Characterization of auditory physiology in FXS mice at critical developmental timepoints

Thesis by

Ishani Ray

In Partial Fulfillment of the Requirements

for the **Honor's College Degree Award**

In Collaboration with

Dr. Elizabeth McCullagh and Lab Team

ABSTRACT

Autism spectrum disorders are strongly associated with auditory hypersensitivity. Fragile X syndrome (FXS), a common monogenic cause of ASD, results from transcriptional silencing of the Fmr1 gene and reduced expression of fragile X messenger ribonucleo-protein (FMRP). FMRP directly impacts myelin proteins and various brain regions show reduced/delayed myelination in FXS, suggesting deficits seen in FXS may be caused by alterations to myelination. FXS is a neurodevelopmental disorder, therefore characterizing when during development auditory dysfunction arises in addition to understanding if these changes are myelin dependent is critical to elucidating the full etiology of FXS. Auditory brainstem response (ABR) measurements record 1-4 waves, each corresponding to part of the ascending auditory pathway; the latency of which could be directly related to myelination of auditory areas. To characterize the physiology of myelination deficits in FXS at developmental time points, ABR measurements were taken for transgenic Fmr1 mice and controls before (P8-10), during (P12-14) or after (P21-23 and adult) hearing onset in mice. This allowed us to study the developmental emergence of auditory disruptions in Fmr1transgenic mice and identify critical windows where underlying auditory pathways are established. We hypothesize that transgenic Fmr1 mice will have increased latencies and decreased amplitudes in their ABR waves compared to the wildtype at different developmental time points. These data will aid in identifying the critical developmental windows of neural circuitry establishment in auditory sensory systems and potential myelination impairments that underly auditory dysfunction observed in patients and mice with FXS.

INTRODUCTION

Autism spectrum disorders (ASD) are strongly associated with hyperacusis (Hagerman, 2008). Fragile X syndrome (FXS), the most common monogenic cause of ASD, results from transcriptional silencing of the Fmr1 gene and reduced expression of fragile X mental retardation protein (FMRP) (Hagerman, 2008). Consequently, FXS has been used as a model for understanding ASD (O'Donnell & Warren, 2002; O'Donnell & Warren, 2002). While the mechanisms by which loss of FMRP leads to FXS and ASD phenotypes are not fully understood, one potential mechanistic approach correlates myelination deficits to phenotypes (Bonaccorso et al., 2015; McCullagh et al., 2020). FMRP targets myelin basic protein whereas various brain regions show reduced/delayed myelination in FXS, suggesting the deficits seen in FXS may be caused by alterations to myelination (Bonaccorso et al., 2015; Ito et al., 2004; (Wang, 2003).

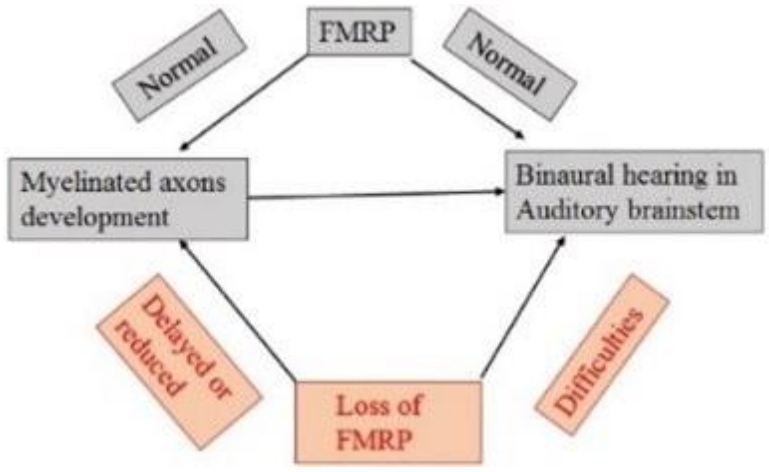

Figure 1: presents a depiction of the mechanistic relationship between FMRP, myelination and auditory phenotypes

Sensory hypersensitivity, particularly in the auditory system, is a prevalent indication in FXS (McCullagh et al., 2020). Auditory phenotypes of auditory hypersensitivity and binaural hearing deficits are conserved between mouse and human FXS, and possibly the consequent myelination deficits (McCullagh et al., 2020). Further, the auditory brainstem, a well-characterized model circuit involved in basic sound processing and localization, is implicated in binaural hearing deficits observed in individuals with FXS and ASD (Hagerman, 2008). Myelination of axons in the auditory brainstem plays a crucial role in the ability to localize sounds, which relies on precise synaptic transmission through binaural circuits (Kim et al., 2013). The auditory brainstem is responsible for initial sound processing and localization and possesses specific features that enable it to encode binaural sound information, including

fast synaptic encoding, tonotopic organization, highly myelinated axons, specialized nuclei, and IID and ITD computation circuits (McCullagh et al., 2020; (O'Donnell & Warren, 2002). Disruptions to sound information processing in the auditory brainstem can lead to higher order processing deficits and neurocognitive disorders, including autism spectrum disorders (ASD) (Hagerman, 2008; Kim et al., 2013; (McCullagh et al., 2020). Some studies also suggest disruptions in the auditory system driven by the Fmr1 gene before hearing onset (Hagerman, 2008). Understanding the underlying causes of ASD and FXS is crucial in determining the etiology of ASD and developing therapeutic interventions, and the auditory brainstem in FXS mouse models provides a useful model to understand the relationship between myelination deficits in the auditory brainstem and higher brain areas in FXS (Kim et al., 2013; (McCullagh et al., 2020).

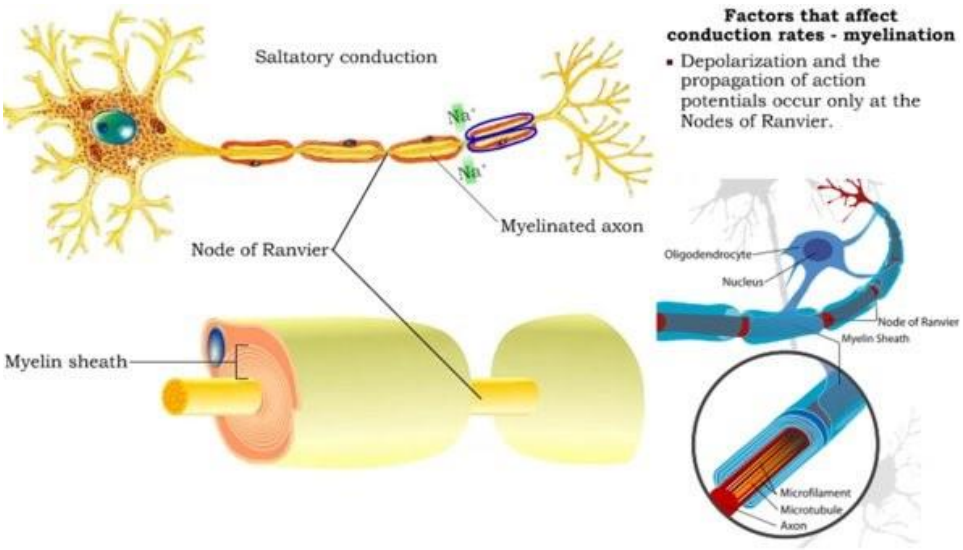

Figure 2: presents a depiction of myelination and its role in impulse conduction and binaural sound processing

Additionally, FXS and ASD are neurodevelopmental disorders which implies that while many phenotypes, biomarkers, and treatments have been generated for adult animals with FXS, it is likely that intervention needs to occur earlier in development to prevent adult impairments (McCullagh et al., 2020). Particularly, critical windows exist in sensory systems during which underlying circuitry is established, and true cure likely requires intervention during these periods (Rotschafer & Cramer, 2017). This makes identification and characterization of critical periods of auditory brain stem development when myelination alterations and consequent phenotypes arise using multifaceted (anatomy, physiology, and behavior) techniques imperative (O'Donnell & Warren, 2002).

Auditory brainstem response (ABR) measurements are a minimally invasive physiological readout of early auditory processing that is also used in humans (ElMoazen et al., 2020). ABR measurements record 1-4 waves in mice, each corresponding to part of the

ascending auditory pathway. The amplitude and latency of these waveforms may potentially correlate to myelination deficits (Ito et al., 2004). Preliminary studies have shown that while waves 1, 3 ABR latencies are not altered, there is a decreased latency for the Binaural Interaction Component (BIC) in adult FXS transgenic mice compared to wildtype (Laumen et al., 2016; Willott et al., 1993). Alternative to the physiological data, anatomical data indicate smaller diameter and thinner myelinated fibers in adult Fmr1 mice compared to controls supporting reduced myelination in FXS brain (Pacey et al., 2013; Phan et al., 2020). There are suggestions of these changes persisting into adulthood.

This study aims to investigate the developmental emergence of FXS physiology by characterizing the physiology of myelination deficits in mouse background strain model of FXS, C57BL/6J, at developmental time points using ABR measurement (Rotschafer & Cramer, 2017). This will allow us to a) study the developmental emergence of auditory disruptions in Fmr1 transgenic mice b) identify critical windows where underlying auditory pathways are established c) investigate the potential myelination mechanisms of FMRP action in this circuit. Furthermore, the study aims to identify potential biomarkers for ASD and FXS by utilizing ABR measurements. The study analyzes monaural and binaural ABR responses in hemizygous males, homozygous females, and heterozygous females compared to wild-type mice at developmental points of 8-10 days, 14 days and 21 days with the aim to fill the gap between basic and translational research (Rotschafer & Cramer, 2017).

The hypothesis of the study is that the loss of FMRP in FXS mice results in delayed or incomplete myelination in the auditory brainstem, which changes the temporal fidelity of timing and level difference computation in this circuit and affects sound localization. The auditory difficulties observed in FXS are specifically caused by the loss of FMRP. Consequently, it is reasonable to predict that sound localization circuit is altered in FXS both in terms of impaired latency & amplitude in ABR waveforms in transgenic mice. More specifically, we expect transgenic Fmr1 mice to have increased latencies and decreased amplitudes in their ABR waves compared to the wildtype, most prominently at P14 developmental time point.

METHODS AND MATERIALS

Non-invasive physiological markers can be utilized to measure myelination deficits and rescue with FMRP in the auditory brainstem (Laumen et al., 2016). The anatomy of myelin changes in the auditory brainstem can lead to physiological and behavioral consequences (Laumen et al., 2016). Auditory brainstem responses (ABR) are a non-invasive representation of the synchronized electrical activity over time of the neurons in the auditory brainstem pathway and the eighth cranial nerve (ElMoazen et al., 2020). It is a pattern of waveforms consisting of electrical potentials, elicited by brief auditory tones or clicks (ElMoazen et al., 2020). In ABR measurements, click stimulation generates a signal consisting of 1-4 waves in rodents. Each wave represents the activity of different regions of the ascending auditory pathway (Laumen et al., 2016). The amplitude and latency of these waves could be directly related to myelination of auditory areas (Long et al., 2017).

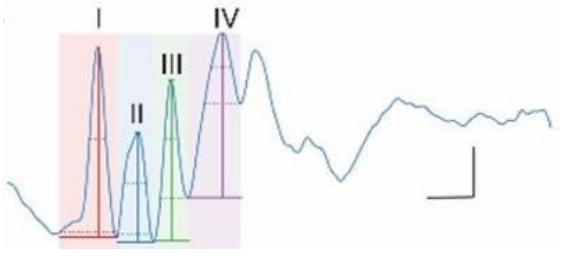

Figure 3: presents a typical mouse monoaural ABR waveform consisting of IV waves

The experimental procedures followed all relevant laws, regulations, and guidelines, including the National Institutes of Health guidelines, and were approved by the Institutional Animal Care and Use Committee of Oklahoma State University. The study was conducted on wild-type C57BL/6J mice and Fmr1 mutant mice, including hemizygous males, homozygous males and females, and heterozygous females. The Fmr1 mice were obtained from the Jackson Laboratory and were bred at Oklahoma State University. Mixed and single genotype mating strategies were used to generate experimental animals, including heterozygotes and littermate controls. Sex was considered as a biological variable, and any differences between sexes were noted in the results.

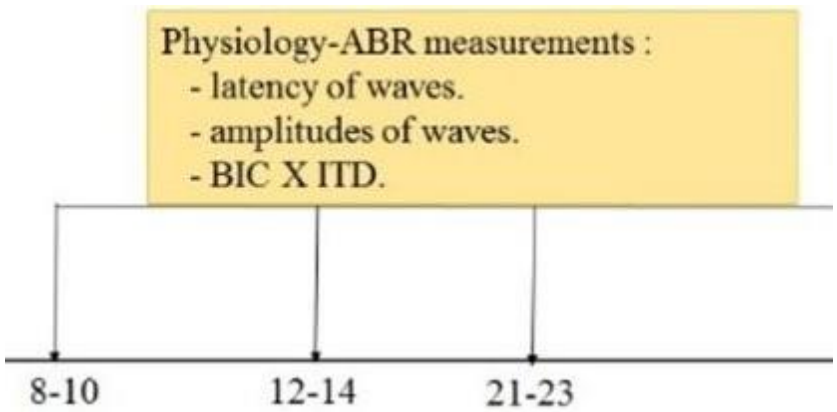

Figure 4: presents a depiction of the experimental design in terms of ABR data collected and the developmental timepoints studied

To characterize the physiology of myelination deficits in FXS at developmental time points, ABR measurements were taken on the transgenic Fmr1 mice and controls at multiple developmental time points representing before (P8-10), during (P12-14) or after (P21-23 and adult) hearing onset in mice before anatomical measurements. In terms of sample size, data was collected from 12/genotype/developmental time point.

ABR protocols outlined in Tolnai & Klump 2020, Grove et al. 2010 were followed to perform recordings of the Auditory Brainstem Response (ABR) in anesthetized animals. The animals were initially induced with a combination of ketamine-xylazine and then maintained using lower doses of the same drugs periodically throughout the experiment and were placed on a heating pad in a sound attenuating chamber throughout the length of the study. Initial anesthesia was followed by a) insertion of earphones into the rodents' ears b) positioning of several subdermal needle electrodes under the skin of the animals' head, neck and leg. Clicks or tones at varying intensities and timing differences between the ears were played to each ear individually and both ears simultaneously. The evoked potentials from the electrodes were amplified and processed using specialized equipment and custom Python software. The sound stimuli were presented to the animal through electrostatic or multifield speakers fitted with probe microphones for in-ear calibration. The sounds were generated using a Real-Time processor and presented at specific intervals. A rejection threshold was set to eliminate high-amplitude heart rate responses from the recorded traces, thereby improving the signal-to-noise ratio.

The following data was collected: monaural waves (left and right - wave amplitude and latency); Binaural Interaction Component or BIC (wave amplitude and latency) across Interaural Time Differences or ITDs; threshold of hearing; range of hearing across genotypes, and sexes at different developmental time points.

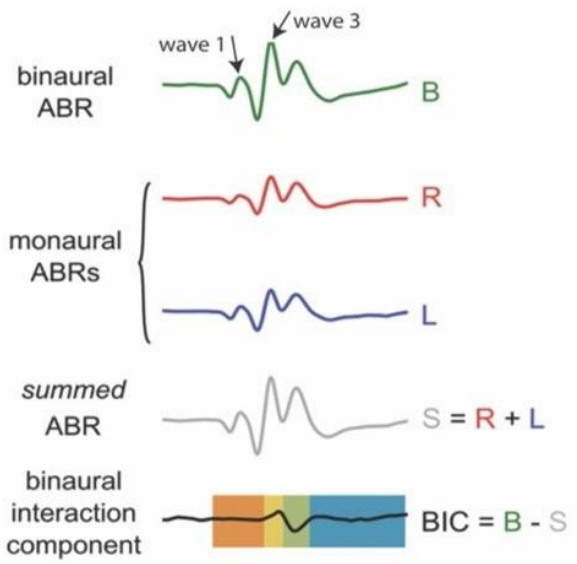

Figure 5: presents a depiction of different ABR components and BIC calculation

Figure 5 depicts the method of quantifying auditory brain stem response (ABR) signals. Monaural ABR amplitudes were determined for each ear as the voltage between the peak of the ABR and the trough of the waveform for waves I-IV. The peak amplitude and latency were measured across the four peaks of the ABR waveform at 90 dB SPL, and data from both ears were averaged. The latency was computed as the time in ms at which the peak of the waveform occurred.

Binaural auditory brain stem responses were generated by presenting broadband click stimuli at 90 dB SPL to both ears simultaneously. The BIC is calculated by subtracting summed monaural responses from the binaural response and results in a negative peak (DN1, Figure 5). The DN1 is the prominent negative peak corresponding with wave IV of the summed and binaural ABR. The BIC amplitude was measured as the voltage at the peak of the DN1 waveform to the baseline of the measurement. ITD computation using the BIC was measured by presenting stimuli with varying ITDs of ± 2 ms in 0.5 ms steps. The BIC is a measure of binaural processing ability in humans and animals whereas BIC x ITD is a measure of sound localization ability in humans and animals (Long et al., 2017).

The study conducted an audiogram to evaluate the hearing range of animals using varying frequencies of sound. Thresholds were measured using a visual detection method or the lowest level of sound intensity where a response could be detected. Tone bursts were used as stimuli in audiogram testing. Monaural auditory brain stem responses were generated by presenting broadband click stimuli to each ear separately. Click threshold was determined by reducing the sound intensity until the ABR waveforms disappeared.

The present study employed custom Python software to analyze evoked potentials of monaural and binaural stimuli. The amplitude and latency of BIC were measured using custom Python software, with amplitude being relative to the zero baselines of the measurement. To ensure accuracy, the software was programmed with automatic peak detection and manual correction capabilities.

Each ABR measurement was followed by the sacrifice of the animal through transcardial perfusion, and dissection of the brains. Using the same animal for the physiological and anatomical measurements allows within individual characterization supporting direct mechanistic approach.

RESULTS

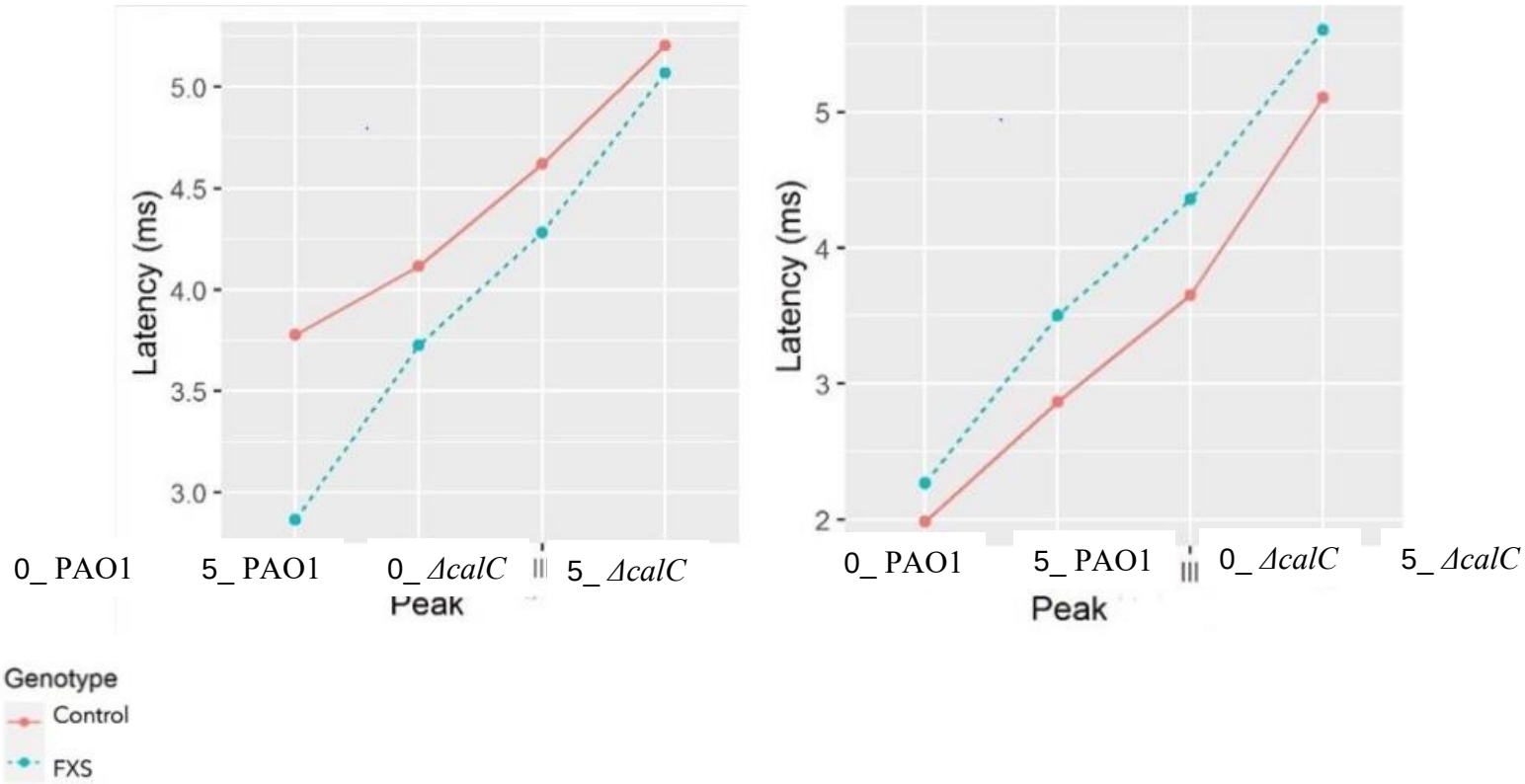

Figure 6: presents the results of ABR monaural hearing tests in Fragile X syndrome (FXS) mice. Latencies for peaks I-IV of monoaural ABR waveforms were recorded for both Fmr1KO and B6 wildtype mice.

Latency (y-axis) for wave I-IV (x-axis) differences in monaural hearing for Fmr1KOs, and B6 wildtype animals at P14 and P21. Latency was measured as time taken to reach peak amplitude in waves. The data showed significant difference in latency of waves between Fmr1KO and B6 wildtype mice at both timepoints. However, surprisingly, there was a flip in the results with Fmr1KO mice ABR waves showing higher latency relative to B6 wildtype at P14 and lower latency relative to B6 wildtype at P20.

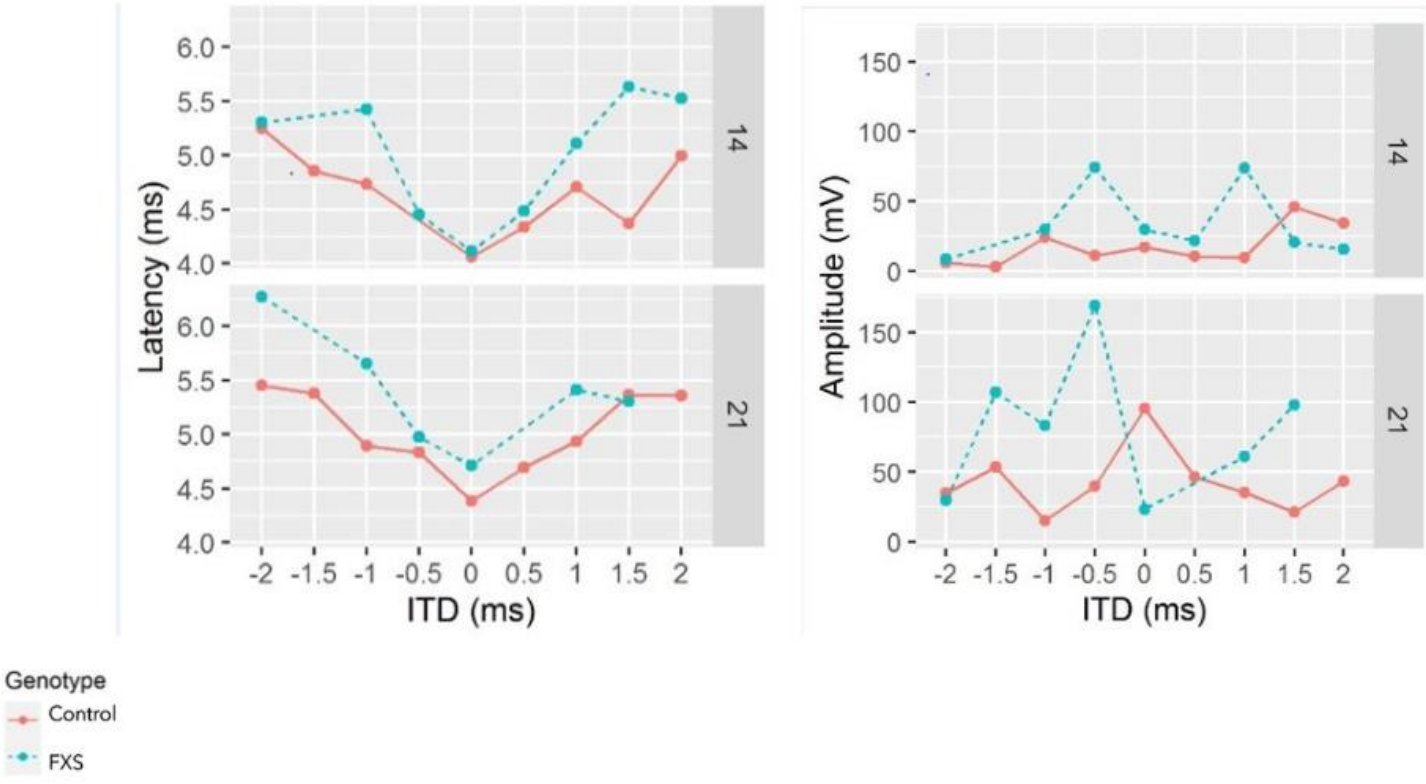

Figure 7: presents our findings on BIC in Fragile X syndrome (FXS) mice. Amplitudes and latencies for the BIC at interaural time differences (ITDs) ranging from -2 to +2 ms in 0.5 ms increments were collected for both Fmr1KO and B6 animals.

Amplitude and latency (y-axis) of BIC at varying ITDs (x-axis) from 2 ms to -2 ms for Fmr1 KOs, and B6 wildtype for P14 and P21 developmental timepoints. The data showed significant difference in latency of the BIC between Fmr1 and wildtype at both P14 and P21. In both cases, the BIC of Fmr1KO mice showed a longer latency relative to the B6 wildtype. Unexpectedly, the data for BIC amplitudes also showed a similar trend with the BIC amplitude for Fmr1KO mice being higher relative to that of B6 wildtype at both P14 and P21 timepoints.

DISCUSSION

This study is the first to investigate the auditory brainstem response (ABR) in the C57BL/6J Fmr1 mouse model of FXS as well as BIC in an FXS-mutant strain at multiple developmental timepoints. The study provides crucial insights into hearing range, monaural ABRs, and binaural integration across Fmr1KO and wildtype mice across development as well as into auditory phenotypes that may have similarities or distinctions among different genetic strains of mice with FXS.

Monaural click stimuli elicit auditory brainstem responses (ABRs) that reflect neural activity along the ascending auditory pathway. Alterations in the amplitude and latency of ABR waves have been reported in previous studies of Fmr1 mutant mice. The latency of monaural waves observed in Fmr1KO mice at P21 is consistent with the majority of work in adult FXS as well as FVB mice. Our study further adds to the knowledge of ABR phenotypes that might be consistent across genotypes. However, we do report some interesting developmental changes that occur that have not been seen or studied previously such as the switch in the relative latency of monoaural ABR waves of Fmr1KO mice from P14 to P21.

In binaural hearing, the monaural ABR is limited in providing information about the binaural integration of sound. Thus, the binaural interaction component (BIC) of the ABR is used to measure the brainstem's ability to process binaural information. The amplitude of the BIC at different interaural time differences (ITDs) did differ between genotypes at both P14 and P21 developmental timepoints with Fmr1KO mice trending towards higher BIC amplitudes relative to B6 wildtype mice. The latency of the BIC was longer in Fmr1KO mice than in B6 wildtype mice.

Sex differences in animal models are important for fully understanding the complexities of disorders such as ASD or FXS, which likely affect females differently than males. In FXS, specifically, being an X-linked disorder, leads to a higher prevalence in males than females, which can undergo X-inactivation on the affected X chromosome. However, mice offer a unique opportunity to measure both heterozygote and homozygous females, giving insight into potential sex differences related to loss of Fmr1 on one or both X chromosomes. It is important to note that ABR data for this study were combined for sexes across genotypes, and the sex differences in the physiological phenotypes across developmental periods were not independently studied.

Our results reveal subtle yet significant differences in the development of auditory brainstem function, specifically for monaural and binaural hearing in B6 mice with either complete or partial mutation. We also identified that binaural sound processing and localization could be related to myelination development in the auditory brainstem, P14 developmental timepoint seems to be the most critical timepoint where monoaural and binaural processing deficits manifest whereas interesting differences in the BIC latency were observed between P14 and P21 Fmr1KO mice which could provide more information on how FXS manifests developmentally.

The results of this study provide preliminary evidence for the involvement of myelination deficits in auditory difficulties observed in individuals with FXS during development and adulthood. It also clarifies the development and mechanism of sound localization and binaural hearing impairments in FXS and perhaps ASD in general, which will provide critical insights helpful in determining future strategies for treatment of these symptoms. In its aim to investigate whether myelination deficits may underlie other aspects of changes to the brain that occur in ASD/FXS, the study further extends the usefulness of the auditory brainstem as a model circuit.

Reliant on the data obtained from the study is the expansion of the project to FMRP rescue of FXS physiological phenotype that will focus on ABR following FMRP viral (Adeno-associated virus mediated) reintroduction at relevant developmental time points to study if ABR BIC latencies can be rescued via restoration of myelin deficits. Alongside the AAV (adeno associated viral) FMRP reintroduction via surgery on P1 mice and collection of ABR measurements with young mice post-surgery at the established developmental time points (P8, P14, and P21), further establishing the utility of ABR as a tool to study FXS, and quantification of myelination development and proliferation at critical timepoints using immunohistochemical experiments are some related areas of expansion. Characterization of anatomical changes will likely be done through the measurement of the diameter and thickness of myelination, spacing and size of sodium channels (nodes and paranodes), as well as the number and type of oligodendrocytes in transgenic mice and littermate controls.

This project might potentially and specifically aid in identifying the critical developmental windows of neural circuitry establishment in auditory sensory systems of FXS when a rescue will likely be most effective as well as whether a viral FMRP-reintroduction rescue of myelination deficits and physiological phenotypes be effective and what the mechanistic implications of the latter might be. It is important to determine if ABR changes are specific to myelination so that they can be used both as a biomarker for other myelination diseases, along with being considered an important component of ASD and FXS.

Some other potential areas for future research may include modeling of FXS anatomical changes, electrophysiological measurements of conduction speed and velocity in FXS, and large-scale myelination changes using micro dice-CT, among others. Further, investigating the behaviorally induced auditory cortical plasticity in the FVB strain could help determine if ABR phenotypes remain consistent across different genetic backgrounds.

CONCLUSIONS

The study concludes that binaural sound processing and localization could be related to myelination development in the auditory brainstem. P14 seems to be the most critical timepoint where monoaural and binaural processing deficits manifest whereas interesting differences in auditory and myelination phenotypes are observed between P14 and P21 which could provide more information on how FXS manifests developmentally.

ACKNOWLEDGEMENTS

I appreciate Dr McCullagh, Amita Chawla and the McCullagh lab team members for dedicating their time and mentorship towards my research training. I am grateful for the Wentz/Purdie Research Scholar Program and the Office of Scholar Development and Undergraduate Research at OSU for their generous funding and program support.

REFERENCES:

Bonaccorso, C. M., Spatuzza, M., Di Marco, B., Gloria, A., Barrancotto, G., Cupo, A., Musumeci, S. A., D'Antoni, S., Bardoni, B., & Catania, M. V. (2015). Fragile X mental retardation protein (FMRP) interacting proteins exhibit different expression patterns during development. *International Journal of Developmental Neuroscience*, *42*(1), 15–23. https://doi.org/10.1016/j.ijdevneu.2015.02.004

ElMoazen, D., Sobhy, O., Abdou, R., & AbdelMotaleb, H. (2020). Binaural interaction component of the auditory brainstem response in children with autism spectrum disorder. *International Journal of Pediatric Otorhinolaryngology*, *131*, 109850. https://doi.org/10.1016/j.ijporl.2019.109850

Gholizadeh, S., Arsenault, J., Xuan, I. C., Pacey, L. K., & Hampson, D. R. (2014c). Reduced phenotypic severity following adeno-associated virus-mediated FMR1 gene delivery in fragile X Mice. *Neuropsychopharmacology*, *39*(13), 3100–3111. https://doi.org/10.1038/npp.2014.167

Hagerman, P. J. (2008). The fragile X prevalence paradox. *Journal of Medical Genetics*, *45*(8), 498–499. https://doi.org/10.1136/jmg.2008.059055

Ito, T., Tokuriki, M., Shibamori, Y., Saito, T., & Nojyo, Y. (2004). Cochlear nerve demyelination causes prolongation of wave I latency in ABR of the myelin deficient (MD) rat. *Hearing Research*, *191*(1–2), 119–124. https://doi.org/10.1016/j.heares.2003.12.019

Kim, J. H., Renden, R., & von Gersdorff, H. (2013). Dysmyelination of auditory afferent axons increases the jitter of action potential timing during high-frequency firing. *The Journal of Neuroscience*, *33*(22), 9402–9407. https://doi.org/10.1523/jneurosci.3389-12.2013

Laumen, G., Ferber, A. T., Klump, G. M., & Tollin, D. J. (2016). The physiological basis and clinical use of the binaural interaction component of the auditory brainstem response. *Ear & Hearing*, *37*(5). https://doi.org/10.1097/aud.0000000000000301

Long, P., Wan, G., Roberts, M. T., & Corfas, G. (2017). Myelin development, plasticity, and pathology in the auditory system. *Developmental Neurobiology*, *78*(2), 80–92. https://doi.org/10.1002/dneu.22538

McCullagh, E. A., Poleg, S., Greene, N. T., Huntsman, M. M., Tollin, D. J., & Klug, A. (2020). Characterization of auditory and binaural spatial hearing in a fragile X syndrome mouse model. *Eneuro*, *7*(1). https://doi.org/10.1523/eneuro.0300-19.2019

McCullagh, E. A., Rotschafer, S. E., Auerbach, B. D., Klug, A., Kaczmarek, L. K., Cramer, K. S., Kulesza, R. J., Razak, K. A., Lovelace, J. W., Lu, Y., Koch, U., & Wang, Y. (2020). Mechanisms underlying auditory processing deficits in fragile X syndrome. *The FASEB Journal*, *34*(3), 3501–3518. https://doi.org/10.1096/fj.201902435r

O'Donnell, W. T., & Warren, S. T. (2002). A decade of molecular studies of Fragile X Syndrome. *Annual Review of Neuroscience*, *25*(1), 315–338. https://doi.org/10.1146/annurev.neuro.25.112701.142909

Pacey, L. K., Xuan, I. C., Guan, S., Sussman, D., Henkelman, R. M., Chen, Y., Thomsen, C., & Hampson, D. R. (2013). Delayed myelination in a mouse model of fragile X syndrome. *Human Molecular Genetics*, *22*(19), 3920–3930. https://doi.org/10.1093/hmg/ddt246

Phan, B. N., Bohlen, J. F., Davis, B. A., Ye, Z., Chen, H.-Y., Mayfield, B., Sripathy, S. R., Cerceo Page, S., Campbell, M. N., Smith, H. L., Gallop, D., Kim, H., Thaxton, C. L., Simon, J. M., Burke, E. E., Shin, J. H., Kennedy, A. J., Sweatt, J. D., Philpot, B. D., … Maher, B. J. (2020). A myelin-related transcriptomic profile is shared by Pitt–Hopkins Syndrome Models and human autism spectrum disorder. *Nature Neuroscience*, *23*(3), 375–385. https://doi.org/10.1038/s41593-019-0578-x

Rotschafer, S. E., & Cramer, K. S. (2017). Developmental emergence of phenotypes in the auditory brainstem nuclei of*fmr1*knockout mice. *Eneuro*, *4*(6). https://doi.org/10.1523/eneuro.0264-17.2017

Wang, H. (2003). Developmentally-programmed FMRP expression in oligodendrocytes: A potential role of FMRP in regulating translation in oligodendroglia progenitors. *Human Molecular Genetics*, *13*(1), 79–89. https://doi.org/10.1093/hmg/ddh009

Willott, J. F., Aitkin, L. M., & McFadden, S. L. (1993). Plasticity of auditory cortex associated with sensorineural hearing loss in adult C57BL/6J MICE. *The Journal of Comparative Neurology*, *329*(3), 402–411. https://doi.org/10.1002/cne.903290310