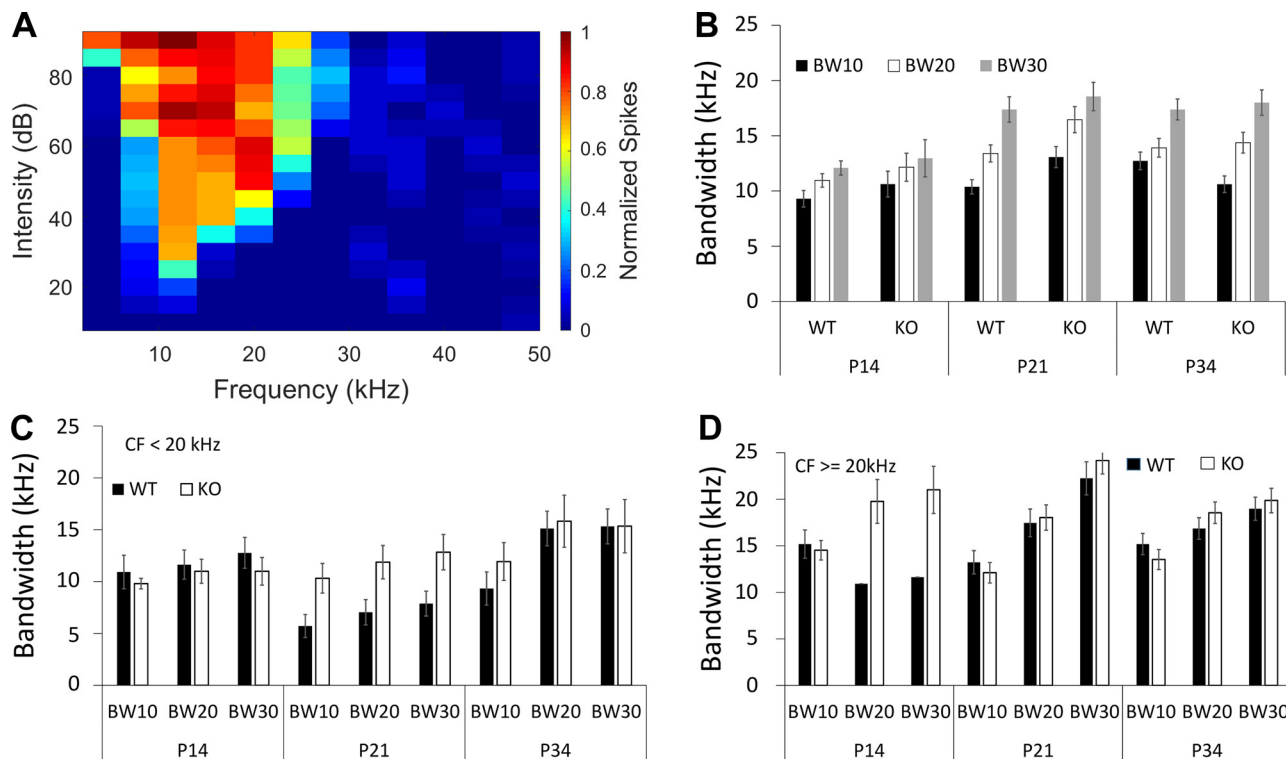


Fig. 8. Frequency tuning was broader in the inferior colliculus (IC) of *Fmr1* knockout (KO) mice compared with wild-type (WT) mice, mainly for neurons with CF <20 kHz. **A**: an example frequency response area with tone frequency on the abscissa and sound intensity on the ordinate. The color scale indicates normalized response magnitude for specific frequency-intensity combinations. The bandwidth of frequency selectivity was quantified at 10 (BW10), 20 (BW20), and 30 (BW30) dB above the minimum threshold of neurons. **B**: when all neurons within each genotype were pooled together, there was no significant main effect of genotype for BW10 ($P = 0.410$) or BW20 ($P = 0.354$). A significant main effect of genotype was present only for BW20 ($P = 0.048$). A significant main effect of age was seen for BW20 ($P = 0.003$) and BW30 ($P < 0.0001$) but not for BW10 (0.079). **C**: For neurons with CF <20 kHz, there was a significant main effect of genotype [BW10 ($P = 0.039$), BW20 ($P = 0.017$), and BW30 ($P = 0.018$)]. There was no genotype $\times$ age interaction [BW10 ($P = 0.175$), BW20 ($P = 0.616$), and BW30 ($P = 0.68$)] or main effect of age [BW10 ($P = 0.944$), BW20 ($P = 0.515$), and BW30 ($P = 0.288$)]. **D**: for neurons with CF $\geq$ 20 kHz, there was no significant main effect of genotype ($P = 0.726$) or age ($P = 0.499$). There was a significant genotype $\times$ age interaction only for BW10 ($P = 0.04$). Error bars show SE.

0.002) and P14 versus P34 ($P = 0.024$) but not P21 versus P34 ($P = 1.00$). There was no significant genotype $\times$ age interaction [$F(2,307) = 1.095$, $P = 0.336$]. For BW30 (Fig. 8B), there was a significant effect of age [$F(2,307) = 12.276$, $P = 0.000007$] with Bonferroni post hoc showing a difference in P14 versus P21 ($P = 0.000018$) and P14 versus P34 ($P = 0.000066$) but not in the P21 versus P34 ($P = 1.00$) groups. There was no main effect of genotype [$F(1,307) = 0.860$, $P = 0.354$] or genotype $\times$ age

interaction [$F(2,307) = 0.032$, $P = 0.968$]. Thus, when all neurons were considered together, genotype difference was seen only for BW20 with *Fmr1* KO neurons showing broader tuning. For BW20 and BW30, we observed a narrowing of tuning curves with developmental age.

When separated by CF, neurons with CF <20 kHz (Fig. 8C and Table 4) were more broadly tuned in the *Fmr1* KO IC compared with WT IC at BW10, BW20, and BW30. For BW10, BW20, and BW30, there was no significant genotype $\times$ age interaction or

Table 4. Statistical analysis of data classified according to CF (<20 kHz vs. $\geq$ 20 kHz)

	CF <20 kHz	CF $\geq$ 20 kHz
BW10		
Genotype	$F(1,118) = 4.346$, $P = 0.039$	$F(1,181) = 0.123$, $P = 0.726$
Age	$F(2,118) = 0.058$, $P = 0.944$	$F(2,181) = 0.699$, $P = 0.499$
Genotype-age interactions	$F(2,118) = 1.771$, $P = 0.175$	$F(2,181) = 3.272$, $P = 0.04$
BW20		
Genotype	$F(1,118) = 5.9$, $P = 0.017$	$F(1,182) = 1.221$, $P = 0.271$
Age	$F(2,118) = 0.487$, $P = 0.515$	$F(2,182) = 2.606$, $P = 0.077$
Genotype-age interactions	$F(2,118) = 0.487$, $P = 0.616$	$F(2,182) = 0.497$, $P = 0.609$
BW30		
Genotype	$F(1,116) = 5.806$, $P = 0.018$	$F(1,180) = 0.225$, $P = 0.636$
Age	$F(2,116) = 1.259$, $P = 0.288$	$F(2,180) = 4.013$, $P = 0.20$
Genotype-age interactions	$F(2,116) = 0.387$, $P = 0.68$	$F(2,180) = 0.702$, $P = 0.497$

BW10, BW20, and BW30, bandwidth at MT + 15 dB, MT + 20 dB, and MT + 30 dB, respectively; MT, minimum threshold; CF, characteristic frequency; BW, bandwidth.

main effect of age. Neurons with CF ≥ 20 kHz (Fig. 8D) showed no main genotype or age effects for BW10, BW20, or BW30. A genotype $\times$ age interaction was only seen for BW10. Thus IC neurons with CF < 20 kHz, but not higher CF neurons, showed a broader tuning in *Fmr1* KO compared with WT mice, which was more pronounced at P21. Yet again, these data indicate that IC neurons with CF < 20 kHz show more genotype differences than neurons with CF ≥ 20 kHz.

IC neurons showed stronger responses to amplitude modulated tones in *Fmr1* KO than WT mice. The rate modulation transfer function (rMTF) and temporal modulation transfer function (tMTF) for responses to sinusoidal amplitude modulated (SAM) tones were compared across age and genotype (Fig. 9). The rMTF was analyzed as the average number of spikes per stimulus presentation over the duration of the stimulus. The tMTF was quantified as the degree of synchronization to the modulations measured as vector strengths. Modulation rates of 5, 10, 20, 50, 100, and 200 Hz with carrier frequency centered at CF were used and the SAM tone was presented at MT + 15 dB.

For rMTF analysis, a two-way ANOVA (genotype and age as factors) was run for each modulation rate. The main statistics are summarized in Table 5, including analyses of all neurons pooled or separated by CF (20 kHz cut-off). In general, Table 5 and Figs. 9 and 10 show a number of significant genotype differences in rMTF driven mostly by increased responses to SAM in the *Fmr1* KO mice. Figure 9 also suggests that the increased responses are more prominent at faster modulation rates with *Fmr1* KO IC neurons showing a peak ~ 50 -Hz modulation rates compared with WT neurons, on average. Neurons with CF < 20 kHz also show more consistent genotype differences across modulation rates (Table 5 and Fig. 10). No consistent patterns were seen for main

effects of age or age $\times$ genotype interactions. Together, we interpret these data to indicate that responses to modulated tones are increased in the *Fmr1* KO mice.

For tMTF (Table 6 and Figs. 9, 11), main effects of genotype were rare (seen only for 10 Hz modulation rate), and the other effects do not show a consistent pattern of specific developmental change or genotype $\times$ age interactions. This suggests that temporal response properties of IC neurons when tested with SAM tones are not different in *Fmr1* KO mice compared with WT and are unlikely to contribute to abnormal auditory sensitivity.

Tonotopy. We quantified the development and possible genotype differences in tonotopy in the IC and observed the expected dorsal to ventral increase in CF representation (Felix and Portfors 2007) at all three developmental ages (Fig. 12) and across genotypes. The CF representation was mostly < 30 kHz at P14 and expanded to include more neurons with CF > 30 kHz at P21 and P34. There was no significant difference in the distribution of CFs across genotypes at any age [P14: $t(159) = 0.831$, $P = 0.406$; P21: $t(164) = 0.788$, $P = 0.431$; and P34: $t(188) = 0.589$, $P = 0.555$]. Together, these data suggest that CF-specific susceptibilities in *Fmr1* KO mice are not due to abnormal development of tonotopy.

DISCUSSION

Based on c-Fos+ cell density analysis, our results indicate that more IC neurons were activated in the *Fmr1* KO mice compared with WT following sound exposure at both P21 and P34. Genotype differences in the density of c-Fos+ cells are also observed in the PP and SGN divisions of the MGB. In vivo single unit recordings show that IC neurons of *Fmr1* KO mice are more responsive to both tone bursts and amplitude-

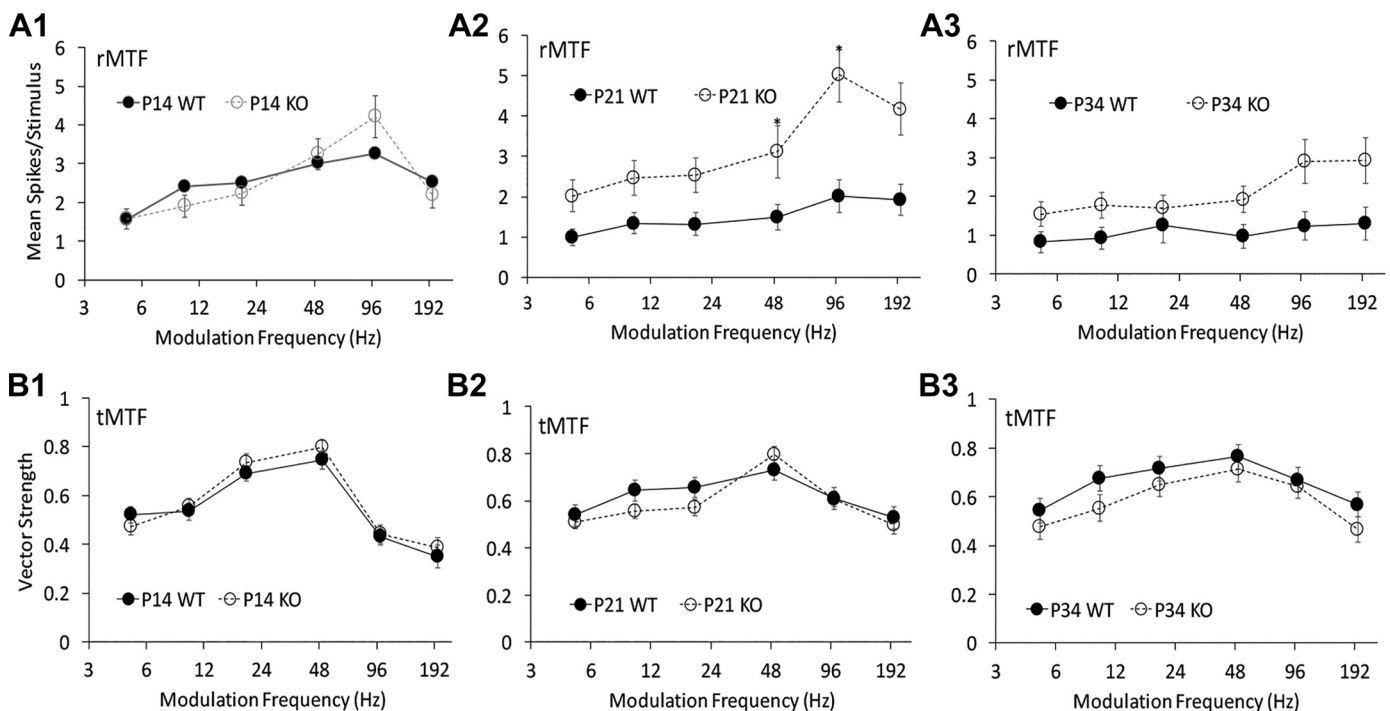


Fig. 9. Inferior colliculus (IC) neurons show hyperresponsiveness to amplitude modulated tones in *Fmr1* knockout (KO) mice, but phase-locking is normal. A1–A3: rate modulation transfer function (rMTF) in the P14, P21, and P34 groups, respectively. B1–B3: temporal modulation transfer function (tMTF) in the P14, P21, and P34 groups, respectively. Dashed lines indicate *Fmr1* KO mice and solid lines indicate WT mice. Error bars show SE. * $P < 0.05$.

Table 5. Statistical analysis of rate modulation transfer functions obtained from WT and *Fmr1* KO mice at the three developmental time points

Modulation Frequency/CF Frequency	Genotype $\times$ Age Interaction	Main Effect of Genotype	Main Effect of Age
5 Hz			
All	$F(2,412) = 1.973, P = 0.140$	$F(1,412) = 2.011, P = 0.157$	$F(2,412) = 0.688, P = 0.503$
CF <20 kHz	$F(2,159) = 1.697, P = 0.187$	$F(1,159) = 7.366, P = 0.007^*$	$F(2,159) = 0.832, P = 0.437$
CF $\geq$ 20 kHz	$F(2,315) = 3.264, P = 0.040^*$	$F(2,315) = 0.001, P = 0.972$	$F(2,315) = 4.279, P = 0.015^*$
10 Hz			
All	$F(2,406) = 1.028, P = 0.359$	$F(1,406) = 0.573, P = 0.450$	$F(2,406) = 1.119, P = 0.328$
CF <20 kHz	$F(2,159) = 1.952, P = 0.145$	$F(2,159) = 2.046, P = 0.155$	$F(2,159) = 0.337, P = 0.715$
CF $\geq$ 20 kHz	$F(2,314) = 1.224, P = 0.296$	$F(2,314) = 0.641, P = 0.424$	$F(2,314) = 2.351, P = 0.097$
20 Hz			
All	$F(2,415) = 0.733, P = 0.481$	$F(1,415) = 5.389, P = 0.021^*$	$F(2,415) = 4.455, P = 0.012^*$
CF <20 kHz	$F(2,159) = 2.732, P = 0.068$	$F(1,159) = 5.786, P = 0.017^*$	$F(2,159) = 1.389, P = 0.252$
CF $\geq$ 20 kHz	$F(2,314) = 0.334, P = 0.717$	$F(1,314) = 2.600, P = 0.108$	$F(2,314) = 11.143, P < 0.0001^*$
50 Hz			
All	$F(2,414) = 1.538, P = 0.216$	$F(1,414) = 16.72, P < 0.0001^*$	$F(2,414) = 3.378, P = 0.035^*$
CF <20 kHz	$F(2,159) = 1.778, P = 0.172$	$F(2,159) = 5.013, P = 0.027^*$	$F(2,159) = 1.008, P = 0.367$
CF $\geq$ 20 kHz	$F(2,315) = 1.387, P = 0.251$	$F(2,315) = 11.473, P = 0.001^*$	$F(2,315) = 6.064, P = 0.003^*$
100 Hz			
All	$F(2,410) = 2.704, P = 0.068$	$F(1,410) = 6.729, P = 0.010^*$	$F(2,410) = 1.292, P = 0.276$
CF <20 kHz	$F(2,159) = 3.724, P = 0.026^*$	$F(2,159) = 6.828, P = 0.010^*$	$F(2,159) = 0.367, P = 0.693$
CF $\geq$ 20 kHz	$F(2,293) = 2.813, P = 0.062$	$F(1,293) = 1.760, P = 0.186$	$F(2,293) = 1.599, P = 0.204$
200 ;Hz			
All	$F(2,401) = 2.282, P = 0.103$	$F(2,401) = 1.845, P = 0.175$	$F(2,401) = 0.262, P = 0.770$
CF <20 kHz	$F(2,159) = 5.217, P = 0.023^*$	$F(2,159) = 5.217, P = 0.024^*$	$F(2,159) = 0.413, P = 0.663$
CF $\geq$ 20 kHz	$F(2,314) = 1.709, P = 0.183$	$F(1,314) = 0.348, P = 0.556$	$F(2,314) = 1.52, P = 0.220$

Data are organized according to the modulation frequency. "All" indicates all the neurons combined. Characteristic frequencies (CF) <20 kHz and CF $\geq$ 20 kHz indicate when data were split according to CF. MT, minimum threshold; WT, wild type; KO, knockout. * $P < 0.05$.

modulated tones and show broader frequency tuning curves than their WT counterparts. In general, genotype differences emerge between P14 and P21, with a stronger effect on neurons with CF <20 kHz, compared with neurons with higher CF. Together, these data suggest that the IC is a major contributor to early developmental auditory hyperresponsiveness in FXS.

Increased density of c-Fos+ cells in the IC of *Fmr1* KO mice at both P21 and P34. The genotype difference in c-Fos+ cell density was apparent in the dorsolateral half of the IC at P21 and shifted to the ventromedial half of the IC (adjacent to the periaqueductal gray) at P34. Chen and Toth (2001) previously examined c-Fos expression in response to sounds in *Fmr1* KO mice using sound levels that caused AGS in some of the mice. When AGS was induced in *Fmr1* KO mice, there was an increase in c-Fos expression in KO compared with WT mice in the dorsal nucleus of the lateral lemniscus, posterior intralaminar nucleus, periaqueductal gray, and MGM. However, because the *Fmr1* KO mice showed seizures and the WT mice did not, motor responses associated with increased AGS-related movement likely contributed to the genotype differences in c-Fos+ cell density (Yang et al. 2020). To overcome this confound, the sound stimulus in our study was in the 80- to 90-dB range, which did not induce AGS in any of the mice used for c-Fos analysis. Under these conditions, we found a genotype difference in the density of c-Fos+ cells in the IC suggesting that the extent of activity in the IC may be a correlate of sensory hypersensitivity in early development.

Dorsomedial IC represents low CFs while ventromedial IC represents higher CFs. Hyperresponsiveness is seen in *Fmr1* KO IC in response to both tone bursts and AM tones for neurons with CF <20 kHz at both P21 and P34. Neurons with

CF $\geq$ 20 kHz do not show considerable differences in response magnitude at either P21 or P34. This lack of spatio-temporal correlation between the c-Fos data and single unit data suggests that the increased density of c-Fos+ cells and increased response magnitudes may contribute independently to hypersensitivity.

Analysis of the density of c-Fos+ cells in the MGB suggests a point of interaction between abnormal sensory processing and anxiety in FXS during early development (Cho et al. 2011). The MGM is a region projecting to all cortical layers and to the amygdala. The SGN is a region that receives multisensory inputs and also projects to the amygdala (LeDoux et al. 1991). The peripeduncular (PP) nucleus integrates auditory, motor, and hypothalamic signals (Arnault and Roger 1987). The recruitment of more cells may underlie a stronger activation of auditory-limbic-motor pathways that may lead to behavioral anxiety phenotypes in response to daily environmental sounds in FXS (Miller et al. 1999; Reinhard et al. 2019).

We did not observe an increase in sound-evoked c-Fos+ cell density in the auditory cortex at P21 or P34. While the IC showed both higher density of activated c-Fos+ cells and increased response magnitude in the *Fmr1* KO mice at this age, the auditory cortex only shows the increased response magnitude (Wen et al. 2018). When *Fmr1* KO mice are most sensitive to AGS (~P21), our data suggest that the IC plays a stronger role than the cortex. This is consistent with the role of IC in AGS generation in rats genetically prone to epilepsy (Faingold 2002) and in the *Fmr1* KO mice (Gonzalez et al. 2019).

Single unit recordings reveal hypersensitivity to sounds in the IC of *Fmr1* KO mice. In vivo electrophysiological recordings in the IC at P14, P21, and P34 showed that the *Fmr1* KO

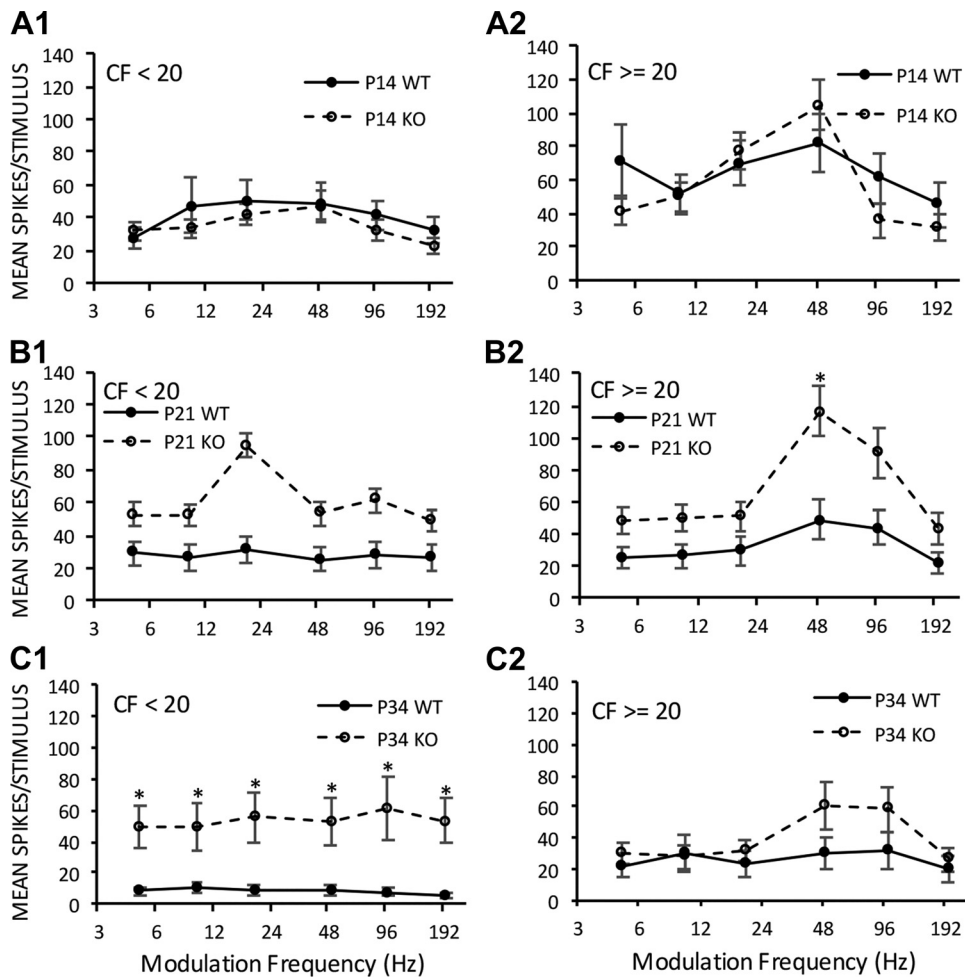


Fig. 10. Rate modulation transfer function (rMTF) subdivided into neurons with characteristic frequency (CF) <20 kHz (A1, B1, and C1) and CF ≥20 kHz (A2, B2, and C2). The rows are arranged by postnatal age (P14, P21, and P34). Error bars show SE. * $P < 0.05$).

neurons produce increased responses to tone bursts and amplitude-modulated tones and had broader frequency tuning curves compared with age-matched WT neurons. Low-frequency tuned neurons (CF <20 kHz) show greater genotype differences compared with neurons with CF ≥20 kHz. Rotschafer and Razak (2013) suggested that a possible mechanism of increased synchrony and hypersensitivity to sounds may be linked to broader frequency tuning curves of individual neurons in *Fmr1* KO mice. This is because more neurons would be activated in response to a single tone frequency if tuning curves were broader and overlapped more. IC neurons show broader tuning curves in *Fmr1* KO mice than in WT, suggesting that more IC neurons will respond synchronously to sounds. These data suggest that the IC is a major source of auditory hyperresponsiveness in FXS during development through increased response magnitudes and a greater number of synchronously activated neurons.

Faingold and Anderson (1991) suggested that abnormal inhibition with increasing sound level in IC neuronal response may lead to AGS in rats genetically susceptible to epilepsy. Therefore, we quantified rate-level responses across age and genotype. In the *Fmr1* KO mice, there were no genotype differences in either the nonmonotonicity of responses or the dynamic range. These data do not support the hypothesis that AGS susceptibility of *Fmr1* KO mice is due to abnormal rate-level relationships in the IC. Given the CF-specific genotype effects observed and because of a previous study that

showed tonotopic gradients of ion channel expression may be affected in the lower auditory brainstem, we hypothesized that development of tonotopy may show genotype differences (Ruby et al. 2015; Strumbos et al. 2010). However, there were no genotype differences in tonotopy indicating that map formation and the underlying guidance cues (Cramer and Gabriele 2014) are relatively normal in the IC of *Fmr1* KO mice. Therefore, increased response to unmodulated and amplitude-modulated tones, broader frequency tuning curves, and increased recruitment of active cells seem to be the major IC phenotypes associated with auditory hyperresponsiveness at P21.

Development of IC responses in WT and *Fmr1* KO mice. Although the major focus of our study was on the *Fmr1* KO mice, the WT data are useful to compare with previous studies of IC development. Our data are consistent with previous findings of shortening latencies, decreasing thresholds, sharpening frequency tuning, and increasing high-frequency representation in the developing IC (Aitkin and Moore 1975; Ehret and Romand 1992; Romand and Ehret 1990; Shnerson and Willott 1979). In addition, we show that both spontaneous and tone-driven response magnitudes of IC neurons decline with age. This was also true for responses to amplitude modulated tones. When IC neurons with CF <20 kHz are considered, there was no main effect of age on the rMTF to AM tones (Table 5). However, IC neurons with CF ≥20 kHz show reduced spike counts with age at multiple modulation rates.

Table 6. Statistical analysis of the temporal modulation transfer functions obtained from WT and *Fmr1* KO mice at the three developmental time points

Modulation Frequency/CF Frequency	Genotype $\times$ Age Interaction	Main Effect of Genotype	Main Effect of Age
5 Hz			
All CF	$F(2,390) = 0.127, P = 0.881$	$F(2,390) = 2.872, P = 0.091$	$F(2,390) = 0.354, P = 0.702$
CF <20 kHz	$F(2,137) = 0.159, P = 0.853$	$F(1,137) = 0.472, P = 0.493$	$F(2,137) = 0.275, P = 0.760$
CF $\geq$ 20 kHz	$F(2,242) = 0.052, P = 0.949$	$F(1,242) = 2.777, P = 0.097$	$F(2,242) = 1.050, P = 0.351$
10 Hz			
All CF	$F(2,383) = 1.989, P = 0.138$	$F(1,383) = 4.912, P = 0.027^*$	$F(2,383) = 2.001, P = 0.137$
CF <20 kHz	$F(2,136) = 0.905, P = 0.407$	$F(1,136) = 0.759, P = 0.385$	$F(2,136) = 3.110, P = 0.048^*$
CF $\geq$ 20 kHz	$F(2,235) = 1.386, P = 0.252$	$F(1,235) = 4.241, P = 0.041^*$	$F(2,235) = 0.695, P = 0.500$
20 Hz			
All CF	$F(2,387) = 2.353, P = 0.096$	$F(2,387) = 1.702, P = 0.193$	$F(2,387) = 4.997, P = 0.007^*$
CF <20 kHz	$F(2,135) = 0.887, P = 0.414$	$F(1,135) = 1.069, P = 0.303$	$F(2,135) = 3.287, P = 0.040^*$
CF $\geq$ 20 kHz	$F(2,240) = 2.087, P = 0.126$	$F(1,240) = 0.599, P = 0.440$	$F(2,240) = 1.734, P = 0.179$
50 Hz			
All CF	$F(2,382) = 2.058, P = 0.129$	$F(1,382) = 0.752, P = 0.386$	$F(2,382) = 0.609, P = 0.544$
CF <20 kHz	$F(2,129) = 0.918, P = 0.402$	$F(1,129) = 0.226, P = 0.636$	$F(2,129) = 0.836, P = 0.436$
CF $\geq$ 20 kHz	$F(2,241) = 0.819, P = 0.442$	$F(1,241) = 0.08, P = 0.778$	$F(2,241) = 1.173, P = 0.311$
100 Hz			
All CF	$F(2,388) = 0.153, P = 0.858$	$F(1,388) = 0.061, P = 0.804$	$F(2,388) = 22.195, P < 0.0001^*$
CF <20 kHz	$F(2,130) = 0.502, P = 0.607$	$F(1,130) = 1.124, P = 0.291$	$F(2,130) = 8.009, P = 0.001$
CF $\geq$ 20 kHz	$F(2,248) = 0.356, P = 0.701$	$F(1,248) = 0.119, P = 0.731$	$F(2,248) = 12.587, P < 0.0001^*$
200 Hz			
All CF	$F(2,352) = 1.356, P = 0.259$	$F(1,352) = 0.932, P = 0.335$	$F(2,352) = 9.349, P = 0.0001^*$
CF <20 kHz	$F(2,120) = 0.433, P = 0.650$	$F(2,120) = 0.027, P = 0.870$	$F(2,120) = 4.008, P = 0.021^*$
CF $\geq$ 20 kHz	$F(2,222) = 1.738, P = 0.178$	$F(1,222) = 1.245, P = 0.266$	$F(2,222) = 4.665, P = 0.010^*$

Data are organized according to the modulation frequency. "All" indicates all the neurons combined. Characteristic frequencies (CF) <20 kHz and CF $\geq$ 20 kHz indicate when data were split according to CF. MT, minimum threshold; WT, wild type; KO, knockout. * $P < 0.05$.

This suggests that AM responses mature more slowly in the high-frequency IC neurons. Taken together, these data are in alignment with both the development of the cochlea and the maturation of inhibitory circuitry that shape frequency tuning and response magnitudes of IC neurons (Fuzessery and Hall 1996; Hurley et al. 2008; Le Beau et al. 1996; Zhang and Kelly 2003).

A number of age $\times$ genotype interactions were observed in this study suggesting that some of the differences may be present only at certain ages. A notably important pattern in the data was that the IC properties are comparable between *Fmr1* KO and WT mice at P14 but begin to diverge at P21. Sound-driven responses begin to occur ~P10–11 in the IC. In the first 2 wk of life, refinement of both extrinsic ascending and intrinsic local connectivity patterns depend mostly on spontaneous activity driven by the cochlea (Gabriele et al. 2000a). Patterns of extrinsic inputs to the IC appear mature at or before hearing onset (Fathke and Gabriele 2009; Gabriele et al. 2000b; Henkel et al. 2007). Our data suggest that this developmental process is relatively normal in *Fmr1* KO mice. Between P14 and P21, sound-driven refinements of excitatory local inputs dominate such that inhibition becomes relatively stronger (E:I ratio declines from P14–21 in WT mice, Sturm et al. 2014). This will sharpen frequency tuning and reduce response magnitudes during development. This process seems to be affected in *Fmr1* KO mice. Abnormal sound-driven refinement may result in elevated response magnitude, broader frequency tuning and consequently hypersensitivity. Grimsley et al. (2013) suggested that the local circuit connectivity may shape responses of IC neurons to increasing sound levels. Therefore, abnormal local IC circuit refinement may also be implicated in AGS. Whether this is due to abnormal refinement

of excitatory connections and/or abnormal development of inhibition remains to be investigated.

Another main observation is that neurons with CF <20 kHz appear to be more hypersensitive than neurons with CF $\geq$ 20 kHz. The underlying mechanisms for this frequency dependence are unclear, but suggest abnormal GABA responses in the IC of *Fmr1* KO mice. Based on the IC tonotopic gradient, neurons with increasing CF are found more ventrally in the IC and most neurons with CF <20 kHz are likely to be within 1,000 μ m from the dorsal surface (~50% of total dorso-ventral depth (Felix and Portfors 2007). IC neurons receive both GABA and glycine inhibitory inputs. Glycine may be more dominant in shaping inhibition in ventral, high-CF regions of the IC (Choy Buentello et al. 2015; Merchán et al. 2005; Sanes et al. 1987). GAD67-labeled inputs appear to dominate more in the dorso-medial regions of the IC (Choy Buentello et al. 2015), suggesting a more prominent role for GABA in the dorsal half of the IC. This may suggest a deficit in GABAergic inhibition, because most deficits were observed in low CF neurons in the dorsolateral region of the IC. GABA_A receptor deficits in the IC are related to AGS in rats that are genetically susceptible to epilepsy (Faingold 2002). In addition, down-regulated tonic GABA_A currents and a decrease in GABA_A receptors were reported in *Fmr1* KO mice (Curia et al. 2009; D'Hulst et al. 2006), suggesting impaired GABA-mediated inhibition in FXS. GABAergic inputs shape firing rates of IC neurons (Palombi and Caspary, 1996). Whether glycinergic inhibition is affected in the IC is unclear, but Garcia-Pino et al. (2017) showed no impact on such inhibition in the lower brainstem of *Fmr1* KO mice. Together, these studies suggest the CF-dependent susceptibility of IC to hyperresponsiveness in early development may be related to GABA dysfunction in *Fmr1* KO mice.

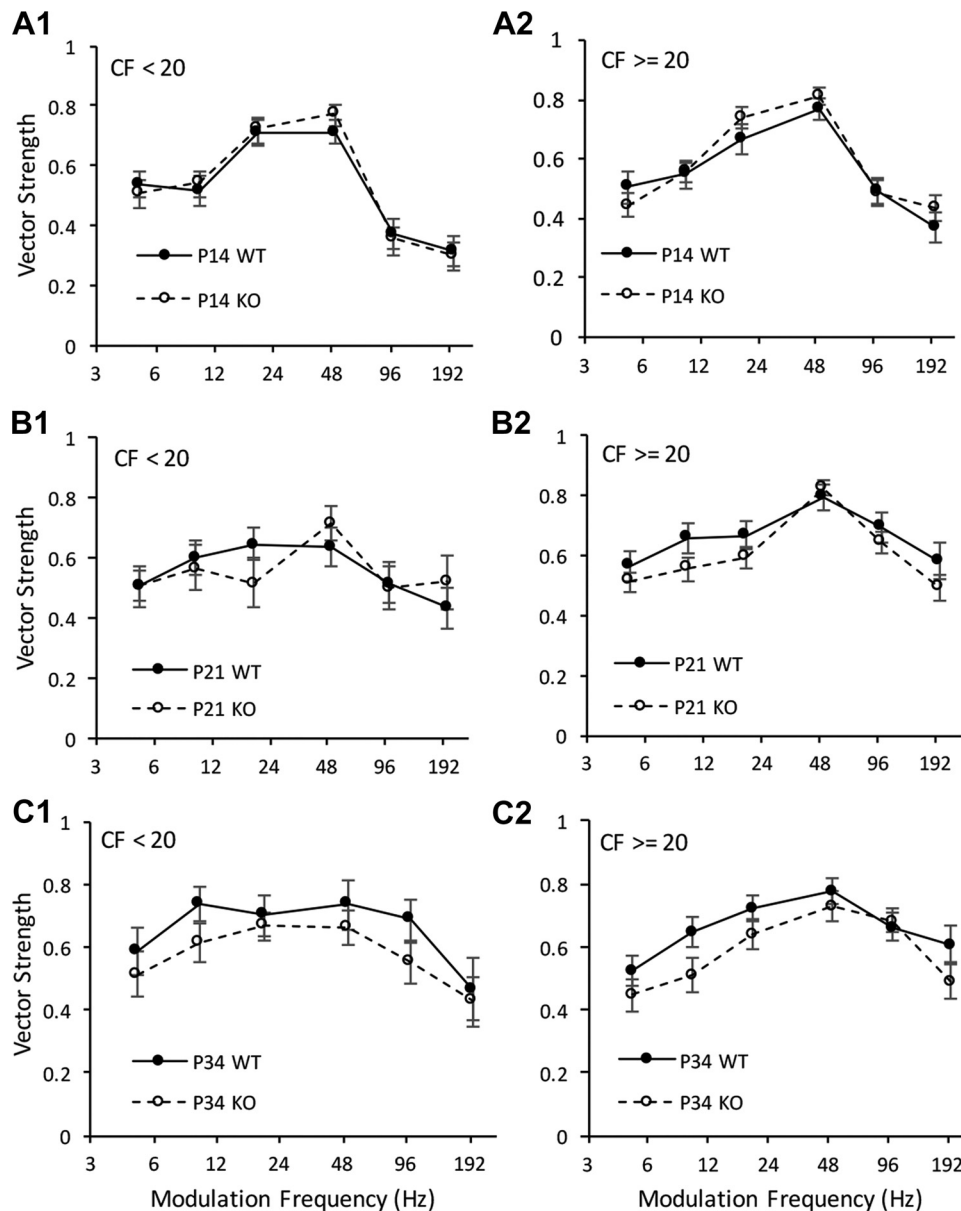


Fig. 11. Temporal modulation transfer function (tMTF) subdivided into neurons with characteristic frequency (CF) <20 kHz (A1, B1, and C1) and CF ≥20 kHz (A2, B2, and C2). The rows are arranged by postnatal age (P14, P21, and P34). Error bars show SE.

System-wide deficits in auditory processing in FXS. Given the consistent and debilitating auditory hypersensitivity in individuals with FXS, there is an increasing interest in understanding the underlying the circuit and cellular pathophysiology across the auditory system and across development (McCullagh et al. 2020). FMRP is expressed at multiple levels of the auditory system from the cochlear nucleus to the auditory cortex. Global deletion of *Fmr1* would affect the development and function of each of these auditory processing stages. Neurons in the lateral superior olive (LSO) of the brainstem show enhanced excitatory synaptic input strength through increased convergence of cochlear nucleus input early in development (Garcia-Pino et al. 2017). LSO neurons showed increased firing rates and broader frequency tuning curves. The abnormal IC responses may, therefore, originate in the lower brainstem. However, this needs to be verified by comparing lower brainstem and IC recordings conducted at similar ages. In the medial nucleus of the trapezoid body (MNTB), one of the major sources of inhibition to the LSO, the

tonotopic gradient of Kv3.1b potassium ion channel is significantly flatter in *Fmr1* KO, compared with WT mice (Strumbos et al. 2010). Modeling (Strumbos et al. 2010) and electrophysiological (Brown et al. 2010) data suggest an impact on temporal precision in MNTB of *Fmr1* KO mice. This may be limited to the MNTB, as our IC data do not reveal any genotype differences in tMTF in response to amplitude modulated tones, suggesting that single neuron phase locking is not affected in the IC. Cell sizes were also reduced and VGAT expression is elevated in the MNTB of the *Fmr1* KO mice suggesting increased inhibitory input and disinhibition of the LSO (Rotschafer et al. 2015).

The auditory cortex has received considerable attention in FXS. Rotschafer and Razak (2013) showed increased response magnitude and broader frequency tuning in the auditory cortex of *Fmr1* KO mice. Wen et al. (2018) showed that such responses are seen at P21, but not at P14, suggesting the origin of hyperresponsivity in this developmental time frame when cortical properties mature (Oswald and Reyes 2008, 2011). The

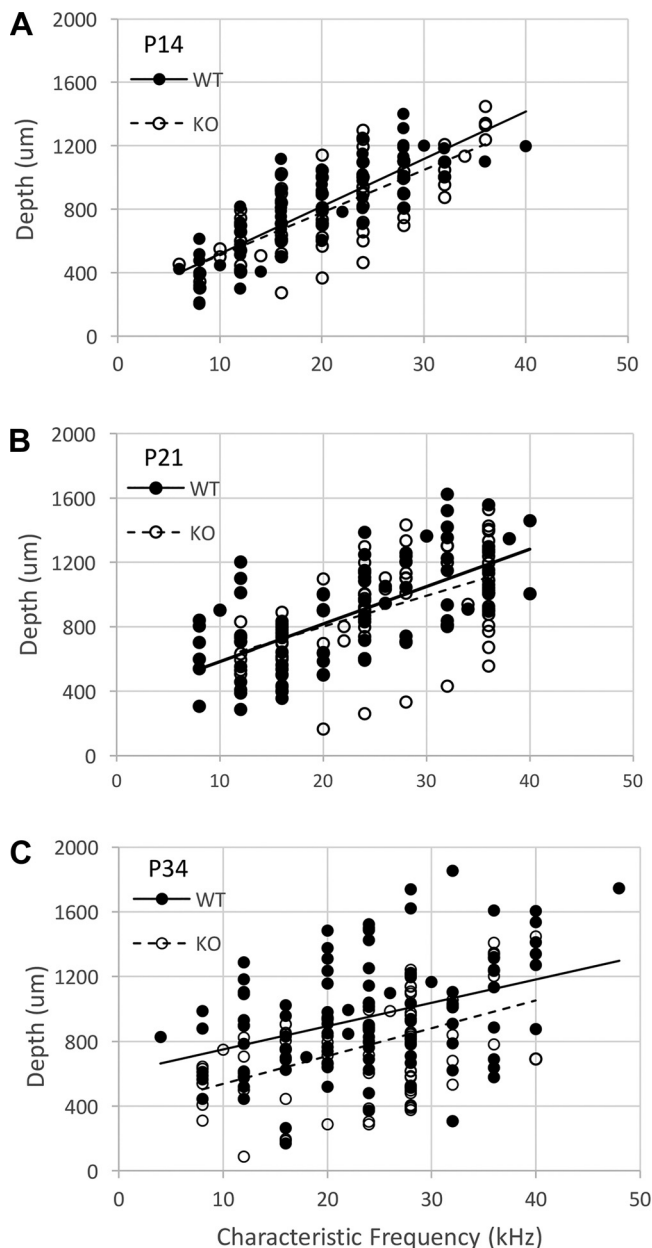


Fig. 12. No genotype differences were observed in inferior colliculus (IC) tonotopy at any age. Distribution of characteristic frequency (CF) along recording depth in the IC. There were no significant differences between wild-type (WT) and *Fmr1* knockout (KO) mice at each age group postnatal day (P)14 (A), P21 (B), and P34 (C).

IC also shows greater genotype difference in response magnitudes at P21, but not P14, suggesting that the cortical hyper-responsivity may be inherited from subcortical sites, including the IC. Similar developmental studies have not been performed in the medial geniculate body.

Consistent with abnormal inhibition, Wen et al. (2018) showed that increased matrix metalloproteinase-9 (MMP-9) in the auditory cortex may lead to abnormal development of perineuronal nets (PNN) and parvalbumin (PV)-positive inhibitory interneurons. Loss of PNNs will reduce excitability of PV cells resulting in reduced network inhibition. This suggests that at least part of the cortical deficit arises due to PV/PNN deficits that are local to cortex and not inherited from the IC. This

notion was further supported by a recent study that showed that removal of *Fmr1* only from forebrain excitatory neurons using the *Nex1* promoter results in enhanced low gamma oscillations in the cortex indicating local cortical circuit deficits (Lovelace et al. 2020). Interestingly, however, enhanced high gamma seen in previous cortical recordings from global *Fmr1* KO mice was not present when *Fmr1* was removed only from forebrain neurons. This suggests a combination of cortical and subcortical contributions give rise to the various auditory processing phenotypes studied in the *Fmr1* KO mice. The present study identifies the IC as a potentially strong hub of hypersensitive responses, at least in early development.

Methodological issues. For the c-Fos analysis, up to four mice were exposed together in a cage. Importantly, they were always of the same genotype and age range when tested together. The control (WT) group and the *Fmr1* KO group were tested with identical methods. Therefore, any group testing effects must affect each genotype similarly. However, any differences in social vocalizations or movement related sounds (walking, running, etc.) across genotypes may potentially affect c-Fos expression in auditory nuclei. Future studies will test one mouse at a time, and quantify movement-related sounds to address these caveats.

No counterstaining was used to distinguish layers of the cortex, divisions of the MGB, or the nuclei of the IC. The distinct areas within each auditory region were identified based on mouse brain atlas. Future studies with cytoarchitecture counterstains are needed to identify specific subnuclei with more precision. Electrophysiological responses were recorded under anesthesia, and this may have reduced the spiking activity of individual neurons. Whether the reported hyperactivity of IC neurons in *Fmr1* KO mice may actually be an underestimate remains to be identified with recordings conducted in awake mice.

We did not perform analysis of age-related changes in the density of c-Fos+ cells to identify any differences in developmental trajectories across the genotypes. The reason for this is the use of different sound levels at the two ages studied. We used the loudest sound levels that did not cause AGS at each age tested. The 85- and 90-dB levels used were ~5 dB below AGS threshold at P21 and P34, respectively. However, they were not matched in absolute sound levels.

Conclusions. We found region-specific deficits in both the density of c-Fos+ cells and response properties in the IC of developing *Fmr1* KO mouse. Most of the deficits were seen at P21, the time of high AGS susceptibility. In addition, the main differences were in dorsolateral IC and in neurons tuned to lower frequencies in the IC. This implies that FMRP affects differently low- and high-frequency regions of IC. Future studies should examine AGS with stimuli that are low-pass or high-pass filtered at ~20 kHz to determine if the *Fmr1* KO mice are more sensitive to low-frequency sounds during development. The lack of electrophysiological deficits at P14 indicates abnormal experience-dependent plasticity between P14 and P21, similar to that seen in the auditory cortex (Wen et al. 2018). In addition, studies on the cognitive and social impacts of early life IC dysfunction are needed to address development of autism related behaviors in *Fmr1* KO mice.

It is important to consider that in neurodevelopmental disorders, it is difficult to disambiguate direct effects of genetic changes, the effects of altered experiences of the animal caused by

the genetic change, and the compensatory plasticity mechanisms generated in the brain (Antoine et al. 2019; Bülow et al. 2019). In fact, many phenotypes observed in FXS (human and mice) are only seen transiently or fluctuate during development (Meredith et al. 2012; Vislay et al. 2013; Wen et al. 2019). Even transient disruptions during early development can have long lasting impact. In the case of auditory hypersensitivity in *Fmr1* KO mice, divergence towards hyperresponsiveness appears to occur between P14 and P21. Our data point to the importance of studies to track the developmental trajectories of phenotypes (Razak et al. In press). This will aid in identifying optimal treatment windows during development for clinical trials in FXS.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.O.N., D.K.B., I.M.E., and K.A.R. conceived and designed research; A.O.N. and K.A.R. performed experiments; A.O.N. and K.A.R. analyzed data; A.O.N., D.K.B., I.M.E., and K.A.R. interpreted results of experiments; A.O.N. and K.A.R. prepared figures; A.O.N., D.K.B., I.M.E., and K.A.R. drafted manuscript; A.O.N., D.K.B., I.M.E., and K.A.R. edited and revised manuscript; A.O.N., D.K.B., I.M.E., and K.A.R. approved final version of manuscript.

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REVIEW

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Developmental studies in fragile X syndrome



Khaleel A. Razak¹, Kelli C. Dominick^{2,3} and Craig A. Erickson^{2,3*}

Abstract

Fragile X syndrome (FXS) is the most common single gene cause of autism and intellectual disabilities. Humans with FXS exhibit increased anxiety, sensory hypersensitivity, seizures, repetitive behaviors, cognitive inflexibility, and social behavioral impairments. The main purpose of this review is to summarize developmental studies of FXS in humans and in the mouse model, the *Fmr1* knockout mouse. The literature presents considerable evidence that a number of early developmental deficits can be identified and that these early deficits chart a course of altered developmental experience leading to symptoms well characterized in adolescents and adults. Nevertheless, a number of critical issues remain unclear or untested regarding the development of symptomology and underlying mechanisms. First, what is the role of FMRP, the protein product of *Fmr1* gene, during different developmental ages? Does the absence of FMRP during early development lead to irreversible changes, or could reintroduction of FMRP or therapeutics aimed at FMRP-interacting proteins/pathways hold promise when provided in adults? These questions have implications for clinical trial designs in terms of optimal treatment windows, but few studies have systematically addressed these issues in preclinical and clinical work. Published studies also point to complex trajectories of symptom development, leading to the conclusion that single developmental time point studies are unlikely to disambiguate effects of genetic mutation from effects of altered developmental experience and compensatory plasticity. We conclude by suggesting a number of experiments needed to address these major gaps in the field.

Introduction

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability and most common single gene cause of autism. Common symptoms of FXS include hyperactivity, repetitive behaviors and cognitive inflexibility, hypersensitivity to sensory stimuli and seizures, social and language impairments, intellectual disability, and increased anxiety. FXS occurs as a result of *Fmr1* gene hypermethylation due to an unstable CGG triplet repeat expansion (> 200 repeats) in the 5' untranslated region of the *Fmr1* gene located on the X chromosome, which leads to gene methylation, inactivation, and resultant loss of fragile X mental retardation protein expression (FMRP; [147]). FMRP functions as a translational regulator,

affecting synthesis of many proteins including those involved in synaptic pruning during development. Additional functions such as modulation of ion channels have also been proposed [15, 29, 39]. A recent meta-analysis estimates the frequencies of individuals with the full mutation FXS allele to be approximately 1 in 7000 males and 1 in 11,000 females [75]. As an X-linked disorder, the phenotypic expression is quite variable in females because of random inactivation and potential compensation by the normal X chromosome [108]. Therefore, the prevalence of females who carry the full mutation allele and display the phenotypic features of FXS is less than 1 in 11,000 [75].

The *Fmr1* knockout (KO) mouse has been the most widely studied animal model of FXS, but other model systems such as drosophila and *Fmr1* KO rats have also contributed valuable information regarding basic biological functions of FMRP and mechanisms of pathophysiology when FMRP is removed. Over the past two decades, considerable progress has been made in terms of our understanding of symptomology and underlying

* Correspondence: craig.erickson@cchmc.org

²Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati, Cincinnati, OH, USA

³Division of Child and Adolescent Psychiatry, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue MLC 4002, Cincinnati, OH 45229, USA
Full list of author information is available at the end of the article



neurobiology in humans with FXS and animal models. Because of the monogenic origin of the disorder and significant overlap with autism, the interest in FXS is at least partly due to the potential for discoveries about FXS to serve as a paradigm for studies of neurodevelopmental disorders more broadly.

While several potential therapeutics show efficacy in reversing symptoms in the *Fmr1* KO mice, they have failed clinical trials [12]. A number of reasons may explain these failures including the validity of the pre-clinical models, validity of outcome measures, insufficient stratification of patients, optimality of dose and duration, and finally, appropriate treatment age. To address optimal treatment ages, we focused on developmental studies of both humans and mice to generate hypotheses based on published data. FXS is a neurodevelopmental disorder. However, there is little understanding of whether and how functions of FMRP change during development. Only a few studies have systematically examined the roles of this protein for normal brain development over time. The developmental trajectories of symptoms and their relationship to changes in underlying mechanisms are only beginning to be understood. In human work, only a handful of studies have examined early childhood development (age < 5). Only a few longitudinal studies with large sample sizes have examined trajectories of developmental change in FXS phenotypes. There is little data-driven guidance on optimal time points in development for clinical tests of potential therapeutics. While studies point to early detection of many phenotypes, whether developmental treatment has longer lasting benefits than adult treatments remains unknown. In the following sections, we summarize the animal and human studies that have bearing on these issues and suggest a number of future experimental approaches that are needed to fill major gaps in our knowledge of FXS development.

Animal Models

Development of mechanisms and phenotypes in animal models of FXS

There is an extensive literature on preclinical animal models of FXS, mostly in mice. Many of these studies have examined phenotypes during early development either due to convenience of methodology (e.g., in vitro slices are easier to record from in very young mice) or to examine developmental trajectory. Almost all of the developmental trajectory studies in animal models use a cross-sectional approach. Such studies conducted before 2014 have been comprehensively reviewed elsewhere [24, 102]. Here, we summarize key studies done in the past few years and focus on the gaps and paradoxes in the developmental

literature on animal models. The major point made by these studies is that many of the phenotypic differences between KO and WT mice are developmentally transient, suggesting the importance of studying trajectories. This is all the more relevant if altered brain responses attributed to an imbalance in excitatory and inhibitory function reflects compensatory plasticity, and is not necessarily the primary defect driven by the genetic mutation causing neurodevelopmental disorders [4, 102, 136].

Developmental regulation of FMRP expression

FMRP levels are developmentally regulated [46] in the mouse brain. Peak FMRP expression during development occurs between P3 and P12 in different brain regions and is reduced in adulthood. This age coincides with the onset of sensory stimulation and developmental critical period plasticity windows, suggesting an important role for FMRP in experience-dependent plasticity. In the auditory and somatosensory cortex, peak FMRP levels are seen between P7 and P12. In the hippocampus, cerebellum, striatum, and brainstem, the peak FMRP expression is between P3 and P7. FMRP is expressed across cell types in multiple brain regions in adults. Whether different cell types in these regions show different developmental profiles of FMRP expression has not been studied.

Such consistently high levels of FMRP expression in early development suggest that the function of FMRP is essential during this time period. FMRP regulates protein translation by association with a vast array of mRNAs [16, 148]. A well-known phenotype in the *Fmr1* KO mouse brain is increased protein synthesis [93, 109]. However, it remains unclear if this phenotype is observed across development and if different brain regions have similar changes in protein synthesis across development. Most studies examine a single or a few time points in specific brain regions. This issue is further complicated in that different cell types may show different proteins whose levels are increased or decreased. Recent studies of protein synthesis in humans with FXS show that at least some of the patients show decreased protein synthesis [81, 114], opposite to that seen in the mouse model. While this naturally leads to questions on the utility of the mouse model, this paradox can be potentially resolved if developmental profiles of protein synthesis in FXS in different brain regions are characterized in rodent models, and if possible, in humans. This remains an outstanding issue.

When adult phenotypes of a genetic disorder are considered, it is difficult to disentangle direct effects of the genetic mutation from effects of altered developmental experience [4]. One approach is to study mechanisms of

pathophysiology early in development before the animal has had considerable time for developmental experience to shape the brain. Such studies show that the lack of FMRP leads to several synaptic, circuit-level and behavioral alterations, many of which are seen very early in development [102]. These studies show that FMRP is clearly involved in brain development, particularly in shaping synaptic connectivity. These deficits most likely impact experience dependent plasticity and lead to permanent changes in brain and behavior. However, in most studies of potential therapeutics, treatment of adult mice or brains shows reversal of phenotypes. This would suggest that the structural and functional brain alterations that develop due to the lack of FMRP in early development could be fixed by focusing on specific molecular pathways in adulthood. It is worth highlighting this as a paradox. Given the number of mRNAs under the control of FMRP, the high expression of FMRP during critical periods of brain growth, and extensive evidence that altered activity early in development causes long-lasting and potentially irreversible changes into adulthood, the ability of adult treatment to reduce a vast range of symptoms in animal models is quite remarkable. This ability of adult treatment to reverse symptoms has major implications for whether the *Fmr1* gene can be reactivated in adults and what the prognosis might be. However, we argue here that two major classes of experiments are needed to fully support the idea that adult treatment alone may be sufficient to treat symptoms of FXS. We also point to the scarcity of such studies in the literature.

1. Test the necessity and sufficiency of FMRP in normal brain development. These experiments, performed in animal models, will entail removing FMRP during an early developmental window, and re-expressing it later in adults. The reverse experiment in which FMRP is removed only in adulthood will address the sufficiency question. In both cases, adult phenotypes should be examined to determine if early normal FMRP is necessary and sufficient to reduce symptoms in adults.
2. To be performed first in animal models, experiments should determine the longevity of benefits in terms of symptoms if early developmental versus late (adult) treatment windows are used. Depending on the results in preclinical studies, clinical trial designs should explore this issue in children.

Currently, only a few studies in the literature have performed the experiments to test the role of FMRP in development, and they point to different outcomes. In the drosophila model of FXS (*dFmr1* KO flies), the small

ventrolateral neurons (sLNv) are GABA-responsive, PDF-peptide expressing neurons involved in circadian pattern generation [44]. *dFmr1* KO flies do not exhibit circadian rhythmicity. sLNv in *dFmr1* KO flies have overgrowth and overextension of synaptic arbor. Overexpression of *dFmr1* specifically in these neurons can reduce synaptic arbor. The expression of *dFmr1* in early development (time of neurogenesis and migration) or in adults does not change synaptic architecture. However, *dFmr1* expression during an intermediate developmental window (pupal days 3, 4) normalize synaptic arbor. This is also the time window when dFMRP levels are normally high in WT flies. While the effects of normalizing synaptic arbor with this brief period of *dFmr1* expression on circadian patterns were not reported, this study shows the sufficiency of normal sLNv *dFmr1* levels during a developmental window in normal synaptic development. Doll et al. [31] also concluded that FMRP function during early developmental critical window is absolutely required for drosophila olfactory circuit organization.

A more recent study in the mouse shows that FMRP in early development is not necessary for the normal expression of a prefrontal cortex (PFC) dependent task, the trace eye-blink conditioning in adult mice [127]. Mice were trained to associate a sound with a puff of air to the eyelid. Eye blinks could be elicited with the sound alone once training is complete. This is called a “trace” conditioning paradigm because of the temporal gap between sound cue and puff of air; the gap makes this task PFC dependent. FMRP deletion only in the adult revealed an increase in the proportion of non-learners and a delay in the onset of learning. This was true in both the full *Fmr1* KO mice and the conditional KO mice in which FMRP was specifically removed from the PFC. Mice without FMRP in PFC in early development, but normal FMRP expression in PFC as adults, did not show learning deficits. This indicates acute roles for FMRP in adult PFC in acquisition and maintenance of eye-blink trace conditioning. Arsenault et al. [5] used viral vectors to express FMRP with the synapsin promoter (for expression in neurons) in mice and determined behavioral outcomes in adults. A single intracerebroventricular injection of adeno-associated viral vector was made in P0–P2 mouse pups. FMRP expression in adult brains was mostly seen in the forebrain. When adult behaviors were examined, hyperactivity, elevated plus, and acoustic startle abnormalities were significantly rescued. While the developmental trajectory of FMRP expression levels is unclear from this study, the results point to the potential utility of post-natal expression of FMRP in reversing symptoms of FXS. Zeier et al. [149] expressed FMRP bilaterally in the hippocampus by injecting the viral vector construct in 5-week-old mice (adolescence/young adult

age). They observed that hippocampal enhanced LTD phenotype is rescued with FMRP expression in the adult hippocampus. This further suggests that FMRP expression in adults is sufficient to restore normal plasticity in hippocampus.

While the mouse and drosophila studies come to different conclusions regarding the necessity of FMRP in early development, a number of differences (even beyond species differences) need to be considered. Terms borrowed from developmental neuroendocrinology may be applicable here to provide a scaffold for future investigations of FMRP in neurobehavioral functions. In the drosophila study, the outcome measured was an “organizational” role of FMRP in terms of synaptic arbor. Whereas in the mouse eye-blink conditioning study, the outcome measured was an “activation” effect of FMRP in terms of experience dependent plasticity. These studies suggest that depending on the outcome measure, the necessity and sufficiency of FMRP in development of FXS symptomology may be different. Perhaps, FMRP plays an early role in synaptic organization during the time of high expression in early development (P3–P12 in mice). The levels decrease into adulthood during which FMRP is necessary for activation of experience dependent plasticity. These results have major implications for the outcome measures that need to be considered in adolescent and adult treatments of patients. Mechanisms and biomarkers that are involved with organizational effects may be irreversibly altered if adult treatment is utilized, while activation effects involved in learning outcomes may be corrected.

Lessons from Angelman and Rett syndromes

The effectiveness of developmental treatment in providing long-lasting benefits has been shown in other disorders such as developmental hearing loss [86] and Angelman syndrome. Particularly pertinent to this review is Angelman syndrome (AS), a neurodevelopmental disorder that has a number of features on the autism spectrum. AS results from the loss of function of maternal UBE3A allele. The pharmacological reactivation of the paternal Ube3a gene in adult mice was not effective in reducing most symptoms of the disorder including epilepsy. However, a systematic examination of critical developmental windows for reactivation of Ube3a in mice revealed that anxiety, repetitive behaviors, and epilepsy were normalized only if reactivation is done from embryonic development [128]. Motor behaviors are normalized if reactivation is commenced at ~P21, but not in adults. Synaptic plasticity (long-term potentiation) in the hippocampus is normal if Ube3a is activated at any time. This is consistent with the *Fmr1* KO mouse study in which even adult expression of *Fmr1* is sufficient for learning related behaviors (“activational effects”). In a

recent paper on an AS mouse model, Gu et al. [53] identified an abnormal accumulation of perineuronal nets in the hippocampus during epileptogenesis and showed that reinstatement of Ube3a from ~P21 reduces PNN and epileptogenesis in the AS model mice, but adult reinstatement did not. Together, these results suggest distinct sensitive windows during which Ube3a reactivation has to occur for different phenotypes. This is in contrast to another neurodevelopmental condition, Rett syndrome, in which both developmental and adult activation of *Mecp2* (mutation in the X-linked MECP2 gene causes Rett syndrome) can successfully reverse Rett syndrome associated pathologies in a mouse model [55]. These studies highlight the importance of understanding functions of Ube3a, *Mecp2*, and *Fmr1* at different developmental ages.

However, the topic of critical windows for treatment or reactivation efficacy has received very little attention in FXS. Only a few studies have directly addressed the efficacy of developmental versus adult treatment in reversing phenotypes. Dansie et al. [27] showed that minocycline treatment in *Fmr1* KO mice was effective in reversing anxiety-like behaviors. When adult mice were treated, a continuous administration of the drug was needed to maintain the effects. However, treatment in young mice has lasting effects after treatment cessation. He et al. [69] showed that inhibiting the chloride cotransporter (NKCC1) with bumetanide treatment during an early developmental period normalizes the development of thalamocortical synapses in the mouse somatosensory cortex. Early inhibition of NKCC1 was sufficient to reduce the size of whisker evoked activity spread in the adult mouse cortex. These studies suggest that early treatment leave a long lasting impact on brain and behavior in *Fmr1* KO mice. Hodges et al. [71] showed that a single seizure in *Fmr1* KO mice during early development can lead to long lasting impairments in cognitive behaviors. Approximately 20–30% of children with FXS exhibit seizures. The potential impact of early life seizures on adult treatment efficacy is unknown in FXS. A systematic evaluation of treatment age and duration of drug efficacy on a number of symptoms is, therefore, a critical need in FXS research and more generally in autism spectrum disorders.

To what extent is developmental compensatory plasticity involved in adult pathophysiology?

An intriguing finding across studies is the consistent observation that many of the phenotypes are either seen only transiently, or show considerable changes in direction and/or strength during development. This seems to be the rule rather than an exception and point to the clear importance of studying developmental trajectories to understand mechanisms of pathophysiology and to

identify optimal treatment points. The topic of fluctuating or transient phenotypes has been reviewed thoroughly by Meredith et al. [102] and Meredith [103]. Additional studies since then continue to show similar trends. For example, when sound driven single unit spiking responses are evaluated in developing and adult auditory cortex, hyper-responsiveness is observed at P21 in adults, but not in P14 and P30, in *Fmr1* KO mice [140]. Hyper-responsivity develops between P14 and P21. A study that examined mouse cortical EEG responses at P21, P30, and adults came to a similar conclusion [142]. The sound-driven non-phase-locked gamma power of EEG responses was significantly elevated in the auditory cortex and frontal cortex of P21 and adult *Fmr1* KO mice compared with WT. However, at P30, the non-phase-locked power was reduced in the *Fmr1* KO mice, again pointing to developmentally fluctuating phenotypes. The resting gamma power was elevated in the *Fmr1* KO mouse frontal cortex at all ages tested pointing to region specific developmental trajectories of different phenotypes.

Wen et al. [141] suggested that the hyperexcitability at P21 may arise due to impaired function of parvalbumin (PV) positive inhibitory neurons due to the altered expression of specialized extracellular matrix assemblies called perineuronal nets (PNN). The altered function of PV cells is also seen during development in the somatosensory cortex [107] and adult visual cortex [48]. PNNs protect PV cells against oxidative stress and also function to increase excitability of these cells. Reduced excitability of PV cells can lead to reduced inhibition in the circuit and hyperexcitability. Abnormal development and dysfunction of GABA neurons is also seen as a key mechanism underlying other neurodevelopmental disorders including Rett [23] and Angelman syndrome. In Rett syndrome model mice, there is an abnormally early maturation of PV neurons in the visual cortex [32]. Notably, daily low-dose Ketamine treatment of *Mecp2* mice can reverse symptoms by modulating NMDA receptors on PV neurons [111]. The treatment that started at ~P30 (onset of regression) was as effective as the treatment that started earlier in development, consistent with findings of Guy et al. [55].

PNNs around PV neurons can be modified by actions of an endopeptidase, matrix metalloprotease-9 (MMP-9), a target of FMRP. In the KO mice, the lack of FMRP leads to increased MMP-9 levels throughout the development and deterioration of PNN. The P14–P21 developmental window is a time of considerable experience dependent plasticity in the mouse auditory system. Abnormal response during this critical period plasticity window is predicted to have consequences for the development of auditory processing [83]. By P30, however, PV/PNN expression and response magnitudes are

normal in the KO mouse auditory cortex consistent with other findings regarding transient phenotypes. In the adult brain as well, PV/PNN expression is normal, but hyper-responsivity is present suggesting potentially novel mechanisms in adulthood, or a “sleeper” effect that arises as a result of developmental alterations. Exposure of developing *Fmr1* KO mice between P5 and P21 to sounds reduces neural correlates of auditory hypersensitivity including a normalization of ERP responses, dendritic spine density, and PV neuron density [87] suggesting the potential use of non-pharmacological treatment strategies either in isolation or in combination with drugs. Together, these studies call for early treatment to observe if adult effects can be prevented. If transient changes in early development can be prevented, does that have long-term beneficial effects in disease progression is a question that has received very little attention in the field.

Human development and FXS

Similar issues emerge when examining human data across the lifespan. FMRP is expressed across cell types in multiple brain regions; we have little to no understanding of changes in FMRP expression across development, and it becomes even more difficult to disentangle the effects of genetic mutation from the effects of altered developmental experience in the human brain. FMRP influences synaptic plasticity (the “activation” effect) as well as the structural integrity (“organizational”) of the brain. How this translates to phenotypic changes and which aspects are most critical for successful intervention are unknown. There is a paucity of human work that links neural (dys) function to both disease phenotype and molecular etiology in a developmental framework. This connection is vital for patient stratification and evaluation of potential biomarkers in future treatment studies in order to optimize outcome measures to correlate with clinically meaningful symptomatic change.

Animal studies have demonstrated the developmental regulation of FMRP expression in neural tissue. In adults, FMRP is widely expressed in epithelial cells, neurons, and glia [11, 30, 47]. We have limited knowledge regarding FMRP expression across development in the human brain. FMRP expression is seen in fetal neural tissue as early as 13 weeks gestation [1], but little to no FMRP expression was noted in a 3- to 7-week embryo [2]. How FMRP expression evolves throughout fetal and early development in humans is unknown. FXS is often not diagnosed until the toddler years; therefore, studies in very young children with FXS are limited but suggest that detectable phenotypic changes occur quite early. Behavioral [119] and neural [133] changes have been identified as early as 6 months old. However, studies have only examined children down to age 5 months, and

longitudinal data is lacking making our understanding regarding the stability of neural and symptomatic changes in FXS limited. We have some information about the relationship between neuroendophenotypes and FMRP levels. Some have probed this question directly by measuring FMRP. Others studies have stratified participants according to sex, mosaicism, and methylation as a proxy for direct FMRP measurements. The studies outlined below suggest complex developmental trajectories across a number of phenotypes in FXS and highlight the need to examine developmental trajectories rather than single time points in relation to molecular changes.

The brain in FXS

Preclinical work suggests wide reaching effects of FMRP on neural structure and function. Human neuropathologic studies demonstrate dendritic spine abnormalities in FXS [78–80] with limited consistent gross pathologic change [1, 51, 121]. Neurophysiologic studies, on the other hand, do show some replicated changes in neural structure and function in FXS across the lifespan.

The earliest changes in the FXS brain to date have been seen in the white matter at 6 months old using diffusion tensor imaging (DTI) [133]. In their study of 27 infants (22 males) with FXS and neurotypical controls ($n = 73$) followed from 6 to 24 months, Swanson et al. [108] found lower fractional anisotropy (FA) across long-range pathways within the brain: those connecting subcortical structures (including the thalamus, basal ganglia, and cerebellum) to the prefrontal cortex, the corpus callosum in areas linking the primary and premotor cortices and in the bilateral uncinate fasciculus. Authors found that the lower FA compared with controls was stable from 6 to 24 months of age and was strongly correlated to IQ in the FXS group. Studies in older individuals with FXS found increased FA in regions that overlap somewhat with regions identified to have decreased FA in the infant work. Green et al. [52] found increased FA in the inferior longitudinal, inferior frontotemporal, and uncinate fasciculi in a group of 40 young adults with FXS (25 female). Hall et al. [59] had very similar results. In contrast, Barnea-Goraly et al. [10] found decreased FA in a small group ($n = 10$) of females (ages 13.1–22.7) with FXS in the frontal-striatal WM, with a small region of increased FA in the superior temporal gyrus. The small number within this study along with the participants being all female may help to explain these differing results. There is also the potential that the regional developmental regulation of FMRP is different in different neural systems which can lead to divergent effects in the adult brain. The discrepancy seen between the infant study and later finding of increased FA in overlapping regions is consistent with a putative

role for FMRP in white matter maturation. As outlined above, FMRP regulates the dendritic translation of a wide variety of proteins critical for synaptic and dendritic function [125]. Dysfunction in this system has a potential effect on pruning and resultant modeling of neural systems. In addition, FMRP has a putative role in the regulation of myelin basic protein (MBP) [47, 110], which plays an important role in the developmental regulation of myelination [13]. Recent work in the mouse model has demonstrated delayed myelination in early development [110].

Neuroimaging studies have suggested a central role for frontal-striatal circuitry in the neuropathology of FXS. Enlargement of the caudate nucleus is one of the most consistent findings in the FXS neuroimaging literature [62, 72, 74, 144] often in association with abnormalities in frontal lobe volume [14, 17, 18, 62]. Caudate abnormalities are seen across MRI modalities, in multiple age groups and in cross-sectional and longitudinal design (e.g., [20, 49, 50, 90]). Haas et al. [56] demonstrated structural changes in frontal-striatal circuits in toddlers with FXS. In a functional imaging study examining frontal-striatal functioning utilizing a go/no go task, Hoeft et al. [73] demonstrated frontal-striatal changes mediated by FMRP and striatal dysfunction. Some studies indicate a more striking increase in males with FXS [35, 49, 50] which may indicate a direct relationship between FMRP expression and caudate structure; however, others have not seen this sex difference [90]. Studies exploring the relationship between FMRP levels and caudate structure more directly support a relationship. Hoeft et al. [74] demonstrated a negative correlation between caudate volume and FMRP in young children with FXS. Gothelf et al. [49] found the same relationship in an older group of participants. FMRP was significantly negatively correlated with caudate volume. Gothelf and colleagues also found FMRP levels to be related to the increased volume of the posterior vermis of the cerebellum, while Hoeft et al. [74] did not see this same relationship in early childhood. Overall, the increased volume of the caudate nucleus appears to be associated with less FMRP and more severe phenotypic expression across different behavioral domains [120, 144]. Stereotypic behavior and inappropriate speech have each been linked to caudate structure in FXS [112, 144]. This is one of the few areas where we start to see a potential molecular-brain-symptom connection emerging that is present in early development emerging in the literature.

We see changes in the striatum in the few longitudinal neuroimaging studies of young children with FXS as well. In a study including 53 male toddlers (18 to 42 months of age) with FXS [68], there was a stable increase in the volume of the caudate, putamen, and globus pallidus, most striking in the caudate nucleus. Bruno

et al. [19] used both cross-sectional and longitudinal approaches to distinguish two groups ($N = 13$ and $N = 24$) of boys (mean age 2.89 years at baseline; mean age = 4.9 years at follow-up) with FXS differentiated by enlarged gray and white matter regions including the caudate, thalamus, frontal lobe, temporal lobe, and cerebellum. The boys with FXS and larger brain volume ($N = 13$) showed reduced adaptive behavior, lower initial IQ, and more ASD symptoms than the comparison FXS group of boys ($N = 24$). Though this group generally had a more severe phenotypic profile, there was no correlation found with FMRP in this cohort.

Additional regions consistently linked to FXS, where changes have been documented early in development, include the amygdala [67, 74, 82] and cerebellum [51, 54, 74, 105, 117, 143]. Each of these structures has been linked to specific features of the FXS phenotype [61, 138]. However, characterization of the influence these brain regions have on the FXS phenotype through development is lacking.

A preliminary look at the FXS brain from a network perspective in older individuals with FXS [17, 18] suggests that there is limited connection between subcortical and cortical systems. Bruno and colleagues [18] found that the caudate and amygdala were less involved in large scale neural networks in FXS. The result of changes in subcortical structures may be a decreased ability to recruit higher order brain regions to modulate behavior.

EEG studies in adolescents and adults with FXS have demonstrated a robust and reliable phenotype of altered gamma band activity [37, 38, 137] that is replicated in the mouse model [99] and correlates with clinical symptoms including sensory hypersensitivities and social/behavioral measures [36–38, 137]. None of these studies have examined the relationship between EEG results and FMRP. When looked at indirectly by comparing males and females, most have seen no difference in these groups, with one exception [36]. There have been no studies examining this phenotype in early development or longitudinally. Given the developmental changes seen in EEG phenotypes with the mouse model, and the central role EEG plays in translational studies of FXS (discussed in more detail below), this will be an important question to address in the near future.

Behavioral and cognitive development

Standardized cognitive and adaptive measures show changes prior to age one. Roberts et al. [119] demonstrated differences as early as 6 months old when examining cross-sectional profiles on the Mullen Scales of Early Learning (MSEL, [106]). Previous studies had demonstrated changes as early as 9 months old in infants with FXS for both cognitive and adaptive measures [21,

118]. Overall, studies examining adaptive and cognitive functioning in FXS suggest a delay followed by steady gain of skills and then what may appear to be a decline, but is more likely a plateau of skills compared with the normative sample used to calculate standard scores [21, 33, 40, 85, 118, 130, 131]. This apparent decline is less severe in females, believed to be due to the expression of some FMRP in females due to their necessary mosaicism [85, 145]. Research directly examining FMRP expression and cognitive behavioral phenotypes in FXS is quite variable. Many have linked FMRP to general cognitive impairments in FXS [7, 8, 96, 97, 130, 134] while others show no relationship [66, 130].

There is some evidence that this pattern in skill development is not uniform across cognitive domains. For instance, Quintin et al. [115] followed cognitive development in 114 males and 70 females with FXS evaluated first at a mean age of 11.3 years with 1–6 years between follow-up points with up to 3 cognitive measures using the Wechsler Intelligence Scale for Children [139]. Over time, a widening of the gap in FXS compared with the normative IQ sample was noted in verbal comprehension, perceptual organization, and processing speed with a narrowing of the gap noted in freedom from distractibility. In a small 1 year follow-up study focused on attention and working memory in FXS, 21 boys with FXS and an equal number of matched typically developing youth (mean age = 8.8 years at first visit), attentional control and working memory were noted to improve over time in the FXS group mirroring developmental change in the control group [25]. This is in contrast to other studies that have demonstrated attention skills similar to mental age matched controls [132] or a relative impairment [123]. There is some limited evidence deficits in FMRP are specifically linked to executive dysfunction in FXS [41, 88, 95, 98], most consistently with working memory and attention. However, some phenotypic studies suggest sparing in this domain [25, 115] which is at odds with this interpretation.

Longitudinal profiles of language development are limited in FXS and predominantly in older individuals. One recent study [116] examined very early language in a small sample of infants and toddlers with FXS using an automated vocal analysis system. Results in this group of 11 males with FXS (ages 17 to 64 months) showed that infants and toddlers with FXS had significantly less vocalizations per hour than typically developing peers, but similar numbers to developmentally age-matched peers suggesting that language in this age group is delayed in proportion to general developmental delay. There was also significant variability in the FXS group in this small study. Martin et al. [101] followed 29 youth with FXS without ASD, 40 youth with FXS and ASD, 34 youth with Down syndrome, and 40 typically developing mental age matched youth ages 3.1 to 16.0 years

over a period of 3 years. In these samples, the rate of language gain was higher in the typically developing group compared with those with FXS regardless of ASD status showing a similar language development trajectory to those with Down syndrome. Together, this research suggests that language development is tightly linked to overall cognitive development in FXS and appears to follow a similar developmental trajectory.

There are mixed results when examining the emergence and development of ASD symptoms over time in FXS [6–9, 42, 65, 91, 122]. However, the majority of these studies have been limited by small sample size and/or cross-sectional design. Two larger longitudinal studies [65, 91] suggest that autism symptoms in FXS may increase with age with the most prominent increase noted in social communication features consistent with ASD. Overall, we see that those children with FXS and comorbid ASD have a more severe presentation. For example, Caravella et al. [21] examined the development of adaptive skills in relation to autism symptoms in male infants with FXS ($N=25$) compared with typical developing male infants ($N=24$) and infant male siblings of persons with ASD ($N=27$) [21]. They found that increased severity of ASD symptoms was related to increased deficit in adaptive skills at 24 months of age. We see a similar pattern of results with ASD symptoms in relation to FMRP. Some have demonstrated a relationship between peripheral FMRP levels and ASD symptoms [65, 70]. Hatton et al. [65] examined the role of FMRP in the expression of ASD symptoms and found a significant relationship with increased ASD symptoms related to lower FMRP levels. Hessel et al. [70] also found a link between FMRP and ASD symptoms, but only in girls. Other studies show no link [7, 8, 63, 94].

In terms of interfering behaviors, Hustyi et al. [76] examined the longitudinal trajectory of interfering behavior in 64 males and 60 females with FXS (ages 2 to 26 years) using two more assessments with the Aberrant Behavior Checklist (ABC [3]); with an average interval of 4 years between evaluations with a mean subject age of 11.6 years at first assessment [76]. In this cohort, irritability (aggression, self-injury, and severe tantrums) and hyperactivity significantly reduced with age and social withdrawal, stereotypic behavior, and inappropriate speech did not change over time as measured by the ABC. This is similar to earlier longitudinal studies examining interfering behaviors [34, 64]. Hall et al. [58] found no relationship between interfering behaviors and FMRP. All studies have examined a rather large age range. As a result, we have some understanding of improvement and stability of symptoms in later childhood, but we have little information about the emergence of interfering behaviors and early modifying factors.

All behavioral phenotypic studies outlined above have similar limitations. Most have small sample sizes and limited time for follow-up. Symptom clusters are often examined in isolation, and there is little exploration of the relationship between phenotypic changes and brain development or FMRP levels.

What about FMRP?

The lack of FMRP is the core deficit of FXS. As noted above, FMRP levels have been linked to a small group of symptoms and neural changes in human studies of FXS; however, this work is limited in sample size and reproducibility. FMRP is difficult to measure, and there are currently many methods used for FMRP quantification with no general consensus regarding the optimal method (reviewed in [89]). As a result, the studies above have relied on a variety of measures making comparison across studies more challenging. There have been no large studies examining test-retest reliability, inter- or intra-individual variability of FMRP measurement in individuals with FXS. Until this hurdle has been overcome, future studies probing the link between molecular and phenotypic changes in FXS will be at a significant disadvantage.

Summary of human data

Human data suggest that very early in life brain deficits exist in FXS and can be captured clinically as well as neurologically. In each domain of development (behavior, cognition, language), limited longitudinal data exists in FXS. Despite the limited nature of the data, the available body of literature does suggest delayed but steady improvement in cognition, adaptive behavior, and language in those with FXS without ASD. In addition, FXS + ASD likely leads to a more severe presentation overall in interfering behaviors and in social-communication deficits. We have little data to link specific brain abnormalities to these phenotypic profiles. Interpretation of these results is impeded by diverse methodology in following subjects with FXS, scarce long-term longitudinal data, and small sample sizes. In addition, this research suggests there are some deficits and behaviors more related to cognitive impairment and global delay, while other changes may be independent of intellectual disability in FXS such as attention and anxiety, though there has been very limited work in young children examining these symptoms in a systematic manner.

It will be important to longitudinally examine both the phenotypic characteristics in FXS, changes in neural structure and function, and changes in FMRP and how they relate. Disentangling the influence of cognitive impairment and global delay will require the inclusion of developmentally matched control groups.

Challenges to integrating human and animal model work

An important challenge in translational neuroscience is the identification and validation of conserved biomarkers across species that can facilitate the treatment development pipeline. This is even more difficult when comparing phenotypes at different developmental ages, because there is no good way to identify equivalent ages across species. Sensory system development may provide an opportunity to effectively address this challenge for multiple reasons. Sensory (particularly auditory) hypersensitivity is present in both humans with FXS and in the *Fmr1* KO mice. There is a rich literature on normal sensory system development in both humans and animals, including mice. This includes a number of studies on the long-lasting effects of neural and behavioral manipulations during critical period plasticity windows, and how these windows can be changed. Equivalent developmental periods (irrespective of chronological age) of increased brain sensitivity to sensory manipulations can be established across species. The brain circuits that shape low-level sensory processing are likely to be more conserved across species compared with circuits involved in complex cognitive and social functions. There is relatively less information on the development of circuits involved in cognitive and social behaviors. Taken together, these considerations suggest that studies of sensory system development offer an avenue to integrate human and mouse work.

Recent studies that were inspired by the above considerations used EEG/ERP recordings and coordinated experimental design to identify remarkably similar auditory response phenotypes in humans with FXS [36–38, 137] and the *Fmr1* KO mice [99, 100, 129, 142]. EEG recordings show that in both species there is increased baseline gamma band power, reduced inter-trial coherence of sound evoked oscillations, enhanced N1 component amplitude of ERP, and reduced habituation of auditory ERP amplitudes. Gamma band abnormalities are correlated with parent report of sensory hypersensitivity and social communication deficits, and a number of clinical ratings in humans, and are sensitive to drug treatment in humans [124] and in mice [129]. In mice, these response abnormalities are present from P21 [142] and may develop between P14 and P21 due to abnormal maturation of PV expressing GABA neurons [141]. Together, these studies suggest EEG/ERP phenotypes can be used as biomarkers for stratification and drug-outcome prediction in humans and to understand pathophysiological mechanisms and drug development in mice.

Despite the wealth of data on adult human EEG/ERP data in FXS, relatively few studies have examined developmental changes in EEGs in children. The lack of

longitudinal and early EEG data is a significant gap in the literature. Therefore, a number of fundamental questions regarding EEG abnormalities in FXS remain unanswered. In neurotypical children, gamma oscillations are detected around 16 months of age and continue to increase until approximately age 5 [135]. During those first 2 years of neurotypical growth, we see significant within network synchronization across functional networks [43]. The emergence of cortical networks and synchronized oscillations are linked. The increased precision with which oscillations are synchronized likely relates to the maturation of the organization—both structurally and functionally—in cortical networks. The emergence of synchronized gamma oscillations is linked to specific cognitive skills, for instance perceptual binding and early gamma changes are related to the later development of cognitive and language skills [43]. Early longitudinal EEG studies are needed in order to understand how the emergence of frequency bands may be altered in FXS and how changes are linked to symptoms. Multimodal studies that incorporate multiple objective measures are required to expand our mechanistic understanding of FXS. Developmental profiles of EEG/ERP changes may be a useful marker to test the outcomes of early versus late treatments in humans. The use of analogous stimulus design and recording methods in mice and humans may serve as a paradigm to identify objective outcome measures specific for different developmental time points. But such studies are currently lacking.

In addition, we need a better understanding of the changes across modalities probing brain function and how these changes relate to both the underlying FMRP deficit and resulting phenotypic presentation. MRI studies have revealed early changes in the FXS brain and suggest that imaging based biomarkers can be used to test the outcomes of early interventions. However, no studies have examined the link between EEG abnormalities and localized brain changes identified in MRI studies. Combining neuroimaging and EEG across development may offer important clues regarding the localization of change in the FXS brain. In FXS, we see abnormalities in functional networks in adolescents [60] but have no understanding regarding when these changes emerge. The amplitude of BOLD signals in functional MRI is closely and positively correlated with the entrainment of neurons into synchronized gamma band oscillations. Therefore, we should see the coincident emergence of functional networks and gamma synchronization across development. In addition, brain changes in both humans and mice should be examined in relation to FMRP levels and phenotypic presentation to disentangle the effect of general developmental delay from specific effects of FXS.

In mice, the relationships between physiological responses and perception should be examined in more detail. Goel et al. [48] showed that reduced neural visual orientation selectivity in *Fmr1* KO mice was related to poor behavioral orientation selectivity. This was shown to be related to abnormal PV cells function, because increasing PV cell activity reduced both neural and perceptual deficits in the KO mice. Interestingly, similar behavioral deficits were also seen in humans with FXS, demonstrating a path towards integrating human and mouse work. In humans, the use of more quantitative and objective measures rather than parent report will add to the richness of phenotypic data and likely lead to a more complete understanding of brain-behavior relationships in FXS. This includes measures such as expressive language sampling, eye tracking, and investigator scored social measures such as the BOSCC (brief observational measure of social communication, [84]). A better understanding of these early brain-behavior changes has implications in how we approach treatment development in FXS.

Multiple lines of evidence in the *Fmr1* KO mouse indicate abnormal GABAergic inhibition across different brain areas. In humans, only a few studies have examined inhibitory mechanisms. D'Hulst et al. [26] showed reduced GABA_A receptor binding using positron emission tomography (PET) scan. Morin-Parent et al. [104] used transcranial magnetic stimulation (TMS) to reveal abnormal functional inhibition in humans with FXS. As seen with mice, these results point to reduced inhibition as a mechanism of hyperexcitability. The human studies were done in adolescents and/or adults. The timing of neural changes can offer insight regarding critical time points for intervention in this system. For instance, in early development, GABA is depolarizing to neurons and then later become hyperpolarizing. Recent work suggests a delay in the GABA reversal is delayed in FXS [69]. This may be linked to changes in plasticity in early development that we may be able to capture. However, this may also be a nonspecific compensatory downstream effect of the FMRP deficit. Delay in GABA reversal is seen in models of other genetic syndromes with intellectual disability, most notably Down syndrome [28, 77]. Early compensatory changes in GABAergic neurotransmission may be related to later changes in the excitatory-inhibitory balance seen in FXS and other related disorders [4]. This E/I imbalance is thought to drive much of the current EEG data, making this perhaps a more nonspecific marker in the population rather than specific to the FXS disease state. Combining EEG, MRI, and TMS may help disentangle changes in excitatory and inhibitory processes in children with FXS [22, 113]. Early studies, like those outlined above with appropriate control groups, are needed to tease these questions apart.

Conclusions and future experiments

While some work has been done to describe the developmental trajectory of FXS in humans, many questions remain. First, limited functional and structural serial brain imaging data is available, and the data is limited to date within the youngest children. Second, while electrophysiological abnormalities have been well characterized in humans with FXS, no developmental or long-term EEG follow-up studies have been conducted in FXS. Third, phenotypic characterization has been limited to primarily standardized measures and have not examined important aspect of the phenotype, specifically anxiety, attention, and sensory dysfunction in depth during early development. These shortcomings will inhibit targeted treatment work in the field because without developmental understanding of brain change over time in FXS it is difficult to evaluate the target engagement of a particular drug or therapy or combination of drug + therapy at specific windows of development. This is of particular importance if a treatment is being administered over time to potentially improve the course of development in FXS and given the advancements in the treatment development field looking at long-term drug + non-drug intervention as a means to enhance developmental gains. Specifically, the FX-LEARN project evaluating the effects of the selective metabotropic glutamate receptor type 5 (mGluR5) antagonist on language learning in young children with FXS (NCT02920892) is an example of a targeted treatment over a long time period that would significantly benefit from a comprehensive foundation of developmental understanding in FXS.

The lack of a firm developmental understanding of the presentation and brain biology of FXS and how that relates to cognition and behaviors impairs clinicians' ability to discuss with families and caregivers what can be expected over time. These deficits are being addressed in part by the Fragile X FORWARD project creating a registry and clinical database describing the natural history of FXS [126]. Despite these efforts, the field still lacks more biologically based supported effort to track developmental change including a lack of understanding of potential change in FMRP expression or brain physiology over time. Given these challenges, new targeted treatment approaches that may seek to enhance FMRP expression [45, 57, 92, 146] will be challenged to define effective target engagement with brain pathophysiology at a specific stage of development. One of the biggest gaps in the literature on FXS is worth reiterating here. Whether early developmental treatment of FXS has long lasting benefits is unclear. In addition, how combinations of treatment either at the same time or staggered at appropriate time points have long lasting benefits has not been tested. Whether reinstatement of FMRP in adults will be useful in

reducing symptoms will depend critically on whether FMRP plays an irreversible brain organizational role during early developmental critical periods. Studies that address these critical period questions, like those done in Angelman and Rett syndromes, are of immediate need in *Fmr1* KO mice.

Abbreviations

ABC: Aberrant Behavior Checklist; AS: Angelman syndrome; ASD: Autism spectrum disorder; EEG: Electroencephalography; ERP: Event-related potential; FMRP: Fragile X mental retardation protein; FXS: Fragile X syndrome; MRI: Magnetic resonance imaging; P: Post-natal; PET: Positron emission tomography; PNN: Perineuronal nets; PV: Parvalbumin; sLNv: Small ventrolateral neurons; TMS: Transcranial magnetic stimulation

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Authors' contributions

KAR and CAE developed the idea and topic of the manuscript. KAR, KCD, and CAE each wrote sections of the manuscript, edited the manuscript, and contributed to the ideas expressed in the manuscript. All authors read and approved the final manuscript.

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Author details

¹Department of Psychology and Graduate Neuroscience Program, University of California, Riverside, USA. ²Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati, Cincinnati, OH, USA. ³Division of Child and Adolescent Psychiatry, Cincinnati Children's Hospital Medical Center, 3333 Burnet Avenue MLC 4002, Cincinnati, OH 45229, USA.

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REVIEWS

Mechanisms underlying auditory processing deficits in Fragile X syndrome

Elizabeth A. McCullagh^{1,2} | Sarah E. Rotschafer^{3,4} | Benjamin D. Auerbach⁵ | Achim Klug¹ | Leonard K. Kaczmarek⁶ | Karina S. Cramer³ | Randy J. Kulesza Jr.⁷ | Khaleel A. Razak⁸ | Jonathan W. Lovelace⁸ | Yong Lu⁹ | Ursula Koch¹⁰ | Yuan Wang¹¹

¹Department of Physiology and Biophysics, University of Colorado Anschutz, Aurora, CO, USA

²Department of Integrative Biology, Oklahoma State University, Stillwater, OK, USA

³Department of Neurobiology and Behavior, University of California, Irvine, CA, USA

⁴Department of Biomedical Sciences, Mercer University School of Medicine, Savannah, GA, USA

⁵Center for Hearing and Deafness, Department of Communicative Disorders & Sciences, SUNY at Buffalo, Buffalo, NY, USA

⁶Departments of Pharmacology and Cellular and Molecular Physiology, Yale University, New Haven, CT, USA

⁷Department of Anatomy, Lake Erie College of Osteopathic Medicine, Erie, PA, USA

⁸Department of Psychology, University of California, Riverside, CA, USA

⁹Department of Anatomy and Neurobiology, College of Medicine, Northeast Ohio Medical University, Rootstown, OH, USA

¹⁰Institute of Biology, Neurophysiology, Freie Universität Berlin, Berlin, Germany

¹¹Department of Biomedical Sciences, Program in Neuroscience, Florida State University, Tallahassee, FL, USA

Correspondence

Yuan Wang, College of Medicine, Florida State University, 1115 West Call Street, Tallahassee, FL 32306, USA.
Email: yuan.wang@med.fsu.edu

Elizabeth A. McCullagh, Department of Integrative Biology, Oklahoma State University, 501 Life Sciences West, Stillwater, OK 74078, USA.
Email: elizabeth.mccullagh@okstate.edu

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Abstract

Autism spectrum disorders (ASD) are strongly associated with auditory hypersensitivity or hyperacusis (difficulty tolerating sounds). Fragile X syndrome (FXS), the most common monogenetic cause of ASD, has emerged as a powerful gateway for exploring underlying mechanisms of hyperacusis and auditory dysfunction in ASD. This review discusses examples of disruption of the auditory pathways in FXS at molecular, synaptic, and circuit levels in animal models as well as in FXS individuals. These examples highlight the involvement of multiple mechanisms, from aberrant synaptic development and ion channel deregulation of auditory brainstem circuits, to impaired neuronal plasticity and network hyperexcitability in the auditory cortex. Though a relatively new area of research, recent discoveries have increased interest in auditory dysfunction and mechanisms underlying hyperacusis in this disorder. This rapidly growing body of data has yielded novel research directions addressing critical questions regarding the timing and possible outcomes of human therapies for auditory dysfunction in ASD.

KEYWORDS

auditory system, autism spectrum disorders, circuit development, Fragile X syndrome, hyperacusis, synaptic transmission

Abbreviations: ASD, autism spectrum disorder; AGS, audiogenic seizures; ASR, acoustic startle response; E/I, ratio of excitation to inhibition; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; *Fmr1*, non-human mammalian *fragile X mental retardation 1* gene; *FMRI*, human *fragile X mental retardation 1* gene; FMRP, Fragile X mental retardation protein; FXS, Fragile X syndrome; GlyT2, glycine transporter 2; ITD, interaural time difference; IID/ILD, interaural intensity/level difference; IPL, interpeak latency; LNTB, lateral nucleus of the trapezoid body; LSO, lateral superior olive; mGluR, metabotropic glutamate receptor; MMP, matrix metalloprotease; MNTB, medial nucleus of the trapezoid body; MSO, medial superior olive; NL, nucleus laminaris; NM, nucleus magnocellularis; PNN, perineuronal nets; PPI, prepulse inhibition; PV, parvalbumin; SON, superior olivary nucleus; VCN, ventral cochlear nucleus; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter; VNTB, ventral nucleus of the trapezoid body.

1 | INTRODUCTION

Communication disorders and sensory hypersensitivity are prominent features of autism spectrum disorder (ASD). ASD is frequently diagnosed in individuals with Fragile X syndrome (FXS), a leading cause of intellectual disability. FXS results from mutations in the *fragile X mental retardation 1* (*FMRI*) gene, which is the most prevalent monogenic cause of ASD.¹ This gene encodes fragile X mental retardation protein (FMRP), an RNA binding protein that regulates expression of a select set of genes. The focus of this review is to understand the effects of *Fmr1* deletion in the auditory pathway, because these effects lie at the heart of the auditory hypersensitivity seen in FXS and likely contribute significantly to communication and other neurological deficits.

A large portion of the recent work discussed here has taken advantage of rodent models of FXS (*Fmr1* knockout mice and rats) and investigated structural, functional, and behavioral phenotypes at multiple stages of central auditory processing. Other studies have examined neuroanatomical and physiological abnormalities of human subjects with FXS. Additionally, the avian auditory brainstem has been used to investigate the FMRP regulation of auditory circuits that encode low frequency sounds, which are essential for acoustic communication in humans.

The significance of studying auditory processing in FXS is twofold. On the one hand, FMRP has the potential to influence a multitude of cellular processes at multiple stages of development. The diversity of FMRP function is of special significance in the auditory system, which carries out unique functions that require exceptionally fast and precise synapses. These requirements are fulfilled with specializations in axon terminations, dendritic branching, and synaptic and intrinsic properties of auditory neurons. Investigation of the roles for FMRP in each of these specializations will advance our understanding of auditory system development and function. Indeed, FMRP is found to be expressed throughout the auditory system and an array of distinct phenotypes has emerged in auditory circuits following FMRP deficiency that may account for auditory hypersensitivity and abnormal processing. At the same time, these findings highlight the auditory system as a powerful model system to study the FMRP function and FXS neuropathology. The

wealth of knowledge on connections and response properties in the healthy brain allows alterations to be relatively easy to characterize, thereby allowing for an in-depth analysis of the underlying etiologies that lead to problems with sensory processing.² Finally, the ability to stimulate neural responses in the auditory system on demand and the fact that many of these responses are independent of cognitive abilities or active participation opens the possibility to use these responses as biomarkers to characterize the effects of potential therapeutic approaches.

2 | FXS AND BIOLOGICAL ROLES OF FMRP

Fragile X syndrome is characterized by many similar symptoms to autism, namely, hyperactivity, anxiety, seizures, learning difficulties, and poor language development (reviewed in^{3,4}). Most cases of FXS are caused by a trinucleotide repeat disorder, in which an increase in the numbers of a CGG motif in the 5' UTR of the *Fmr1* gene (>200 repeats) leads to transcriptional silencing and the consequent loss of its product, FMRP. As a monogenetic disorder, FXS has come to be at the frontier of developing treatments for neurodevelopmental disorders. Loss of FMRP underlies the etiology of FXS, and most drug development has centered around rescuing specific biological effects caused by loss of FMRP. Evidence supporting the importance of FMRP in FXS includes (i) FMRP protein level is associated in a dose-dependent manner with brain anatomy, sensory function, autistic behaviors, and cognitive function of individuals with FXS,⁵⁻⁸ (ii) genetically reintroducing FMRP rescues cellular and behavioral deficits in *Fmr1* knockout mice,⁹ and (iii) point mutations in the *Fmr1* coding sequence lead to FXS symptoms similar to those of CGG expansion.^{10,11} Thus, examining FMRP knockouts presents a suitable avenue for exploring FXS etiology.

FMRP regulates many aspects of RNA biology from RNA transport, stability, to mRNA translation.^{12,13} The best-described biological role of FMRP in neurons is to bind a subset of mRNAs and to suppress their translation.^{14,15} Appropriate stimulation of neurons can reverse this suppression, most likely by altering the phosphorylation state of FMRP, leading to increased synthesis of the protein products

of these mRNAs. There are on the order of ~1000 mRNAs that bind FMRP and these are termed targets of FMRP.^{16,17} Thus, one central phenotype of *Fmr1* knockout animals is an increase in levels of some of these proteins, together with a lack of activity-dependent translation. This role for FMRP is commonly termed its canonical role. However, only a limited number of FMRP targets have been validated so far in specific circuits and FMRP binds to different sets of RNA targets in an age-dependent and cell type or brain region specific manner. As evidence, strikingly distinct sets of proteins exhibit altered expression in *Fmr1* knockout mice between the hippocampus and neocortex of adult brains,¹⁸ and between the developing and adult neocortex.¹⁹ In addition to RNA binding, FMRP directly binds proteins that control other aspects of neuronal activity. These include proteins required for RNA editing, RNA splicing, cytoskeletal rearrangement, regulation of protein kinases, as well as direct binding to ion channels that determine neuronal excitability.²⁰ In some cases, it is possible to link auditory phenotypes in *Fmr1* knockout animals to changes in mRNA targets or to other biological actions of FMRP. Indeed, key findings in FXS animal models include disturbances in circuit development, imbalanced inhibitory, and excitatory neuronal circuits, and altered synaptic plasticity.²¹⁻²³

3 | FMRP REGULATES THE DEVELOPMENT OF SYNAPTIC TRANSMISSION IN THE AUDITORY BRAINSTEM

The auditory brainstem is where fundamental steps of auditory processing take place, including binaural

computation for sound localization. Sounds arriving at our two ears from various locations along the azimuth create discrete interaural time and level differences (ITDs and ILDs).²⁴ These two binaural cues are first computed in the brainstem. In mammals, the major auditory nuclei involved in this process include the ventral cochlear nucleus (VCN), the lateral, ventral, and medial nucleus of the trapezoid body (LNTB, VNTB, and MNTB), and the medial and lateral superior olive (MSO and LSO; Figure 1). In reptiles and birds, nucleus magnocellularis (NM), and nucleus laminaris (NL) are structurally and functionally similar to the mammalian VCN and MSO, respectively.

Traditionally, the brainstem was thought to express low levels of FMRP^{25,26} and received little attention in the field of FMRP research. Recent cellular analyses, however, reported strong expression of FMRP in auditory brainstem cell groups across a number of vertebrate species.²⁷ Quantitative analyses across the entire adult (5-6 weeks) mouse brain further confirmed that auditory neurons in the brainstem and thalamus have levels of FMRP immunoreactivity comparable to those in cortical neurons.²⁸ It is worth noting that FMRP is also expressed in spiral ganglion neurons, which convey acoustic signals from the inner ear to the brainstem, in both birds and mammals. In contrast, many cell groups in the midbrain and hypothalamus exhibit low FMRP levels.²⁸

In this section, we first describe the expression and function of FMRP in the ITD circuit (3.1) and then discuss how *Fmr1* deletion affects the cell groups involved in ILD computation (3.2). Finally, we summarize effects of FMRP loss on auditory brainstem responses (ABRs), a noninvasive physiological output of the auditory brainstem (3.3).

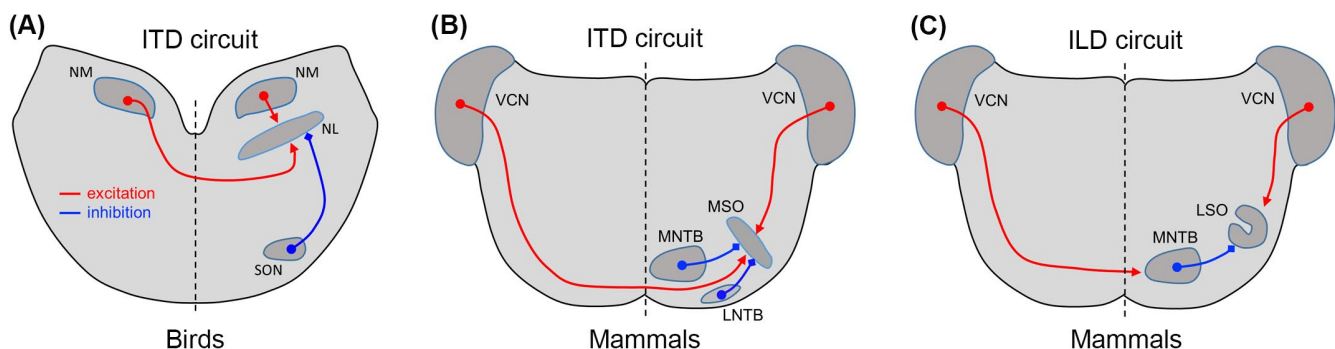


FIGURE 1 Sound localization circuits. Sound localization information is integrated in the auditory brainstem using interaural time and level differences (ITDs and ILDs). A-B, The ITD circuit is structurally and functionally comparable between birds (A) and mammals (B). The circuit is composed of spherical bushy cells in NM and VCN and fusiform bipolar cells in NL and MSO. The NL also receives an inhibitory input from the superior olive nucleus (SON). The MSO receives inhibitory inputs from MNTB and LNTB. C, In mammals, ILDs are computed in LSO through an excitatory projection from globular bushy cells in VCN and an inhibitory projection from MNTB

3.1 | FMRP regulates dendritic development and morphology in the ITD circuit

In the brainstem, ITD computation for low frequency sounds takes place in MSO and NL (Figure 1A,B). The MSO and NL are best developed in humans and other animal species most sensitive to low frequency sounds including reptiles, birds, some rodents such as gerbils, and primates. Dendrites of MSO and NL neurons segregate into two domains; each domain receives excitatory input from the ipsilateral or contralateral ear via VCN and NM (Figure 2A). This anatomic segregation, along with specialized synaptic and intrinsic physiology, enables MSO and NL neurons to detect ITDs in the microsecond range.²⁹

Strong FMRP expression is detected in the VCN-MSO circuit across taxa, including gerbils,²⁷ rats,³⁰ and humans³¹ (Figure 2B). Similarly, almost all neurons of NM and NL in alligator and chicken brainstems contain exceptionally high levels of FMRP.²⁷ Perhaps surprisingly, postmortem human brains were found to have more variation of FMRP expression depending on the cell type.³¹ In the human VCN (2 males, 5 females; average age 79 years), the vast majority (85%-95%) of bushy cells and octopus cells are FMRP immunoreactive, while only about 68% of stellate neurons express FMRP. It is

not clear whether this difference results from technical variations associated with postmortem tissue processing of human brains or reflects varied degrees of FMRP regulation in functionally distinct human VCN cell types.

Strong FMRP expression in normal developing MSO neurons is consistent with the remarkable changes seen in FMRP-deficient MSO. A morphological study of the human MSO was conducted in an age-matched (29-32 years of age, all males) series of subjects (one neurotypical subject, one subject with ASD, and one subject with FXS).³² MSO neurons were significantly smaller in ASD and FXS. In fact, MSO neurons in the FXS subject were significantly smaller than both the control and ASD subjects, suggesting a direct link between FMRP and MSO development, although additional FXS patients are certainly needed for investigation. In control subjects, the MSO is composed of fusiform and stellate neurons, with only a small population of round/oval neurons. In the FXS patient, the MSO contained significantly more round/oval neurons, reflecting an immaturity of MSO neurons in this subject. Finally, in control subjects, individual MSO neurons are arranged with their long axis perpendicular to the dorsoventral axis of the brainstem. This orientation of neurons is lost in both ASD and FXS patients, implicating disrupted dendritic branching in MSO. Although only one human FXS subject has been examined so far, very similar cellular changes

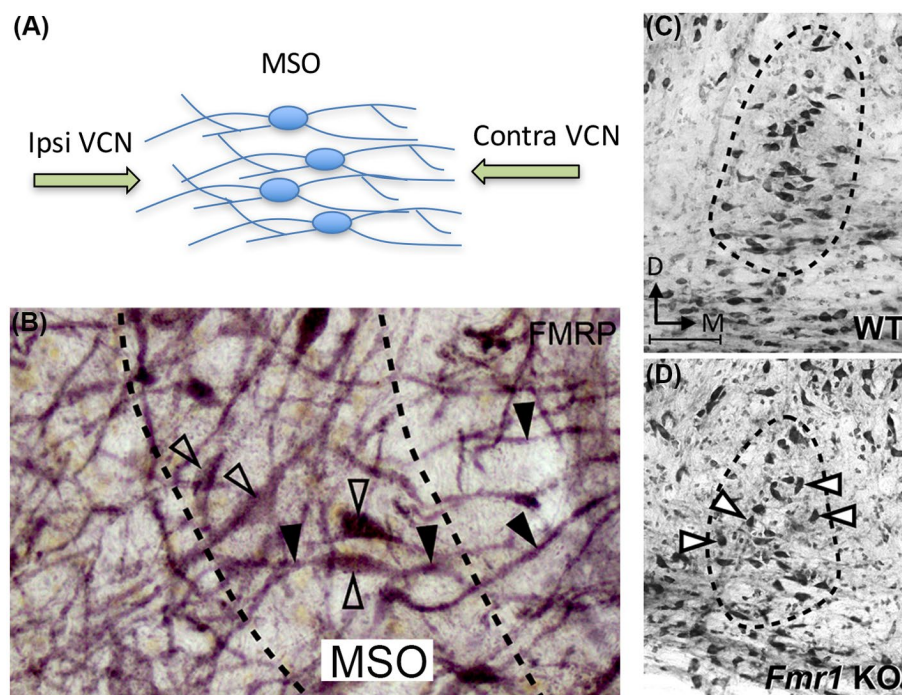


FIGURE 2 FMRP deficiency leads to altered cytoarchitecture in MSO. A, Schematic drawing of the normal organization of MSO. B, FMRP expression in the human MSO. Empty and solid arrowheads point to cell bodies and dendritic branches, respectively. C-D, Altered organization of MSO in *Fmr1* knockout rats (D) as compared to wild type (C). In *Fmr1* knockout animals, MSO somata were smaller, more round/oval, and more vertically oriented (arrows). Abbreviation: VCN, ventral cochlear nucleus; MSO, medial superior olive

have been detected in the MSO of a *Fmr1* knockout rat strain⁽³⁰⁾; Figure 2C,D), strongly supporting the notion that these MSO changes are common phenotypes in FXS.

These morphological studies in humans and rats, along with reports of altered dendritic arborization and their dynamics in nonauditory neuronal cell types in *Fmr1* knockout mice,³³⁻³⁷ warranted further studies of the role of FMRP in regulating dendrites in the VCN-MSO circuit. High-resolution imaging studies demonstrated intense dendritic localization of FMRP, particularly in dendritic branch points and distal endings, in NL (chicken and alligator) and MSO (gerbil and human).²⁷ In the chicken NL, the branching points are thought to be a site for initiating activity-dependent dendritic reorganization.³⁸ A proteomic study specifically targeting the chicken NL identified a number of dendritic proteins whose mRNAs are potential FMRP targets.³⁹ One of these is RhoC, a member of the Rho family of GTPases that is involved in developmental regulation of neuronal dendrites,⁴⁰ and that has been validated as an RNA target of FMRP.³⁹ Together, these results support a role of FMRP in dendritic development and regulation of MSO and NL neurons.

FMRP regulation of dendritic development has been studied in the chicken NM.⁴¹ In NM and VCN, presynaptic auditory axons form large axosomatic endbulb synapses on cell bodies of postsynaptic bushy neurons. Normally, bushy neurons in NM grow extensive dendrites at early stages and retract these dendrites when endbulbs begin to form. Following FMRP knockdown with an shRNA approach, NM neurons exhibit a remarkable delay in branch retraction, failing to provide necessary somatic surface for timely formation and growth of large endbulbs. Importantly, these structural changes are coupled to functional changes in neurotransmission, specifically smaller amplitudes and slower kinetics of spontaneous and evoked excitatory postsynaptic currents (eEPSCs).

In summary, FMRP is localized in high concentrations in neuronal dendrites that are specialized for ITD computation in the brainstem. FMRP deficiency disrupts dendritic maturation, which negatively affects the development of synaptic connectivity and integration. Sound localization plays a crucial role in our ability to separate multiple simultaneous sound sources into distinct auditory streams (reviewed in 42). Disrupted dendritic function in the ITD circuit may, therefore, impair hearing in noise environments in individuals with FXS.

3.2 | *Fmr1* deletion disrupts the balance between excitation and inhibition in the ILD circuit

Interaural level differences, also known as interaural intensity differences (IIDs), are encoded by neurons in LSO for high

frequency sounds (Figure 1C). The balance of glutamatergic excitation from VCN and glycinergic inhibition from MNTB allows LSO neurons to compute the ILD, which is used to determine the location of the sound source. Disturbances in the ratio of excitation to inhibition (E/I) in the brain, generally resulting in hyperexcitability, are well described in FXS and ASD.^{21,22,43-45} These alterations in E/I balance may account for the auditory hypersensitivity and impaired sound location in noisy environments reported in individuals with FXS and ASD.⁴⁶⁻⁴⁸ Neuroanatomical and physiological studies of *Fmr1* knockout rodents have uncovered a number of cellular alterations that potentially contribute to a disrupted E/I balance in the ILD circuit.

3.2.1 | Synaptic distribution is altered but neurotransmission is preserved in the MNTB

MNTB neurons receive glutamatergic excitation from the VCN through a giant synapse called the calyx of Held, and glycinergic and GABAergic inhibition from collateral projections and VNTB.^{49,50} The GABAergic component declines through adulthood resulting in mostly glycinergic inhibition in the adult⁵¹⁻⁵³. The glycinergic component of inhibition is fast and large, capable of following rapid stimulus trains (several 100 Hz) that match and suppress firing of MNTB neurons despite the extremely large calyceal excitatory input.^{53,54} Disruptions to glutamatergic, GABAergic or glycinergic presynaptic inputs to MNTB could have profound effects on ILD encoding.

Immunocytochemical studies of various cellular and synaptic markers have suggested that excitatory and inhibitory inputs to MNTB may be altered, but the direction of this alteration is not yet clear. A study using *Fmr1* knockout rats revealed that the MNTB contains significantly fewer terminals immunoreactive for calretinin, a marker for calyx terminals from VCN.³⁰ Two studies have reported presynaptic alterations in inhibitory inputs to MNTB in *Fmr1* knockout mice.^{55,56} McCullagh et al⁵⁶ showed that the number of GABAergic and glycinergic inhibitory inputs to the MNTB is decreased, as indicated by glutamate decarboxylase (GAD67) and the glycine transporter 2 (GlyT2) antibody staining.⁵⁶ Interestingly, these changes are specific to the tonotopic location within the nucleus, with lateral MNTB showing decreased GABAergic inhibition while medial MNTB showing decreased glycinergic inhibition. In contrast, Rotschafer et al⁵⁵ suggested *strengthened* inhibition by showing increased immunoreactivity for the vesicular GABA transporter (VGAT), a marker for both GABAergic and glycinergic terminal boutons.⁵⁵ This discrepancy could be explained by different markers for inhibition (VGAT vs GlyT2 and GAD67), differences in mouse strain (FVB vs B6 background), or location/tonotopic specific changes in MNTB. These immunocytochemical studies do not, however, resolve whether FMRP loss

induces a net change in the ratio of excitatory and inhibitory inputs to MNTB.

Direct in vitro slice recordings of synaptic transmission in MNTB of *Fmr1* knockout mice suggest that changes in synaptic excitation are subtle.⁵⁷ The maximal synaptic strength and kinetics of the glutamatergic input remain unchanged but the way the synapse responds to afferent stimulation with gradually increasing intensity becomes altered. In wild-type neurons, a typical all-or-none response pattern of evoked eEPSCs is observed in the majority of cells. Proportionally fewer *Fmr1* knockout than wild-type neurons showed this pattern. In *Fmr1* knockout animals, a smaller proportion of calyces in the MNTB display simple morphology,⁵⁸ which is associated with synaptic depression.⁵⁹ Additionally, in vivo juxtacellular recordings detected only minor changes in excitatory spiking activity.⁵⁸ In vitro slice physiology on the inhibitory inputs has also revealed no significant changes in most of the parameters examined for both glycinergic and GABAergic transmission.⁵⁷ The discrepancy between anatomy and physiology suggests that the anatomical changes in the presynaptic terminals may not be sufficient to lead to significant functional changes. Alternatively, the electrophysiological changes may be attenuated by multiple anatomical alterations that balance out the overall effect on neurotransmission (for example, a decrease in GABA synthesis and an increase in GABA transport). A lack of change in synaptic strength at inhibitory synapses, measured by electrophysiological techniques, despite clear anatomical changes, has been also observed in other brain systems (eg,^{60,61}).

One of the core mechanisms that have been proposed to account for the deficits in FXS in some parts of the nervous system is the “mGluR (metabotropic glutamate receptor) theory,” specifically that group I mGluR activity is exaggerated.⁶² While multiple transmitter systems may regulate auditory neurons, no significant differences have been reported in mGluR-mediated neuromodulation of the inhibitory inputs to MNTB.⁶³ Activation of group I mGluRs affected the inhibitory transmission in the same manner in both wild-type and *Fmr1* knockout neurons, enhancing spontaneous glycine release but suppressing the evoked inhibitory postsynaptic currents (eIPSCs), and suppressing the evoked GABAergic IPSCs without changing the spontaneous GABA release. These observations in MNTB contrast the more dramatic changes observed in other brain regions, supporting the view that the effects of FMRP on neural functions vary across brain regions, and potentially across development.

3.2.2 | Ion channels are regulated in a different way in the MNTB

Hyperexcitability in a circuit can result from changes in the intrinsic excitability of neurons, as well as changes in

synaptic E/I ratio. Direct recordings of the firing patterns of MNTB neurons in acute slice preparations have shown that loss of FMRP renders these neurons hyperexcitable. In wild-type mice, the normal response of MNTB neurons to a sustained depolarization is to fire only a single action potential at the onset of the depolarization. In MNTB neurons from *Fmr1* knockout animals, however, the same depolarization results in repetitive firing throughout the current pulse⁶⁴ (Figure 3A). Such changes in the intrinsic excitability of neurons produced by loss of FMRP must reflect changes in the numbers and types of ion channels expressed in neurons. This can be accomplished by at least two quite distinct mechanisms. The first is by changes in the rates of translation of mRNAs that encode channels.^{65,66} The second is by altering the amplitude, gating or trafficking of the channel subunits that normally bind FMRP directly.

Ion channels are relatively minor cellular constituents compared to cytoplasmic and organellar proteins, and of the approximately 90 genes that encode Na⁺, Ca²⁺, or K⁺ channel subunits, only very few of their mRNAs have been shown to bind FMRP.¹⁶ A surprisingly high proportion of those that do bind FMRP are, however, expressed in neurons of the auditory system. Potassium channels that play major roles in regulating the intrinsic excitability of auditory brainstem neurons and whose mRNAs are targets of FMRP include the Kv3.1, Kv3.3, Kv1.2, Kv1.1.3, and K_{Na}1.1 channels.⁶⁷⁻⁷⁵ The sodium channel Nav1.6⁷⁶ and the calcium channels Cav2.1, Cav2.2, and Cav 2.3⁷⁷ are also expressed in MNTB and their mRNAs are targets of FMRP.¹⁶ These links between FMRP and its channel targets that directly determine intrinsic excitability have allowed analyses of how loss of FMRP contributes to changes in firing patterns such as those of Figure 3.

One clear example of how a change in the rate of synthesis of an ion channel caused by loss of FMRP disrupts auditory processing is provided by the Kv3.1 channel in MNTB neurons, as well as in the VCN. Messenger RNA for Kv3.1 was one of the very first mRNAs found to bind to FMRP.^{17,78} This channel is absolutely required for neurons to fire at the very high rates required for phase-locking to physiological sound stimuli (up to 800 Hz).⁷² Kv3.1 is normally expressed in a gradient along the tonotopic axis of MNTB, with highest levels in neurons that respond optimally to high-frequency sounds.^{79,80} This gradient is absent and overall levels of this Kv3.1 protein and Kv3.1 currents are significantly higher in mice lacking FMRP (Figure 3B,C).^{64,78} Moreover, levels of Kv3.1 in VCN and MNTB are increased by physiological increases in auditory stimulation in vivo.⁸¹ The ability of auditory stimulation to alter the already high level of Kv3.1 protein in these nuclei is abolished in *Fmr1* knockout mice.⁷⁸ These findings are consistent with a role for FMRP in the control of sound-induced translation of Kv3.1 mRNA, and with the enhanced firing rate of MNTB neurons in *Fmr1* knockout mice.

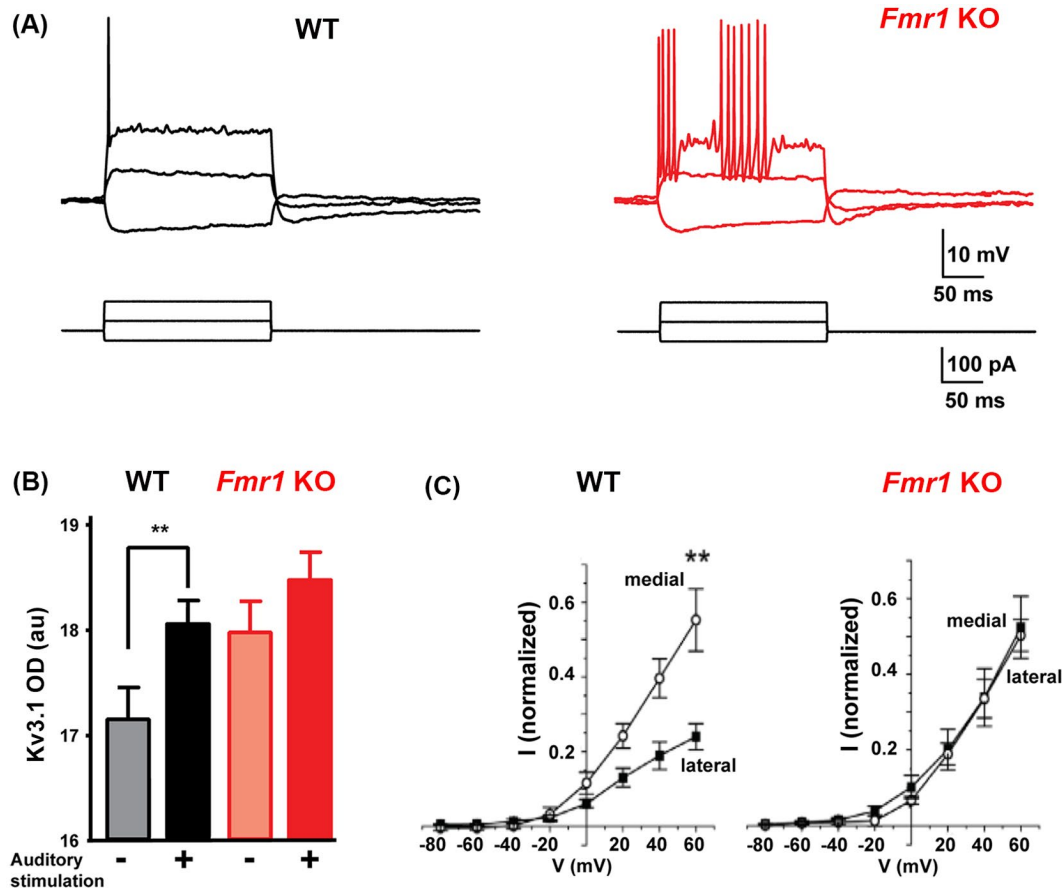


FIGURE 3 MNTB neurons from *Fmr1* knockout mice are hyperexcitable. A, Current-clamp recordings of action potentials in MNTB neurons from wild-type and *Fmr1* knockout mice in response to hyperpolarizing and depolarizing current pulses (modified from 64). B, Bar graphs showing quantification of Kv3.1 immunoreactivity in MNTB in silence or after 30 minutes of exposure to physiological sound stimulation. C, Mean current-voltage relations for high-threshold Kv3.1 currents in patch clamp recordings of neurons in lateral and medial aspects of MNTB. The tonotopic organization of currents present in wild-type neurons is absent in neurons from *Fmr1* knockout mice (B and C modified from 78). WT, wild type; KO, knockout

A second mechanism by which FMRP regulates neuronal excitability is through direct protein-protein interactions with ion channels subunits.^{67,82-87} In each case, the interaction with FMRP alters the amplitude, kinetic behavior, or trafficking of the channel. Binding of FMRP to channels was first demonstrated for $K_{Na}1.1$ channels, also termed Slack channels,^{67,82} and has also been documented for Kv1.2⁸⁷ and Cav2.2,⁸⁵ all of which are expressed in MNTB. The two potassium channels, Kv1.2 and $K_{Na}1.1$, that are regulated in this way play a very different function in the excitability of auditory brainstem neurons from that of the Kv3.1 channel. Both channels activate at negative membrane potentials close to the resting potential and contribute to the so-called low-threshold potassium currents. These are required for the high degree of temporal accuracy with which principal neurons of MNTB lock their action potentials to incoming stimuli.^{68,75} In both cases, the direct binding of the channel subunit to FMRP promotes channel opening, increasing current amplitude.^{67,82,87} Accordingly, the amplitude of

the low-threshold potassium currents is reduced in MNTB neurons from *Fmr1* knockout mice, promoting the abnormal firing patterns shown in Figure 3.⁶⁴ Novel drugs that alter the voltage-dependence of Kv3.1 channels have been shown to rectify these abnormal firing patterns by increasing low-threshold currents and decreasing high-threshold currents in *Fmr1* knockout animals.⁶⁴ These agents, therefore, represent a potential new therapy for auditory hypersensitivity in FXS patients

Loss of FMRP affects the levels and activity of several other types of ion channels both by translational mechanisms and by direct protein-protein interactions.⁶⁵ These include KCa1.1 (BK), Kv4.2, and HCN channels, which shape the excitability of many types of neurons in the auditory system, but their roles in auditory processing in FXS have not been investigated. Because FXS is a neurodevelopmental disorder, it is likely that there exist compensatory mechanisms that act over time to alter the excitability and synaptic transmission in the patients and in animal models of the disease.⁵⁸

3.2.3 | Synaptic distribution is altered and excitation is enhanced in the LSO

In LSO, alterations in synaptic E/I balance directly translate into a measurable shift in ILD sensitivity. There is evidence of increases in both excitatory and inhibitory inputs as indicated by a larger area of LSO containing the vesicular glutamate transporter (VGLUT1 or 2) and VGAT.^{55,88} Consistent with this, in vitro electrophysiological recordings from LSO neurons in acute brain slices of *Fmr1* knockout mice showed that maximal EPSCs evoked by stimulating the ipsilateral incoming fibers from VCN are more than twice as large in mature *Fmr1* knockout compared to wild-type animals (Figure 4A,B). This disproportionate increase in EPSCs occurs in the first week after hearing onset (Figure 4C). This increase is paralleled by a large increase in spontaneous EPSC frequency with no change in amplitude, and a similar increase in the number of synaptic endings labeled with VGLUT1 and VGLUT2.⁸⁸ Inhibitory synaptic transmission from MNTB to LSO is mainly unaltered.^{56,88} These results suggest that LSO neurons of *Fmr1* knockout mice receive more synaptic inputs and input fibers from VCN, whereas the strength of each individual synapse is unaffected. One possible mechanism may be that the loss of FMRP disturbs the developmental pruning of excitatory inputs to LSO which in

wild-type animals results in a sharpening of frequency tuning in these neurons.^{89,90}

Single unit recordings from LSO of anesthetized *Fmr1* knockout mice show specific changes in their spike responses to monaural and binaural acoustic stimulation. The number of spikes in response to stimulation at each neuron's characteristic frequency is increased and more neurons respond throughout the stimulus presentation⁸⁸ (Figure 4D). This is consistent with the larger number of excitatory input fibers and more excitatory synapses as observed in the in vitro experiments. Binaural stimulation at the characteristic frequency with different ILDs reveal that the E/I balance in LSO neurons in *Fmr1* knockout mice is shifted. On average, a louder stimulus at the contralateral, inhibitory ear is needed to cause complete suppression of spikes evoked by ipsilateral sound stimulation (Figure 4E). In addition, the slope of the ILD function, a measurement for how many decibels the sound at the contralateral ear has to change to go from no spiking to maximal spiking, was shallower. Shallower ILD slopes are considered to provide less accurate information on the azimuthal location of a sound.²⁴ Whether *Fmr1* knockout animals exhibit sound localization deficits needs to be tested in future experiments. Moreover, generating frequency response maps, where neurons are stimulated with a large range of different

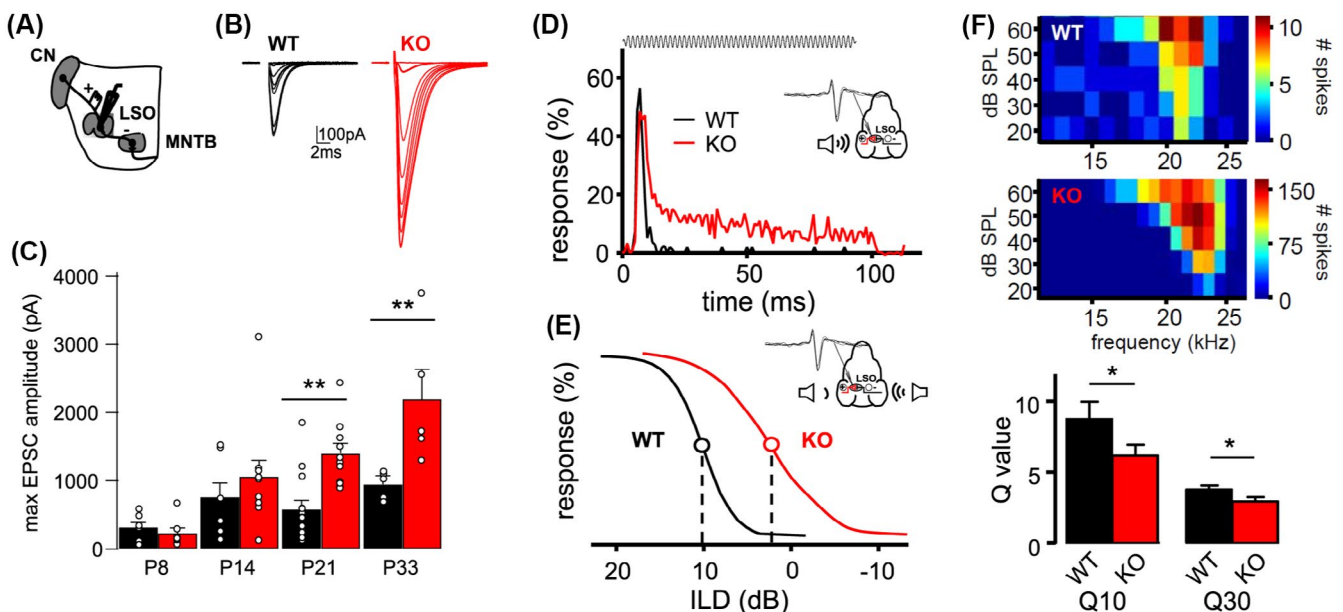


FIGURE 4 Enhanced excitation, altered E/I balance and broadened frequency tuning in LSO neurons of *Fmr1* knockout mice. A, Schematic of patch-clamp recordings from LSO neurons in acute brain slices. B, Examples of excitatory postsynaptic currents (EPSCs) evoked by stimulation of AVCN-LSO fibers with different intensities in wild-type (black) and *Fmr1* knockout mice (red). C, Averaged EPSC amplitudes evoked by maximal stimulation for different developmental stages (P8 to P33) in wild-type (black) and *Fmr1* knockout mice (red). D, Averaged spike poststimulus time histograms of wild-type and knockout LSO neurons in response to monaural, ipsilateral sound stimulation. E, Schematic of ILD function in wild-type and *Fmr1* knockout mice. Circles represent points of half-maximal inhibition. F, Frequency response areas of two representative LSO neurons, each patch showing the total number of spikes of four repetitions. Q-values as a measure for frequency tuning sharpness, expressing the bandwidth of the tuning curve 10 and 30 dB above threshold. Smaller Q10 and Q30 values indicate broader tuning in *Fmr1* knockout animals. Modified from 88 with permission

frequencies and intensities, show that frequency tuning of LSO neurons is broadened in *Fmr1* knockout compared to wild-type animals (Figure 4F).

In summary, current electrophysiological and immunostaining studies indicate that, despite clear changes in morphology and intrinsic excitability at the level of the VCN and MNTB, overall changes in the balance of excitation and inhibition in *Fmr1* knockout animals only become fully evident at the LSO where synaptic excitation is enhanced while the inhibitory input remains largely unchanged in strength. The disturbed E/I balance in LSO neurons may thus underlie broadened frequency tuning as well as shifted ILD responses in *Fmr1* knockout animals.

3.3 | FMRP loss affects ABR measurements in FXS and *Fmr1* knockout mice

Noninvasive measures of brainstem activity can be performed in both humans and animal models. ABRs are

electrophysiological representations of the population activity of auditory structures in the peripheral and central nervous system.⁹¹ Each wave in an ABR corresponds to specific structures along the ascending auditory tract, with some differences between humans and rodents. Generally, the waves of the ABR correspond to the ascending auditory pathway through the areas of the brainstem responsible for sound localization (reviewed in 92,93).

Whether FMRP loss affects human ABRs is controversial. Early studies on patients with FXS showed significantly delayed wave peaks and prolonged interpeak latencies (IPLs; Table 1). Among the changes consistently detected, the absolute latency of peak III is delayed and the I-III IPL is prolonged.^{96,97,99} Prolonged I-III IPL could be explained by a delay in the transmission of the auditory signal from the periphery to the brainstem nuclei, or a delay in the activation of the superior olivary complex itself. In these studies, the participants were not screened for sensorineural hearing loss and, because individuals with FXS are prone to otitis media, presumably had a wide range of

TABLE 1 ABR measurements in FXS

Study	Participants	Abnormal ABR/BAEP?	Results
Wisniewski et al ⁹⁴	n = 6 All male Mean age = 13.3 years	No	Normal ABRs; methodology was vague
Gillberg et al ⁹⁵	n = 10 All male Mean age unknown*	6 of 7 were abnormal	Longer latencies; ABR methodology was vague
Ferri et al ⁹⁶	n = 8 Males and females Mean age = 13.8 years	Yes	Delayed peak III; Prolonged I-III IPL; Shorter III-V IPL
Ferri et al ⁹⁷ ; Ferri ⁹⁸	n = 10 All male Mean age = 18.4 years	Yes	Delayed peak III; Prolonged I-III IPL; Shorter III-V IPL
Arinami et al ⁹⁹ ; Arinami ¹⁰⁰	n = 12 All male Mean age = 32.2 years	Yes	Longer peak V latencies; increased I-V and III-V IPL
Wisniewski et al ¹⁰¹	n = 12 All male Mean age unknown**	5 of 12 were abnormal	Ambiguous late waves (peaks III-V) prolonged peak III-V IPL
Miezejeski et al ¹⁰²	n = 13 All male Mean age = 16.5 years	No	5 of 13 individuals with FXS sedated*** during ABR testing; Prolonged ABR latencies attributed to sedation
Roberts et al ¹⁰³	n = 23 All male Mean age = 11 years	No	No difference in FXS absolute or interpeak latencies

*7 participants were sampled from a pool of 10 FXS patients, mean age = 10.9 years.

**12 participants were sampled from a pool of 62 FXS patients, mean age = 23.1 $\pm$ 14.3 years.

***Agents of sedation: chloral hydrate, thiopental, fentanyl citrate, diazepam, or droperidol. Sedation lasted less than 1 h.

peripheral hearing function.^{104,105} It is thus possible that the detected ABR alterations may have resulted indirectly from impaired middle ear function. Indeed, more recent studies from FXS individuals without middle ear infection did not find any differences in the absolute peak latencies and IPLs of ABRs.^{102,103,106} However, one should be careful in attempting to interpret this result as implying that ABRs and FMRP expression are independent. Because more than half of FXS children have otitis media,¹⁰⁵ individuals without this peripheral pathology present only a specific population of FXS who are probably less sensitive to FMRP loss in both the periphery and the central nervous system. Other work compared children with FXS, with or without receiving sedation, during ABR testing and argued that differences seen in ABR testing across studies may be caused by the administration of sedatives.¹⁰⁷ However, this study may have over-sampled from high-functional patients who may have been more tolerant of the ABR test.

Studies with mice are not confounded by some of the variables that affect human studies. Several ABR alterations have been identified by comparing *Fmr1* knockout mice and age-matched wild types under anesthesia. Similar to human studies, peak latencies appear to be a subject of FMRP loss in FVB mice,¹⁰⁸ but not in B6 mice.^{55,64} Interestingly, although there is no report of altered ABR wave amplitudes in human FXS, the amplitudes of several ABR peaks are altered in *Fmr1* knockout mice, including decreased peak I and increased peak IV, suggesting impaired peripheral and central auditory systems. As the methodology for collecting ABRs can be quite variable, future work using similar experimental protocols on mice and humans may be useful for further characterization of the auditory brainstem phenotype in FXS.

4 | FMRP LOSS ALTERS AUDITORY CORTICAL ACTIVITY

Consequences of *Fmr1* loss at the cortical level have been primarily studied using physiological recordings at the single cell and population levels. These studies have made three main contributions to our understanding of sensory phenotypes in FXS. First, auditory cortex exhibits increased sound evoked responses, and one potential mechanism for this involves changes in levels of matrix metalloprotease-9 (MMP-9), a protein whose mRNA is a target of FMRP. This finding points to a potential therapeutic avenue that targets MMP-9 for treating FXS. Second, deficits in cortical processing are generated early in development. Third, humans with FXS and *Fmr1* knockout mice exhibit remarkably similar changes in their electroencephalogram (EEG), which may prove a useful biomarker in FXS translational studies.

Studies in humans with FXS reveal a number of electrophysiological differences in auditory cortex compared to control participants.² Increased activation of left hemispheric circuitry, including superior temporal gyrus, was observed in FXS subjects during an auditory temporal discrimination task.⁴⁶ FXS subjects also show reduced synchronization of neural responses to time-varying signals, suggesting impairments in temporal processing.¹⁰⁹ Rojas et al¹¹⁰ showed that tone-evoked responses measured using magnetic fields are higher in the auditory cortex of individuals with FXS.¹¹⁰ EEG recordings revealed increased gamma frequency band power in resting state EEG of FXS patients.¹¹¹ Auditory event related potential (ERP) studies report abnormally high amplitudes of the N1 wave in response to tones and reduced habituation to repeated sound in FXS.^{110,112-115} These data indicate a noisy resting state of auditory cortex in FXS that may lead to abnormal amplitude and synchronization of evoked responses.

Electroencephalogram recordings from the auditory cortex of the *Fmr1* knockout mouse show remarkably similar phenotypes to those seen in humans.^{116,117} The gamma frequency band power is increased in resting state EEG in auditory cortex of *Fmr1* knockout mice. The N1 amplitude of ERP is elevated and shows reduced habituation to repeated sounds. The auditory cortex shows a reduced ability to synchronize responses to time-varying signals. In fact, almost every EEG phenotype that have been measured in both *Fmr1* knockout mice and humans with FXS has shown a similar change in both species, indicating a potential use of these deficits as biomarkers to facilitate the translation pipeline. Moreover, EEG deficits in *Fmr1* knockout mice are present from early in development.¹¹⁸ At the single neuron level, Rotschafer and Razak¹¹⁹ showed that adult mouse cortical neurons produce a stronger response to brief tones in *Fmr1* knockout mice compared to wild-type mice.¹¹⁹ Developmental studies further demonstrated that the auditory cortex develops hyper-responsiveness between P14 and P21, and that this is maintained in adulthood.¹¹⁸ In addition, the adult knockout neurons showed broader frequency tuning (similar to LSO – see above) and reduced selectivity for frequency modulated (FM) sweeps suggesting abnormal spectrotemporal processing.¹¹⁹

In addition to potential changes in synaptic transmission and ion channel regulation similar to those described for the auditory brainstem, there is evidence that an alteration in MMP-9 dependent regulation of perineuronal nets (PNNs) may contribute to hyperactivity of the auditory cortex, as well as perhaps lower brainstem structures. PNNs are extracellular matrix components that are often associated with parvalbumin (PV)-expressing cortical cells and regulate their development and functions.¹²¹⁻¹²⁴ MMP-9 is a secreted endopeptidase that regulates PNN formation and organization.¹²⁵ FMRP negatively regulates MMP-9 translation and MMP-9 levels in neurons, including those in the auditory cortex, are

elevated in FXS.^{120,126-128} Consistently, the auditory cortex exhibits delayed development of PNNs and PV cells in *Fmr1* knockout mice.¹²⁰ Genetic reduction of MMP-9 in *Fmr1* knockout mice restored the formation of PNNs around PV cells and rescued spontaneous and sound-driven responses to normal level in the auditory cortex.¹²⁰ Together, these studies demonstrate that elevated MMP-9 levels contribute to the development of auditory processing deficits by influencing the development of PNNs and PV cells in the auditory cortex of *Fmr1* knockout mice (Figure 5). Whether altered synaptic transmission and ion channel dysregulation as described in the auditory brainstem is also present in the auditory cortex requires further investigation.

Given that FMRP is expressed across multiple auditory system nuclei, the question arises as to whether abnormal cortical responses reflect local circuit dysfunction. This is a complex issue because of the nature of ascending and descending connections in the auditory system. To begin to evaluate the relative contribution of subcortical inputs and local cortical circuits to observed changes in cortical recordings, a conditional knockout in which FMRP was deleted specifically from forebrain excitatory neurons using the *Nex1* promoter was generated.¹²⁹ Similar to global knockout animals, this conditional knockout strain exhibits elevated gamma frequency band power in EEGs as well as delayed development of PNN and PV cells, indicating that these deficits arise locally in the cortex. However, the deficit in temporal synchronization of responses to time

varying stimuli was absent when FMRP expression is preserved at subcortical levels, demonstrating that the change following global *Fmr1* knockout is a secondary consequence of altered subcortical inputs. Together, these observations suggest that FMRP deficiency at both subcortical and cortical levels contribute to the physiological deficits recorded from the auditory cortex.

5 | AUDITION-ASSOCIATED BEHAVIORAL CHANGES IN FXS

Three main groups of auditory-associated behavioral phenotypes have been described in *Fmr1* knockout rodents: (i) alterations to the acoustic startle response (ASR) and/or prepulse inhibition (PPI) of the ASR, (ii) increased audiogenic seizure susceptibility, and (iii) impaired auditory-cued fear conditioning.

5.1 | Altered ASR and PPI

Deletion of *Fmr1* impacts acoustic processing in both rodents and humans, as evaluated by altered ASR and PPI.¹³⁰ ASR is a full body reflexive response elicited by a loud sound stimulus. PPI is thought to occur when a weak nonstartling sound presented immediately before a startling sound that suppresses the ASR. ASR provides a robust and high throughput

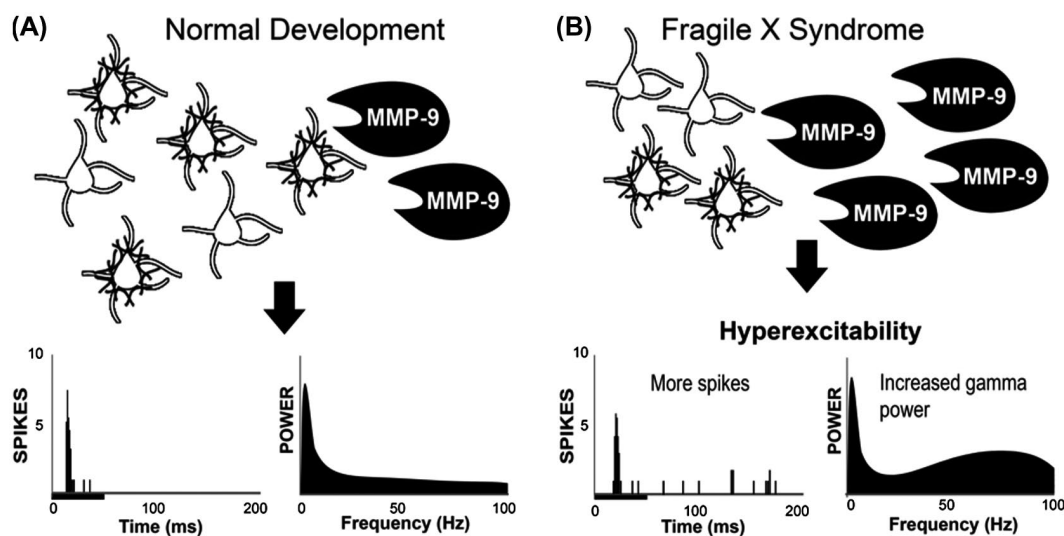


FIGURE 5 Mechanisms underlying cortical hyper-responsiveness in FXS involve local circuit deficits. A, In normal development, MMP-9 provides a certain level of control over PNN integrity around PV cells leading to normal balance between inhibition and excitation, auditory cortical responses as shown below with the poststimulus time histogram and power spectral density plots in the lower left panels. B, In FXS, the MMP-9 level is increased leading to greater breakdown of PNN around PV cells leading to increased spiking and increased resting EEG gamma band power. MMP-9/PV/PNN defects are present in global *Fmr1* knockout mice, as well as mice in which FMRP is removed only from forebrain excitatory neurons. This suggests that local PV neuron functional deficits can lead to cortical hyper-responsiveness. Moreover, even though FMRP was affected only in excitatory neurons, deficits were observed at the network level involving PV cells and PNN that surround them. Image modified from 120

assay for measuring sound sensitivity, but it is difficult to differentiate changes to sound perception from generalized hyperactivity or motor abnormalities.^{131,132} Additionally, ASR only occurs for high-intensity sounds, which limits its sensitivity for identifying perceptual abnormalities at moderate sound levels. On the contrary, PPI can be used to assess behavioral responses to softer stimuli that do not elicit a startle response themselves. Enhanced or reduced PPI indicates increased or decreased sensitivity to a sound stimulus and implicates sensorimotor gating disruptions that are specific to the auditory system.

In contrast to the variability in human measurements of the ABR, studies of ASR and PPI in humans have given clear-cut results. Two clinical studies have compared ASR and PPI responses between individuals with FXS and control subjects.^{130,133} Both studies agree on unchanged threshold and magnitude of the baseline ASR but a reduced PPI in FXS individuals. Interestingly, studies of *Fmr1* knockout mice show mixed results, perhaps because of the very different hearing range of humans and mice. Many studies found reduced ASR to startle sounds at the level of 90–120 dB, along with enhanced PPI, as compared to age-matched wild-type mice.^{130,134–145} Other studies reported unaltered, enhanced ASR, or reduced PPI.^{134,138,139,142} Potential contributors to these discrepancies include, but are not limited to, the genetic background,^{140,146} sex (147; but also see 137), age,¹⁴⁵ and the sound level for triggering ASR.¹⁴⁰ Particularly, strain-specific differences have been reported in a variety of behavioral phenotypes following *Fmr1* deletion¹⁴² and it is known that different mouse strains exhibit varying degrees of ASR in wild-type mouse strains.¹⁴⁸ Despite the variability in ASR and PPI phenotypes, ASR changes in *Fmr1* knockout mice are developmentally regulated¹⁴⁵ and can be rescued with re-introduction of the *Fmr1* gene,¹⁴¹ supporting a direct involvement of *Fmr1* gene in these auditory-mediated behaviors. Because their hearing range overlaps with that of humans, it is likely that *Fmr1* rats will prove a better model for studying these auditory phenotypes.

5.2 | Increased audiogenic seizure susceptibility

A high proportion of FXS patients have childhood seizures, which typically abate with development.¹⁴⁹ In *Fmr1* knockout mice, a continuous loud sound lasting longer than 30 seconds leads to audiogenic seizures (AGS).^{136,150} AGS are generalized convulsive seizures characterized by wild running, loss of righting reflex, generalized tonic-clonic seizing and, in some cases, death of the animal. As the seizure phenotype is robust, it is frequently used as a marker for testing drug efficacy in mouse models of FXS. Indeed, many therapeutics for FXS have been shown to reduce or abolish AGS in *Fmr1* knockout mice

(metformin: ¹⁵¹; CTEP: ¹⁵²; Lovastatin: ^{153,154}; Baclofen: ¹⁵⁵; PAK inhibitor: ¹⁵⁶). Similar to ASR and PPI, there are strain-specific differences in both susceptibility and developmental timing of AGS, with the most robust seizure phenotype seen in *Fmr1* knockout mice on the FVB background.^{136,150} Animals from the C57BL/6 background are most susceptible to seizures during a restricted developmental window around postnatal day 21–25.¹³⁷ Determining the contribution of genetic background to audiogenic seizure susceptibility in *Fmr1* knockout mice could potentially help explain epilepsy variability seen across individuals with FXS.¹⁴⁹

Cell type specific *Fmr1* deletion demonstrates that lack of FMRP in subcortical regions is sufficient to cause AGS. Moreover, restoration of FMRP expression solely in the inferior colliculus is sufficient to prevent AGS in the global knockout mouse strain.¹⁵⁷ At the molecular level, generation of AGS likely involves the common signal pathways underlying other FXS phenotypes, because the frequency of AGS in *Fmr1* knockout mice is reduced by correcting mGluR5 signaling^{143,158} or ERK1/2 activity.^{159,160} Although the AGS in *Fmr1* knockout mice are frequently compared to epilepsy in FXS individuals, it is not clear that the underlying seizures are similar in humans and mice.¹⁴⁹ Moreover, the relationship between AGS and sound tolerance issues in FXS individuals has not been established, presenting questions for future research.

5.3 | Perceptual learning and decision making

Recent studies have provided evidence for perceptual learning or discrimination deficits in *Fmr1* knockout mice in both the tactile^{161,162} and visual domains.¹⁶³ Although fewer studies have examined auditory perceptual learning in FXS models, there is evidence for impaired auditory-cued fear conditioning in *Fmr1* knockout mice.^{164,165} It is not clear, however, whether this impairment is more cognitive than perceptual in nature, because *Fmr1* knockout mice exhibit impairment on a variety of associative learning paradigms that are not dependent on auditory cues.¹⁶⁶ Examination of auditory learning and/or discrimination in *Fmr1* knockout mice has the potential to shed light onto the mechanistic basis of sound sensitivity and processing deficits in FXS, as well as fuel development of behavioral paradigms that can be directly translated to human studies.

6 | CONCLUSIONS AND FUTURE DIRECTIONS

The studies of the auditory system in FXS in humans and animal models represent one of the most detailed examinations

across a sensory system in an ASD. The results discussed here demonstrate that FMRP deficiency affects the auditory system at multiple stages along the ascending pathway, starting with the spiral ganglion and very first nuclei in the brainstem and extending all the way to auditory cortex. This wide-spread involvement strongly supports the notion that FXS is not purely a cognitive condition but alters fundamental sensory information processing that is required for a subject to perceive, separate, and localize sounds. Interestingly, striking changes in morphology or intrinsic neuronal excitability caused directly by loss of FMRP may be compensated by other mechanisms, such that changes in overall synaptic strength may be little changed at lower levels but become progressively clearer at higher levels. In some cases, studies of auditory neurons have also revealed new potential therapeutic strategies for hypersensitivity to sounds in FXS, including treatments that alter the activity of potassium channels or metalloproteases.

Fmr1 knockout rodents have provided numerous insights into specific cellular and molecular changes that contribute to the auditory problems of FXS patients. Similar genetic approaches are likely to provide even more insights in the future. It is important to note that although *Fmr1* knockout mice recapture some phenotypes of the auditory system of human FXS, large variations and even completely opposite changes are frequently encountered across mouse studies as well as between mouse and human studies. In part, these problems may arise because some of the observed changes are the result of compensatory neuronal plasticity rather than true FMRP-dependent biological pathways, and may therefore, differ with age, sex, and genetic background. More systematic future studies of the role of FMRP at different developmental states in multiple auditory nuclei may, therefore, resolve some of these issues.¹⁶⁷

Finally, it may be possible to “borrow” useful approaches in other fields for studying FMRP regulation of auditory processing. For example, hearing loss often results in auditory perceptual disruptions, including hyperacusis, where moderate-intensity “everyday” sounds are perceived as intolerably loud or aversive.¹⁶⁸ While sound hypersensitivity due to hearing loss or FMRP deficiency are likely to be mechanistically distinct, there are well-developed assays for assessing sound sensitivity following hearing loss that can be applied to FXS and ASD models. Potential approaches include operant conditioning paradigms that assess perceptual decision making,¹⁶⁹⁻¹⁷¹ which likely engage a more extensive range of auditory structures than startle-based measures, or innate place-preference paradigms that reflect the aversive nature of sounds without extensive training or conditioning.^{172,173}

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing and editing of the manuscript and have given final approval for all edits. E.A. McCullagh and Y. Wang organized and edited the final versions of the manuscript in addition to writing and editing the main text.

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