eNeuro

Research Article: Confirmation | Disorders of the Nervous System

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https://doi.org/10.1523/ENEURO.0300-19.2019

Cite as: eNeuro 2020; 10.1523/ENEURO.0300-19.2019

Received: 31 July 2019
Revised: 1 December 2019
Accepted: 20 December 2019

Title: Characterization of auditory and binaural spatial hearing in a Fragile X Syndrome mouse model

Abbreviated title: Mouse auditory spatial acuity in Fragile X Syndrome

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7 Figures, word count: Abstract 164 words, Introduction 800 words, Discussion 2,014 words

Conflict of Interest Statement: The authors declare no competing financial interests.

Acknowledgements

We would like to acknowledge Dr. John Peacock for his assistance in troubleshooting the various components of the behavioral set up. We would also like to acknowledge Dr. Peter McCullagh and Dr. Alex Kaizer for their assistance with the statistical models used in this paper.

Funding Sources: Supported in part by NIH R01 DC017924 (Klug). Dr. McCullagh was funded by FRAXA and NIH 3T32DC012280-05S1.

Author Contributions: All authors helped write and revise the manuscript. EAM, SP, NTG, DJT, AK developed the ideas and methods. EAM and SP collected the data for the manuscript. MMH provided the *Fmr1* animals and controls. EAM performed the statistical analyses and figures for the manuscript.

Abstract:

The auditory brainstem compares sound-evoked excitation and inhibition from both ears to compute sound source location and determine spatial acuity. Although alterations to the anatomy and physiology of the auditory brainstem have been demonstrated in Fragile X Syndrome (FXS) it is not known whether these changes cause spatial acuity deficits in FXS. To test the hypothesis that FXS-related alterations to brainstem circuits impair spatial hearing abilities, a reflexive prepulse inhibition (PPI) task, with variations in sound (gap, location, masking) as the prepulse stimulus, was used on *Fmr1* knockout mice and B6 controls. Specifically, *Fmr1* mice show

decreased PPI compared to wildtype during gap detection, changes in sound source location, and spatial release from masking with no alteration to their overall startle thresholds compared to wildtype. Lastly, *Fmr1* mice have increased latency to respond in these tasks suggesting additional impairments in the pathway responsible for reacting to a startling sound. This study further supports data in humans with FXS that show similar deficits in PPI.

Significance Statement:

This is the first study to characterize auditory spatial acuity in a mouse model of FXS. We saw minor differences in *Fmr1* mice compared to B6 mice in several measures of auditory acuity as measured by inhibition of the startle response. *Fmr1* mice had increased latency to startle for almost all conditions compared to B6 mice suggesting altered timing to acoustic cues. These experiments further show that, consistent with patient report and anatomical/physiological data, the auditory system is altered in a mouse model of FXS, though with some potential compensation leading to a subtle behavioral impact.

Introduction

Fragile X Syndrome (FXS) is the leading monogenetic cause of autism (Penagarikano et al., 2007). FXS is caused by a mutation in the gene *Fmr1* that encodes Fragile X Mental Retardation Protein (FMRP). One of the hallmark symptoms of FXS, among many other cognitive symptoms, is auditory hypersensitivity (reviewed in Rotschafer and Razak, 2014). An imbalance of neural excitation/inhibition (E/I) is thought to underlie many pathologies in FXS (Contractor et al., 2015) including those leading to auditory symptomology (Keine et al., 2016).

E/I imbalances in FXS extend to the auditory brainstem circuits responsible for sound localization, as well as auditory cortical areas (Garcia-Pino et al., 2017; McCullagh et al., 2017; Rotschafer et al., 2015; Rotschafer and Cramer, 2017). FMRP is highly expressed in the auditory brainstem (Ruby et al., 2015; Wang et al., 2014; Zorio et al., 2017) leading to changes in potassium channel distribution (Brown et al., 2010; Strumbos et al., 2010) that underlie changes in synaptic function *in vitro* (Curry et al., 2018; El-Hassar et al., 2019; Garcia-Pino et al., 2017; Lu, 2019; Wang et al., 2015). In addition, studies have shown alterations to the auditory brainstem response (ABR), an *in vivo* measure of auditory brainstem activity, in *Fmr1* mice that are potentially caused by these underlying changes to E/I balance and physiological activity (El-Hassar et al., 2019; Rotschafer et al., 2015).

Binaural hearing and spatial acuity not only used for sound localization per se but are also essential for communication in busy acoustic environments where several sound sources are active at the same time - in the literature often labeled “cocktail party situations” (Cherry, 1953). In such situations, the sound localization pathway in the auditory brainstem associates these various sounds with their respective spatial channel, thereby providing the foundation for our ability to follow a particular sound of interest when competing sounds are present. This separation is dependent on an intricate E/I balance that starts in the auditory brainstem (reviewed in (Bronkhorst, 2015; Grothe et al., 2010)). Basic encoding of sound location information is also relayed through the precise balance of E/I and encoded as interaural level and timing differences (ILD and ITD respectively)(Grothe et al., 2010; Pollak et al., 2002). ITDs and ILDs are dependent on timing and level information from the two ears that becomes excitation or inhibition within the auditory brainstem (Caird and Klinke, 1983; Goldberg and Brown, 1969; Moore and Caspary, 1983; Thompson and Schofield, 2000). Therefore, impairments in the E/I balance of the auditory brainstem that occur in FXS are expected to lead to impaired ability to

function in complex noisy acoustic environments and ITD and ILD encoding. As a signal is displaced further in space from a distracting background noise, it becomes easier to discriminate from the noise, this effect is termed spatial release from masking (SRM)(reviewed in Feng and Ratnam, 2000). Despite the substantial alterations to the auditory brainstem in FXS, it has never been clearly shown, beyond patient report and surveys, that mice or humans with a mutation in *Fmr1* have impairments in their ability to localize sound in normal listening or complex acoustic environments (Baranek et al., 2008, 2002; Rogers et al., 2003).

This study tests the hypothesis that mice with a mutation in the *Fmr1* gene have a functional deficit in binaural hearing despite a normal range of auditory hearing ability. Binaural hearing ability was assessed using a reflexive prepulse inhibition (PPI) paradigm, where a change in the sound source location served as the prepulse, a method described previously for mice (Allen and Ison, 2010) and guinea pigs (Greene et al., 2018). The acoustic startle response is a reflexive whole-body response elicited by a very brief, but loud impulse noise. PPI consists of modification of the acoustic startle response by pairing the startle eliciting stimulus with a preceding stimulus, the prepulse, that inhibits the startle response by providing a cue to the impending startle (Young and Fechter, 1983). PPI is a useful tool for measuring deficits in cognitive disorders such as autism, FXS, or schizophrenia since it is reflexive and independent of cognitive ability (Young and Fechter, 1983). It has been shown previously, with conflicting results, that mice and humans with *Fmr1* mutations have impaired PPI (Chen and Toth, 2001; Frankland et al., 2004; Hessl et al., 2009; Nielsen et al., 2002; Thomas et al., 2012; Veeraragavan et al., 2012) suggesting altered sensorimotor gating. We use a similar PPI paradigm, but with cues to specifically target spatial hearing ability (gap in sound, speaker swaps and spatial release from masking), to test the hypothesis that behavioral impairments result from altered signaling in the brainstem sound localization circuits. We show subtle changes in *Fmr1* mice responses to spatial auditory stimuli, quantified as reduced PPI, reduced detection of prepulses compared to wildtype animals, and increased response latencies to startling sounds.

Materials and Methods

All experiments complied with all applicable laws, NIH guidelines, and were approved by the University of Colorado Anschutz IACUC.

Subjects

All experiments were conducted in either C57BL/6J background (wildtype) or hemizygous male and homozygous *Fmr1* knockout strain maintained on the background (commercially available through Jackson Laboratory, Bar Harbor, ME, *Fmr1* stock number 003025, B6.129P2-*Fmr1*^{tm1Cgr}/J (Consorthium et al., 1994), C57BL/6J stock number, 000664). Forty-six total mice were used, exact number of animals used per experiment are listed in the figure legend and corresponding results sections. The results are based on experiments conducted in both male (N = 30) and female (N = 16) wildtype and *Fmr1* knock out mice. No significant differences were observed between sexes, and data for males and females were combined. Animals were genotyped regularly using Transnetyx (Cordova, TN). Mice used in these experiments were all adult animals and varied in age between 55 and 167 days old. Age was not significantly different between the two groups ($p = 0.96$, *Fmr1* mice 83.1 ± 4.3 days old, wildtype mice 83.4 ± 4.7 days old). Each animal was weighed after completion of data collection. Animal weights varied between 13-37 g, and generally increased with age. On average, *Fmr1* mice tended to weigh less (23.5 ± 0.75 g) than wildtype animals (25.8 ± 0.43 g) ($p = 0.01$). Pinna morphology was

measured using methods as described in Anbuhl et al., 2017 by measuring the height and width of each ear and estimating the effective diameter (square root of the height x width). There was no significant difference in pinna morphology between B6 and *Fmr1* animals ($p = 0.9026$, diameter 8.79 ± 0.36 B6, 8.84 ± 0.21 *Fmr1*).

ABR Audiogram

ABR recordings were conducted using methods similar to those described previously (Anbuhl et al., 2017). Briefly, a devoted cohort of mice (7 mice of each genotype 132 ± 2.8 days old B6, 117.9 ± 4.3 days old *Fmr1* of both sexes) were anesthetized (60mg/kg Ketamine + 10mg/kg Xylazine for initial anesthesia and 25mg/kg Ketamine + 12 mg/kg Xylazine), and placed on a heating pad in a small sound-attenuating chamber (ETS-Lindgren). Stimuli presentation and generation as well as evoked potentials recording were conducted through custom MATLAB (Mathworks Inc. Natick, MA) software interfacing with an RME (Haimhausen, Germany) Fireface UCX sound card (operating at a sampling rate of 44.1 kHz). Stimuli were presented through calibrated (Beutelmann et al., 2015) Etymotic (Elk Grove Village, IL) ER10B+ ear coupling tubes, and presented by ER2 earphones using standard sound-delivery tubes. Evoked potentials were made with platinum subdermal needle electrodes (F-E2-12 electrodes; Grass Technologies, West Warwick, RI) simultaneously at the apex, and behind each pinna (active), referenced to the nape of the neck, with a hind leg ground. Signals were amplified and digitized by a Tucker Davis Technologies low impedance headstage (RA4LI) and preamplifier (RA4PA), further amplified digitally (10,000x), and output as an analog signal by a multi-I/O processor (RZ5). ABRs were recorded in response to 1000 repetitions of short (5 ms) tone-burst (4, 8, and 16 kHz) stimuli, gated on and off with 1 ms linear ramps, and presented at a rate of ~14 per second. Frequencies of the ABR stimuli spanned the effective bandwidth of the auditory stimuli used for behavioral testing (see next section). Stimuli included interleaved presentations of left and right monaural, as well as binaural stimulus presentations. Responses were initially recorded for 90 dB SPL stimulus presentations, then for progressively lower levels (in 10 dB SPL steps) until threshold was found. Threshold was determined as the average between when a waveform was present and the next dB SPL step in which a discernable ABR signal appeared. Responses were analyzed by assessing for the lowest level eliciting a detectable evoked potential in any of the three recorded channels.

Apparatus

Experimental conditions and apparatus are previously described in Greene et al., 2018 but are also briefly described here. All experiments were conducted in a double-walled sound attenuating chamber (IAC Bronx, NY) lined with acoustical foam to reduce echoes. The animal was snugly placed, in order to ensure the animal was forward-facing, in a custom-built acoustically transparent steel-wired cage attached to a polyvinyl chloride post anchored to a flexible polycarbonate platform with an accelerometer (Analog Devices ADXL335 Norwood, MA) to capture startle responses. All animals were tested in the dark using a closed-circuit infrared (IR) camera to monitor movement and proper orientation of the animal. When the animal was placed snugly into the chamber with the steel lightly compressed around its body, the animal maintained its forward-facing position and was unable to turn around. The cage with the animal was then always oriented towards the center loudspeaker. A diagram with the experimental apparatus is shown in (Greene et al., 2018). The chamber consisted of an array of 25 loudspeakers (Morel MDT-20 Ness Ziona, Israel) placed horizontally in a 1 m radius

semicircular boom at 7.5° intervals from -90° (right) to +90° (left) in front of the animal from which pre-pulse stimuli were presented. Startle stimuli were presented from a Faital Pro HF102 compression driver (Faital S.p.A. San Donato Milanese, Italy) placed ~ 35cm (from the base of the platform) directly above the cage and amplified using an Alesis RA150 (Cumberland, RI).

Stimuli were generated and responses recorded from three Tucker-Davis Technologies (TDT Alachua, FL) RP 2.1 Real-Time processors using custom written MATLAB (Mathworks Natwick, MA) software. The startle stimuli were 20ms broad-band noise bursts generated by one of the RP2.1 processors and presented at 110dB SPL (unless otherwise stated such as in the startle threshold experiments). Carrier stimuli (CS) were broadband-noise generated by a second RP2.1 and presented continuously (unless otherwise noted) during testing. In the speaker swap experiment the broad-band noise was high-pass filtered (4 kHz cutoff) with a 100^{th} order FIR filter. Because the Morel MDT-20 loudspeakers begin to roll off at 20 kHz the effective bandwidth of the noise stimuli was ~4-20 kHz. The CS was presented from one speaker at a time and had the ability to be switched by two sets of TDT PM2Relay power multiplexers controlled by the RP2.1s. Attenuation of the signals and startle stimuli was achieved using TDT PA5 programmable attenuators. Vertical movement of the polycarbonate plate on which the accelerometer was mounted was detected as the voltage output, sampled at 1kHz by one of the RP2.1s. Startle response amplitude was calculated as the root mean square (RMS) of the accelerometer output in the first 100ms after the delivery of the startle stimuli, and startle latency was calculated as the delay between the startle stimulus onset and the time at which the accelerometer output exceeded three standard deviations of the 100 ms immediately preceding the startle stimulus presentation.

Experimental Conditions

Four types of experiment were conducted on most of the animals, startle threshold, gap detection, speaker swap, and spatial release from masking (similar to experiments performed in (Allen and Ison, 2010; Greene et al., 2018). The first repetition within a condition was excluded from analysis and all conditions were presented at least four times per experiment with most experiments containing six or more trials per condition. Most of the animals were tested once in each experiment; however, some mice were not subjected to acoustic startle threshold testing to reduce overall data collection time. The order of each of the four types of experiments was pseudo-randomized for each animal and each animal was only tested once per experiment (startle, gap, etc.). Total time of testing was around three hours per animal, and all conducted on the same day. The inter-trial interval (ITI time between trials) was uniformly distributed between 15-25 s in 1 s increments to prevent the animal from acclimating to the time of startle. For all experiments excluding the startle threshold, the startle stimulus was presented at 110 dB SPL. Order of prepulse conditions were pseudo randomly presented for each experiment.

Experiment 1: Startle Threshold

Startle threshold was assessed by varying the intensity (for most animals between 60-120dB SPL in 10dB steps) of the startle eliciting stimulus, presented with the overhead startle speaker and recording their acoustic startle response (ASR) through the cage-mounted accelerometer. Startle responses were assessed in the presence of a 70dB SPL background noise played continuously from the speaker directly in front of the animal (0°). Presentation of these conditions were limited to three to five repetitions to ensure that the animal had a robust startle response while minimizing the duration of testing.

Experiment 2: Gap Detection

The ability of animals to detect a short quiet period in a continuously noisy background was similarly assessed by presenting a broadband noise from the speaker directly in front of the animal (0°). A 20 ms gap in the noise (the pre-pulse) was introduced before the startle eliciting stimulus with interstimulus-intervals (ISI, time between the stimulus (gap) and startle eliciting stimulus)(Figure 3A) of 1, 2, 5, 10, 20, 40, 80, 160 and 240 ms from the onset of the gap. A subset of animals were only tested with 10, 20, 40, 80, 160 and 240 ms ISIs. Responses were assessed for ten repetitions of each ISI, presented pseudo randomly in a block-wise fashion. Two control condition trials, consisting of the continuous broadband noise with no gap preceding the startle-eliciting stimulus, were included in each repetition block.

Experiment 3a: Fixed 90° angle speaker swap with variable inter-stimulus interval (ISI)

The optimal ISI for speaker swap detection was assessed by swapping the source speaker (the prepulse) of a continuous broadband noise (70 dB SPL) 90° symmetrically across the midline (Figure 4A). The background noise was initially played from the speaker -45° (right) with respect to the animal and swapped with to the speaker +45° (left) of the animal some ISI prior to the startle-eliciting stimulus. Startle responses were assessed for five repetitions of 1, 2, 5, 10, 20, 30, 40, 80, 100, 150, and 300 ms ISIs, presented randomized in a blockwise fashion. Two control conditions, where no speaker swap occurred (i.e. the noise was continuously played from the initial speaker at -45°), were included in each repetition block. The ISIs for gap and speaker swap detection may be different as well as number of repetitions needed and therefore different ISIs and number of repetitions were presented for experiments 2 and 3a.

Experiment 3b: Variable angle speaker swap with 20 ms fixed ISI

Minimum audible angle was similarly assessed using a speaker swap paradigm. The animal orientation was maintained at 0° (center) as described above to test responses to sounds swapped across the midline and assess minimum audible angle detection ability. The prepulse was a change in the source of a high-pass noise (cut off below 4kHz) between two matched speakers separated by 7.5°, 15°, 30°, 45°, and 90° symmetrically (except for 7.5°) across the midline, in both directions (left to right and right to left). ISI was set at 20 ms between presentation of the prepulse (speaker swap) and startle-eliciting stimulus. The ISI was set in this experiment to reduce the length of the overall experimentation time and based on the results from experiments 3a and 2 showing that an ISI of 20ms is optimal for eliciting PPI. Startle responses were assessed for eight presentations of each condition (N = 10 since swap angle and direction co-vary), and one control condition (the high pass noise presented from the starting speaker, but no swap to the matched speaker) for each starting speaker (N=10), were presented randomized within repetition blocks.

Experiment 4a: Detection threshold for Spatial Release from Masking

The detection threshold of the signal used in a spatial release from masking (SRM), i.e. the ability of mice to detect a signal in a continuous 70 dB SPL broadband masking noise, presented from the center speaker (0°), was assessed by varying the intensity of the "signal" speaker presented adjacent to the center speaker (7.5°, SRM threshold, Figure 6A). The ISI was set at 20 ms from the onset of the prepulse to the startle eliciting stimulus. The "signal" was a 100 ms duration multi-tone complex with a 4kHz fundamental frequency and overtones at octave

spacing up to 32kHz (4 octaves). The intensity of the signal was varied by decreasing the signal level by 9, 12, 15, 18, 21, 24, and 27 dB attenuation relative to the full scale (~83.5 dB SPL), with a TDT PA5 programmable attenuator. Two control conditions (in which the masking noise was presented continuously with no "signal" prepulse presented) were included in each of five randomized trial blocks.

Experiment 4b: Speaker Swap Spatial Release from Masking

SRM was assessed by varying the location of the signal speaker at two levels determined based on preliminary results from the detection threshold task. In this task, the same "signal" as in Experiment 4a was presented at 15 or 24 dB attenuation (from ~83.5 dB SPL, Figure 7A), from speakers at 7.5°, 15°, 30°, 45°, and 90° (to the left or right) relative to center (0°) at a constant ISI of 20 ms. Two control conditions (in which the masking noise was presented continuously with no "signal" prepulse presented) were once again included in each randomized repetition block (of five).

Data Analysis

The ASR was assessed as the RMS output of the accelerometer, amplified by 25dB in the 100 ms following the startle eliciting stimulus presentation. The units of the ASR are reported as arbitrary voltage units that are proportional to meters/second since the output of the accelerometer was not explicitly calibrated (though it was held constant throughout data collection). The mean ASR was calculated for each animal, with the first presentation of each condition excluded to exclude initial adaptation to the startle. Most responses were quantified as prepulse inhibition (PPI), calculated as 1 minus the ratio of the mean prepulse ASR during each prepulse condition (ASR_p) to the mean ASR during the corresponding control condition(s) (ASR_c), recorded for each session: $PPI = 1 - [ASR_P/ASR_c]$. A PPI of 0 corresponds to an ASR_p equal to ASR_c suggesting no detection of the prepulse, whereas both positive PPI, indicating a reduction in ASR, and negative PPI, indicating an increase in ASR, suggest that the prepulse was detected and modified the animal's startle response. Figures were generated in R (R Core Team, 2013) using ggplot2 (Wickham, 2016). Data were analyzed using a mixed effects model to account for repeat observations within one animal (lme4 (Bates et al., 2014)) with genotype and conditions (dB SPL, ISI, angle, ABR threshold, etc.) as fixed effects, and animal as a random effect. It was expected that there would be no differences between some conditions where the prepulse was not detectable. Therefore apriori, independent of results from fixed effects (i.e. no difference in main effect of genotype), it was determined that estimated marginal means (*emmeans*, (Lenth, 2019)) were going to be used to make pairwise comparisons between genotype and condition or replicate and condition. A Tukey method for multiple comparisons was implemented for these contrasts using *emmeans*. A zero-intercept model was used to compare genotypes to a PPI value of zero (to determine detection of the sound prepulse). T-tests used Satterthwaite's method for comparing the multiple levels of ISI across genotype (Kuznetsova et al., 2017). Animal weight and age were compared between the two genotypes using a two-tailed t-test and data are presented as mean ± standard error. Tables 1-3 show mean, standard error, median, interquartile range (IQR) and p-values for each experiment. Red values in Table 2 indicate conditions where PPI was greater than zero. Where values are indicated as statistically significant between the two genotypes, * indicated a p-value of < 0.05, ** = $p < 0.01$, and *** = $p < 0.0001$. Figures were prepared for publication using Adobe Photoshop and Adobe Illustrator (Adobe, San Jose, CA).

Results

ABR audiogram

Hearing range for *Fmr1* and B6 mice was determined through presenting tones of varying frequency (4, 8, 16 kHz) and recording the threshold of the auditory brainstem response (ABR). These frequencies span the bandwidth of the noise stimuli used for behavior. There was a main effect of genotype ($p = 0.043$) indicating that the ABR thresholds were different between *Fmr1* and B6. Posthoc analysis showed that the two genotypes were no different for 4 and 8 kHz but that *Fmr1* mice had increased ABR thresholds at 16 kHz compared to wildtype (Figure 1, Table 1).

Experiment 1: Startle Threshold

Animals were initially tested to determine their response threshold to acoustic startle stimuli. This was done both to characterize the responses of *Fmr1* mice and ensure that all animals had a robust startle response with increasing intensity of sound. Startle amplitude is reported in units of arbitrary volts (output of the accelerometer) that are an uncalibrated measure proportional to acceleration. Startle sounds were 20 ms in duration and varied in intensity between 60 and 120 dB SPL presented randomly in 10 dB SPL steps, in the presence of continuous 70dB SPL broadband noise (presented from the speaker directly in front of the animal) (Figure 2A). All animals were tested with intensities ranging from 80-120 dB SPL. Two additional levels (60 and 70 dB SPL) were tested in a subset of animals (6 B6, 8 *Fmr1*) to ensure that animals are not startled at lower intensity sounds. There was no difference between genotypes for either startle amplitude (Figure 2B, $p = 0.4074$) or latency (Figure 2D, $p = 0.8331$). Startle responses increased and latency decreased with increasing stimulus level in both genotypes, indicating that animals had no trouble detecting the startle stimulus and had a robust startle response. The magnitude of the startle responses for both B6 and *Fmr1* animals plateaued (threshold) around 100dB SPL and therefore the startle stimulus was set at 10 dB above this threshold (110 dB SPL) for the remainder of the experiments.

When individual animal weights were used to calculate the force of each startle response (in arbitrary units proportional to Newtons), significant differences were observed between genotypes at 100 ($p = 0.0321$) and 110 ($p = 0.0224$) dB SPL with *Fmr1* mice showing reduced startle force compared to wildtype (Figure 2C). This could be due to a reduced muscle tone in *Fmr1* animals or some other factor, neither of which are explored further in this study. To account for differences in animal weight and reduced startle force, responses are normalized to the baseline startle amplitude when calculating PPI (see methods).

Experiment 2: Gap Detection

Recent data has shown that impairments in gap detection may be caused by underlying changes to excitation-inhibition balance (E/I) in the inferior colliculus (IC) (Sturm et al., 2017), suggesting that gap detection may be used to probe the E/I balance in the auditory system. This E/I balance is known to be altered in FXS (Garcia-Pino et al., 2017; McCullagh et al., 2017; Rotschafer et al., 2015). We tested 42 animals (20 B6 and 22 *Fmr1*) in a gap detection paradigm with a 20 ms quiet gap in broad band noise (prepulse) followed by a startle eliciting stimulus at varying inter-stimulus interval (ISI) times (1-240 ms) between the prepulse and startle eliciting stimuli (Figure 3A, B). A subset of animals were only tested at 10-240 ms ISIs (8 B6, 8 *Fmr1*). There was no main effect of genotype ($p = 0.2011$) at all durations of ISI, however there were

significant differences between B6 and *Fmr1* animals at 10 ms ($p < 0.05$) and 20 ms ($p < 0.01$) ISIs (Figure 3B). In addition, *Fmr1* mice were slower to startle at all ISIs (as indicated by increased startle latency, (main effect of genotype, $p = 0.00000255$)), except 10 ms and 20 ms, compared to B6 mice (Figure 3C). Both genotypes did not show PPI significantly different from zero for ISI of less than 1 ms ($p = 0.45$ B6 and $p = 0.91$ *Fmr1*), and additionally at 2 ms ($p = 0.14$) for *Fmr1* mice suggesting a lack of detection of the prepulse with these short ISIs. These data suggest that not only do *Fmr1* mice have decreased PPI compared to wildtype at optimal ISIs but are also consistently slow to startle under most conditions, where in contrast, B6 mice show modulations to latency based on ISI.

Experiment 3a: Varying ISI with 90° Speaker Swap

The gap detection test suggests that *Fmr1* mice demonstrated deficits in temporal auditory processing, next we wanted to determine if *Fmr1* mice also have deficits in spatial auditory processing. The first step to determining whether *Fmr1* mice have spatial hearing deficits is to establish the optimal ISI for detection of a spatial speaker swap. In this task, the prepulse was a speaker swap of broadband noise from one speaker 45° to the right of the animal to the symmetrical speaker 45° to the left of the animal (90° total angle), with varying ISIs between the prepulse and startle eliciting stimuli (Figure 4A). There was no main effect of ISI on genotype ($p = 0.1068$). However, *Fmr1* mice had reduced PPI of their startle compared to B6 mice at 20 ms and 30 ms ($p < 0.05$) ISI after the 90° speaker swap (12 B6, 14 *Fmr1* mice, Figure 4B). In addition, *Fmr1* mice showed an increased latency to startle compared to B6 at all ISIs except 1 ms and 2 ms (Figure 4C, main effect $p = 0.0000007913$). These data suggest that at ISIs that elicited some of the highest PPI for B6 animals (also similar ISIs that showed a deficit in the gap detection test), *Fmr1* mice showed reduced PPI compared to wildtype. Neither genotype demonstrated PPI significantly different than zero for ISIs less than or equal to 5 ms (1, 2, or 5 ms ISIs), suggesting lack of detection at these ISIs (B6: 1 ms $p = 0.50$, 2 ms $p = 0.51$, 5 ms $p = 0.07$; *Fmr1*: 1ms $p = 0.13$, 2 ms $p = 0.50$, 5 ms $p = 0.27$). *Fmr1* animals did not have PPI significantly different from zero for ISIs of 10 ($p = 0.079$), 20 ($p = 0.05$), 30 ($p = 0.068$), 40 ($p = 0.06$), and 300 ($p = 0.059$) ms also suggesting potential impaired detection at these ISIs. In addition, *Fmr1* mice showed increased latencies to startle at ISIs that elicited a PPI above zero, suggesting that addition of a detectable prepulse actually slowed responses in comparison to B6 mice. Based on the results of this task and the gap detection, it was determined that the optimal ISI for spatial tasks for B6 mice is 20 ms consistent with other studies (Allen and Ison, 2010).

Experiment 3b: Minimum Audible Angle Detection with a fixed ISI

To determine whether *Fmr1* mice have impairments in spatial acuity, we measured and compared minimum audible angle detection for *Fmr1* and wildtype mice. The minimum audible angle is defined as the smallest change in speaker source location that the animals could just detect via the PPI metric (PPI significantly different than zero). In this task, the angle of the speaker swap across the midline was varied as the prepulse to the startle, with a constant ISI of 20 ms as established by the previous experiments. 25 mice (11 B6 and 14 *Fmr1*) were tested with angle swaps (to the left and right of the animal) of 7.5, 15, 30, 45 and 90° across the midline (i.e. with the animal oriented towards 0°; Figure 5A). Data were comparable for left to right and right to left directional swaps, therefore the data were pooled for both directions (Figure 5B, C). There was no main effect of genotype in this task ($p = 0.7255$). *Fmr1* mice showed less PPI than B6 mice only at the 90° angle speaker swap suggesting minimum audible detection was comparable

in the two groups (Figure 5B). Neither genotype showed PPI significantly different than zero for angles of 30° or lower (except for at 15° where *Fmr1* mice show PPI > 0, p = 0.026) suggesting that at these angles the animals could not detect the speaker swap, and that the animal's minimum audible angles were < ~45° (B6: 7.5° p = 0.75, 15° p = 0.19, 30° p = 0.89; *Fmr1*: 7.5° p = 0.15, 30° p = 0.41). These data suggest that both genotypes have poor angular discrimination abilities as assessed in this task; however, *Fmr1* mice had longer latencies to startle at all angles compared to B6 mice (Figure 4C, main effect p < 0.0001), consistent with results in the previous varying ISI experiment.

Experiment 4a: Auditory Spatial Release from Masking Threshold- Signal Detection in Noise

Listening to sounds in a complex auditory environment with competing sound sources (spatial release from masking) more naturally replicates real-world listening environments, in which both people and mice with FXS experience difficulties. We used a PPI-based task to replicate this experience, and to determine whether *Fmr1* mice have impairments in spatial release from masking (SRM). First, we determined the signal attenuation required for animals to no longer be able to distinguish signals from the background. Signal detection thresholds were measured in 32 animals (16 B6 and 16 *Fmr1*) placed in the chamber with a 70 dB SPL masker sound presented from the 0° speaker and a the prepulse cue centered around 4 kHz was played at varying attenuated levels (from ~83.5 dB SPL) from the adjacent speakers (7.5° to the left or right; Figure 6A). A tone-based sound was chosen because it is audible to the animal while not eliciting a social or emotional response (such as vocalization sounds etc.) that might elicit an unexpected behavioral response. In this task, there was no main effect of genotype (p = 0.2786). At the loudest levels (9-15 dB attenuation) the 4 kHz sound elicited a robust PPI in both genotypes, although *Fmr1* mice showed less PPI of their startle response compared to B6 mice (Figure 6B). Both genotypes did not have PPI greater than zero at 27 dB attenuation (B6: p = 0.31, *Fmr1*: p = 0.22) and B6 animals also at 24 dB attenuation (p = 0.22), suggesting that mice did not detect the prepulse at these levels, and that their detection threshold was < 21 - 24 dB attenuation. Similar to latencies in the gap detection task, B6 mice showed modulation of their latency based on condition, whereas *Fmr1* mice had consistent slower latencies across conditions (Figure 6C, main effect genotype p = 0.001189).

Experiment 4b: Spatial Release from Masking Varying Angle

Next, in order to determine if *Fmr1* mice had impairments in their spatial release from masking, the angular separation between masker and prepulse signal were varied. In this task, 31 animals (15 B6 and 16 *Fmr1*) were presented with the same 4 kHz signal (prepulse), at 2 attenuation levels (15 and 24 dB attenuation), at varying angles relative to a masker noise presented from the 0° speaker (Figure 7A). There was no main effect of genotype at either 15 dB attenuation (p = 0.1267) or 24 dB attenuation (p = 0.6325). Consistent with the speaker swap task, *Fmr1* mice only showed a difference in PPI relative to B6 mice at the largest angle (90°), and only at the louder (15 dB attenuation) level (p < 0.05, Figure 7B, C). Both *Fmr1* and B6 mice showed PPI greater than zero at all angles for the louder signal (15 dB attenuation, p>0.05), suggesting the signal was readily detected above the masker at all angles, and only showed PPI greater than zero for the largest angle (90°) for the quieter signal (24 dB attenuation)(p < 0.05 at 90° and p > 0.05 for all other angles for both genotypes). Similar to the above experiments, *Fmr1* mice had longer latencies to startle compared to B6 mice at several angles (7.5 and 30° at 15 dB attenuation and 7.5, 22.5, 30, 45° at 24 dB attenuation Figure 7D, E, main effect genotype p =

0.0001). These data do not indicate substantial deficits in SRM; however, the longer latencies seen here, and in other experiments, suggests altered timing of startle responses in *Fmr1* mice compared to wildtype.

Habituation of the Startle Response

Previous studies have shown that *Fmr1* mice have impaired habituation (decreased startle response or PPI in later presentations of a replicate) during PPI tasks (Nielsen et al., 2002). Therefore, we used the replicate number per condition (ISI, dB, etc.) as the main effect variable per genotype. In contrast to previous studies, we found no habituation in either the B6 or *Fmr1* knockout mice in any of the experiments tested (startle threshold, gap detection, speaker swaps, or spatial release from masking)(Nielsen et al., 2002). This was indicated as no change in startle amplitude or PPI as a result of replicate, in particular the second replicate compared to the last replicate per condition block ($p > 0.05$ in all tests).

Discussion

We characterize the binaural and spatial hearing ability of *Fmr1* mice using a reflexive PPI task. In addition, we measured the audiogram of mice using the auditory brainstem response (ABR). Surprisingly, *Fmr1* mice appear to show spatial hearing ability comparable to wildtype, with only subtle differences noted in some prepulse conditions. *Fmr1* mice showed similar "detection" ability (PPI greater than zero) of prepulses under most conditions compared to wildtype, except for varying ISI with 90° swap. In contrast, *Fmr1* mice had increased latency to startle in all experiments except when determining the startle threshold. These data suggest that perhaps *Fmr1* mice do not have a severe spatial hearing deficit but do show impairments in timing of responses. Lastly, we did not see any short-term habituation response within experiments.

Increased high frequency ABR thresholds

Fmr1 mice had somewhat increased ABR thresholds at 16 kHz compared to B6 indicating potential high-frequency hearing loss. Previous studies examining ABR in *Fmr1* mice, saw no change in latency of the ABR waveforms and a reduction in wave I amplitude (El-Hassar et al., 2019; Rotschafer et al., 2015). One study saw an increase in ABR threshold (though across all frequencies) in *Fmr1* mice (Rotschafer et al., 2015), while the other saw no change in ABR threshold based on frequency (El-Hassar et al., 2019). However, both of these previous ABR studies were performed in the *Fmr1* FVB knockout strain, which is one potential reason to explain the difference in results with our study. In addition, the mice used in our study were slightly older, average age of 125 days old for both genotypes, though we did not see any obvious age-related hearing loss in either genotype (as indicated by reduced thresholds overall across frequencies). Lastly, these ABR experiments were performed independently of the PPI experiments described elsewhere in this study, therefore it is difficult to know if the results found here are directly related to changes we see in PPI.

No change in overall startle threshold

Our results indicate that there is no difference in the overall acoustic startle response between *Fmr1* mice and B6 mice. However, startle force, accounting for the weight of the mice, did show differences at 100 and 110 dB SPL suggesting that *Fmr1* mice startle with less force. Accounting for mass of an animal could help interpretation of data across experimental methods

and apparatuses (Grimsley et al., 2015). None of the previous studies account for weight or force of the animal and the results in *Fmr1* mice are equivocal, where some studies show an increase in the ASR (Arsenault et al., 2016; Nielsen et al., 2002), while others show a decrease in the ASR (Baker et al., 2010; Chen and Toth, 2001; Frankland et al., 2004; Paylor et al., 2008; Spencer et al., 2006; Thomas et al., 2012; Veeraragavan et al., 2012; Yun et al., 2006; and Nielsen et al., 2002), and others show no change in overall ASR (Ding et al., 2014) consistent with our study. The cause of these discrepancies is not clear; however, one possible explanation is the use of different mouse strains between previous studies (Bullock et al., 1997). Additional differences across reports include experimental set up and method of measuring the ASR.

Studies comparing patients with FXS and neurotypical human subjects did not find any differences in startle magnitude (Hessl et al., 2009; Frankland et al., 2004) consistent with our results. Lastly, studies have shown that the ASR is related directly to FMRP expression (Yun et al., 2006) and can be rescued with addition of the *Fmr1* gene (Paylor et al., 2008) suggesting that some aspects of the ASR are directly related to loss of FMRP.

Fmr1 mice show decreased PPI during 10 and 20 ms gaps

Gap detection ability is thought to be directly related to E/I balance in the auditory system, particularly in the inferior colliculus (IC, Sturm et al., 2017). E/I imbalances have been found in *Fmr1* mice in the auditory brainstem, particularly the medial nucleus of the trapezoid body (MNTB, McCullagh et al., 2017; Rotschafer et al., 2015) and lateral superior olive (LSO, Garcia-Pino et al., 2017). These areas also contribute to the PPI and ASR pathways as they convey sound location information to higher areas such as the IC (Koch, 1999). Our data show that *Fmr1* mice show lower PPI at short gap lengths (10 and 20ms) suggesting that *Fmr1* mice have impairments in their inhibition of the startle response. However, gap detection is dependent on high-frequency hearing ability, and as indicated by the ABR audiogram, *Fmr1* mice have higher ABR thresholds at 16 kHz compared to B6 (Fitzgibbons and Gordon-Salant, 1987). High frequency hearing difficulties could help explain some of the deficits that we see in the *Fmr1* knockout mice. In addition, our study examined latency to startle, and interestingly, *Fmr1* mice did not show the reduction in startle latency at gap ISIs longer than 20ms observed in wildtype mice.

Fmr1 mice show decreased PPI to 90° speaker swap

This is the first study to measure the minimum audible angle detection of *Fmr1* mice. *Fmr1* mice showed less PPI at any ISI with a 90° speaker swap indicating that they had overall lower magnitude startle responses even at such a large angle than wildtype (only significantly different from wildtype at 20 and 30 ms). In addition, when we kept the ISI at 20 ms and varied the angle, *Fmr1* mice again showed lower PPI values at 90° speaker swaps compared to B6 mice. There was no difference in pinna morphology, which is one possible explanation for the differences seen in 90° speaker swaps. However, both genotypes showed low PPI values for other speaker angles suggesting that these mice exhibit poor minimum audible angle ability. Other studies have shown higher PPI values for mice at smaller angle swaps than we report, however differences in background strain (CBA/CaJ and CBA/129) and stimuli presented (wide-band noise vs. high-pass noise (> 4 kHz)) may explain the differing results (Allen and Ison, 2010; Lauer et al., 2011). Moreover, the upper frequency of noise we used was ~20 kHz. In addition, consistent with other experiments reported here, the *Fmr1* mice responded with longer latencies to startle at almost all angles compared to B6. This indicates that not only do *Fmr1*

mice have difficulties in inhibiting their startle response, *Fmr1* mice may have longer processing speeds than B6 mice.

Fmr1 mice show alterations to spatial release from masking

Spatial release from masking involves detecting a signal in a noisy background. We show that even at loud signals compared to background, *Fmr1* mice show less PPI of their startle response than B6. In addition, when varying the location of the signal, at the louder sound, mice again had deficits at 90°, but this time off to the side of the animal. Lastly, similar to the speaker swap experiments, *Fmr1* animals also had longer latencies under most conditions to respond to the startle speaker compared to B6, suggesting again not only impairments in detection, but also reaction ability/time.

Other studies have examined PPI while varying the intensity of a prepulse signal above an ambient noise level. While not exactly the same as the SRM task discussed here, in contrast to our results, most studies found that *Fmr1* mice had increased PPI compared to wildtype (Baker et al., 2010; Chen and Toth, 2001; Frankland et al., 2004; Nielsen et al., 2002; Paylor et al., 2008; Veeraragavan et al., 2012), however see also (Spencer et al., 2006; Thomas et al., 2012). These discrepancies could be due to the prepulse eliciting a startle response in these other studies, which would cause increased PPI during the actual startle, and in particular since these studies did not explore latency to startle, the prepulse startle response could be delayed coinciding with the startle-eliciting speaker. Lastly, most of the other studies do not discuss where the signal is coming from, which could impact the inhibition of the startle response in these animals and is likely different from our experiments. Interestingly, our results are consistent with data from patients with FXS who show reduced PPI under similar conditions (Frankland et al., 2004; Hessl et al., 2009). Consistency with human data implies that our assay may be a better measure of PPI that could apply to drug rescue experiments and be more applicable to the human FXS condition.

Mice in these experiments did not habituate

Often animals habituate to the startle stimulus, meaning that as the animal continues to be exposed to a loud sound stimulus, they will no longer startle as robustly as the earlier presentations of the stimulus. Habituation can also limit the length of experiments since animals may not respond as robustly after several hours of testing. Interestingly, we did not see any habituation to the startle in either *Fmr1* or B6 animals, as seen by a change in PPI or startle amplitude between early and later presentations of the same stimulus for any of the tasks presented. Other studies have examined habituation and shown that *Fmr1* mice do not habituate, though their results were not consistent between an F1 cross of genotypes and *Fmr1* mice on a B6 background suggesting that their results might be a result of background genotype (Nielsen et al., 2002). Mice typically show less habituation than other animals, responding robustly and consistently to many stimulus presentations, and habituation can be extinguished with a few minutes rest between experiments (Valsamis and Schmid, 2011). Our results suggest also that mice can tolerate several hours (we kept total testing time under three hours) of testing without a concern for habituation to the startle response, in particular in the B6 background strain tested here.

Latency versus Startle

In contrast to PPI and ASR responses that differed between *Fmr1* and B6 mice, where we saw specific impairments under certain conditions, there was an overall trend for *Fmr1* mice to

have increased latency to startle under a variety of conditions. This could be due to impairments in a different circuit that causes the response to the startle, i.e. when versus how much to startle in *Fmr1* mice. There has been one study which examined latency to react in patients with FXS after an acoustic startle, and they saw no differences between neurotypical controls and FXS patients (Roberts et al., 2013). This study, however, did not use PPI as a measure or look at EMG responses to the acoustic startle. None of the other studies examining PPI and ASR in *Fmr1* mice or humans examined the latency to respond making it difficult to know if our results are comparable. More in depth gap detection experiments and a more thorough ABR characterization could shed light on the latency changes seen here in this study, though Rotschafer et al 2015 did not see any changes to latency of the ABR (however in a different background strain- FVB). In addition, the circuit underlying latency to respond to ASR or PPI is not well understood and is an interesting area for further exploration.

Conclusions

Several recent studies described anatomical alterations in the sound localization pathway of *Fmr1* mice which lead to altered physiological properties (Brown et al., 2010; Curry et al., 2018; El-Hassar et al., 2019; Garcia-Pino et al., 2017; Lu, 2019; McCullagh et al., 2017; Rotschafer et al., 2015; Rotschafer and Cramer, 2017; Strumbos et al., 2010). These alterations include differences in synaptic strength and connectivity, differences in postsynaptic ion channels, postsynaptic input resistance, altered firing properties, alterations in action potential shape, and differences in macroscopic physiological properties such as auditory brain stem responses (ABRs). In a circuit in which amplitude, kinetics and timing of excitation and inhibition are balanced very precisely to perform sound localization, these alterations should have dramatic effects on the animal's localization ability. Surprisingly, the observed effects were smaller than expected and do not support the view of a degraded localization circuit "across the board". However, our results are consistent with observations from human FXS patients, suggesting that the *Fmr1* mouse model can recapitulate the human FXS condition well, at least as far as the sound localization circuit is concerned.

References

Allen PD, Ison JR (2010) Sensitivity of the mouse to changes in azimuthal sound location: Angular separation, spectral composition, and sound level. Behav Neurosci 124:265–277.

Anbuhl KL, Benichoux V, Greene NT, Brown AD, Tollin DJ (2017) Development of the head, pinnae, and acoustical cues to sound location in a precocial species, the guinea pig (Cavia porcellus). Hear Res 356:35–50.

Arsenault J, Gholizadeh S, Niibori Y, Pacey LK, Halder SK, Koxhioni E, Konno A, Hirai H, Hampson DR (2016) FMRP Expression Levels in Mouse Central Nervous System Neurons Determine Behavioral Phenotype. Hum Gene Ther 27:982–996.

Baker KB, Wray SP, Ritter R, Mason S, Lanthorn TH, Savelieva KV (2010) Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments. Genes Brain Behav 9:562–574.

Baranek GT, Chin YH, Hess LMG, Yankee JG, Hatton DD, Hooper SR (2002) Sensory processing correlates of occupational performance in children with fragile X syndrome: preliminary findings. Am J Occup Ther Off Publ Am Occup Ther Assoc 56:538–546.

643 Baranek GT, Roberts JE, David FJ, Sideris J, Mirrett PL, Hatton DD, Bailey DBJ (2008)
644 Developmental trajectories and correlates of sensory processing in young boys with
645 fragile X syndrome. Phys Occup Ther Pediatr 28:79–98.
646 Bates D, Mächler M, Bolker B, Walker S (2014) Fitting Linear Mixed-Effects Models using
647 lme4. arXiv.org.
648 Benichoux V, Ferber A, Hunt S, Hughes E, Tollin D (2018) Across Species "Natural Ablation"
649 Reveals the Brainstem Source of a Noninvasive Biomarker of Binaural Hearing. J
650 Neurosci 38:8563–8573.
651 Beutelmann R, Laumen G, Tollin D, Klump GM (2015) Amplitude and phase equalization of
652 stimuli for click evoked auditory brainstem responses. J Acoust Soc Am 137:EL71–
653 EL77.
654 Bronkhorst AW (2015) The cocktail-party problem revisited: early processing and selection of
655 multi-talker speech. Atten Percept Psychophys 77:1465–1487.
656 Brown MR, Kronengold J, Gazula V-R, Chen Y, Strumbos JG, Sigworth FJ, Navaratnam D,
657 Kaczmarek LK (2010) Fragile X mental retardation protein controls gating of the
658 sodium-activated potassium channel Slack. Nat Neurosci 13:819–821.
659 Bullock AE, Slobe BS, Vizquez V, Collins AC (n.d.) Inbred Mouse Strains Differ in the
660 Regulation of Startle and Prepulse Inhibition of the Startle Response 8.
661 Caird D, Klinke R (1983) Processing of binaural stimuli by cat superior olivary complex
662 neurons. Exp Brain Res 52:385–399.
663 Chen L, Toth M (2001) Fragile X mice develop sensory hyperreactivity to auditory stimuli.
664 Neuroscience 103:1043–1050.
665 Cherry EC (1953) Some Experiments on the Recognition of Speech, with One and with Two
666 Ears. J Acoust Soc Am 25:975–979.
667 Consorthium TD-BFX et al. (1994) Fmr1 knockout mice: A model to study fragile X mental
668 retardation. Cell 78:23–33.
669 Contractor A, Klyachko VA, Portera-Cailliau C (2015) Altered Neuronal and Circuit Excitability
670 in Fragile X Syndrome. Neuron 87:699–715.
671 Curry RJ, Peng K, Lu Y (2018) Neurotransmitter- and Release-Mode-Specific Modulation of
672 Inhibitory Transmission by Group I Metabotropic Glutamate Receptors in Central
673 Auditory Neurons of the Mouse. J Neurosci 38:8187–8199.
674 Ding Q, Sethna F, Wang H (2014) Behavioral analysis of male and female Fmr1 knockout mice
675 on C57BL/6 background. Behav Brain Res 271:72–78.
676 El-Hassar L, Song L, Winston TJT, Large CH, Alvaro G, Santos-Sacchi J, Kaczmarek LK
677 (2019) Modulators of Kv3 potassium channels rescue the auditory function of Fragile X
678 mice. J Neurosci 0839–18.
679 Feng AS, Ratnam R (2000) Neural Basis of Hearing in Real-World Situations. Annu Rev
680 Psychol 51:699–725.
681 Ferber AT, Benichoux V, Tollin DJ (2016) Test-Retest Reliability of the Binaural Interaction
682 Component of the Auditory Brainstem Response. Ear Hear 37:e291-301.
683 Fitzgibbons PJ, Gordon-Salant S (1987) Temporal gap resolution in listeners with high-
684 frequency sensorineural hearing loss. J Acoust Soc Am 81:133–137.
685 Frankland PW, Wang Y, Rosner B, Shimizu T, Balleine BW, Dykens EM, Ornitz EM, Silva AJ
686 (2004) Sensorimotor gating abnormalities in young males with fragile X syndrome and
687 Fmr1-knockout mice. Mol Psychiatry 9:417–425.

688 Garcia-Pino E, Gessele N, Koch U (2017) Enhanced Excitatory Connectivity and Disturbed
689 Sound Processing in the Auditory Brainstem of Fragile X Mice. J Neurosci Off J Soc
690 Neurosci 37:7403–7419.
691 Goldberg JM, Brown PB (1969) Response of binaural neurons of dog superior olivary complex
692 to dichotic tonal stimuli: some physiological mechanisms of sound localization. J
693 Neurophysiol 32:613–636.
694 Greene NT, Anbuhl KL, Ferber AT, DeGuzman M, Allen PD, Tollin DJ (2018) Spatial hearing
695 ability of the pigmented Guinea pig (Cavia porcellus): Minimum audible angle and
696 spatial release from masking in azimuth. Hear Res 365:62–76.
697 Grimsley CA, Longenecker RJ, Rosen MJ, Young JW, Grimsley JM, Galazyuk AV (2015) An
698 improved approach to separating startle data from noise. J Neurosci Methods 253:206–
699 217.
700 Grothe B, Pecka M, McAlpine D (2010) Mechanisms of sound localization in mammals. Physiol
701 Rev 90:983–1012.
702 Hessl D, Berry-Kravis E, Cordeiro L, Yuhas J, Ornitz EM, Campbell A, Chruscinski E, Hervey
703 C, Long JM, Hagerman RJ (2009) Prepulse inhibition in fragile X syndrome: Feasibility,
704 reliability, and implications for treatment. Am J Med Genet B Neuropsychiatr Genet
705 150B:545–553.
706 Keine C, Rübsamen R, Englitz B (2016) Inhibition in the auditory brainstem enhances signal
707 representation and regulates gain in complex acoustic environments. eLife 5.
708 Koch M (1999) The neurobiology of startle. Prog Neurobiol 59:107–128.
709 Kuznetsova A, Brockhoff PB, Christensen RHB (2017) **lmerTest** Package: Tests in Linear
710 Mixed Effects Models. J Stat Softw 82.
711 Lauer AM, Slee SJ, May BJ (2011) Acoustic Basis of Directional Acuity in Laboratory Mice. J
712 Assoc Res Otolaryngol 12:633–645.
713 Lenth R (2019) emmeans: Estimated Marginal Means, aka Least-Squares Means.
714 Lu Y (2019) Subtle differences in synaptic transmission in medial nucleus of trapezoid body
715 neurons between wild-type and Fmr1 knockout mice. Brain Res 1717:95–103.
716 McCullagh EA, Salcedo E, Huntsman MM, Klug A (2017) Tonotopic alterations in inhibitory
717 input to the medial nucleus of the trapezoid body in a mouse model of Fragile X
718 syndrome. J Comp Neurol 262:375.
719 Moore MJ, Caspary DM (1983) Strychnine blocks binaural inhibition in lateral superior olivary
720 neurons. J Neurosci Off J Soc Neurosci 3:237–242.
721 Nielsen DM, Derber WJ, McClellan DA, Crnic LS (2002) Alterations in the auditory startle
722 response in Fmr1 targeted mutant mouse models of fragile X syndrome. Brain Res
723 927:8–17.
724 Paylor R, Yuva-Paylor LA, Nelson DL, Spencer CM (2008) Reversal of sensorimotor gating
725 abnormalities in Fmr1 knockout mice carrying a human Fmr1 transgene. Behav Neurosci
726 122:1371–1377.
727 Penagarikano O, Mulle JG, Warren ST (2007) The Pathophysiology of Fragile X Syndrome.
728 Annu Rev Genomics Hum Genet 8:109–129.
729 Pollak GD, Burger RM, Park TJ, Klug A, Bauer EE (2002) Roles of inhibition for transforming
730 binaural properties in the brainstem auditory system. Hear Res 168:60–78.
731 R Core Team (2013) R: A Language and Environment for Statistical Computing. Vienna,
732 Austria: R Foundation for Statistical Computing.

733 Roberts JE, Long ACJ, McCary LM, Quady AN, Rose BS, Widrick D, Baranek G (2013)
734 Cardiovascular and Behavioral Response to Auditory Stimuli in Boys With Fragile X
735 Syndrome. J Pediatr Psychol 38:276–284.
736 Rogers SJ, Hepburn S, Wehner E (2003) Parent reports of sensory symptoms in toddlers with
737 autism and those with other developmental disorders. J Autism Dev Disord 33:631–642.
738 Rotschafer SE, Cramer KS (2017) Developmental Emergence of Phenotypes in the Auditory
739 Brainstem Nuclei of Fmr1Knockout Mice. eNeuro 4:1–21.
740 Rotschafer SE, Marshak S, Cramer KS (2015) Deletion of Fmr1 Alters Function and Synaptic
741 Inputs in the Auditory Brainstem. PLoS One 10:1–15.
742 Rotschafer SE, Razak KA (2014) Auditory processing in fragile x syndrome. Front Cell
743 Neurosci 8:19.
744 Ruby K, Falvey K, Kulesza RJ (2015) Abnormal neuronal morphology and neurochemistry in
745 the auditory brainstem of Fmr1 knockout rats. Neuroscience 303:285–298.
746 Spencer CM, Serysheva E, Yuva-Paylor LA, Oostra BA, Nelson DL, Paylor R (2006)
747 Exaggerated behavioral phenotypes in Fmr1/Fxr2 double knockout mice reveal a
748 functional genetic interaction between Fragile X-related proteins. Hum Mol Genet
749 15:1984–1994.
750 Strumbos JG, Brown MR, Kronengold J, Polley DB, Kaczmarek LK (2010) Fragile X Mental
751 Retardation Protein Is Required for Rapid Experience-Dependent Regulation of the
752 Potassium Channel Kv3.1b. J Neurosci 30:10263–10271.
753 Sturm JJ, Zhang-Hooks Y-X, Roos H, Nguyen T, Kandler K (2017) Noise Trauma-Induced
754 Behavioral Gap Detection Deficits Correlate with Reorganization of Excitatory and
755 Inhibitory Local Circuits in the Inferior Colliculus and Are Prevented by Acoustic
756 Enrichment. J Neurosci 37:6314–6330.
757 Thomas AM, Bui N, Perkins JR, Yuva-Paylor LA, Paylor R (2012) Group I metabotropic
758 glutamate receptor antagonists alter select behaviors in a mouse model for fragile X
759 syndrome. Psychopharmacology (Berl) 219:47–58.
760 Thompson AM, Schofield BR (2000) Afferent projections of the superior olivary complex.
761 Microsc Res Tech 51:330–354.
762 Valsamis B, Schmid S (2011) Habituation and Prepulse Inhibition of Acoustic Startle in
763 Rodents. J Vis Exp.
764 Veeraragavan S, Graham D, Bui N, Yuva-Paylor LA, Wess J, Paylor R (2012) Genetic reduction
765 of muscarinic M4 receptor modulates analgesic response and acoustic startle response in
766 a mouse model of fragile X syndrome (FXS). Behav Brain Res 228:1–8.
767 Wang T, de Kok L, Willemsen R, Elgersma Y, Borst JGG (2015) In vivo synaptic transmission
768 and morphology in mouse models of Tuberous sclerosis, Fragile X syndrome,
769 Neurofibromatosis type 1, and Costello syndrome. Front Cell Neurosci 9:234.
770 Wang Y, Sakano H, Beebe K, Brown MR, de Laat R, Bothwell M, Kulesza RJ, Rubel EW
771 (2014) Intense and specialized dendritic localization of the fragile X mental retardation
772 protein in binaural brainstem neurons: A comparative study in the alligator, chicken,
773 gerbil, and human: FMRP localization in NL/MSO dendrites. J Comp Neurol 522:2107–
774 2128.
775 Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.
776 Young JS, Fechter LD (1983) Reflex inhibition procedures for animal audiometry: a technique
777 for assessing ototoxicity. J Acoust Soc Am 73:1686–1693.

Yun S-W, Platholi J, Flaherty MS, Fu W, Kottmann AH, Toth M (2006) Fmrp is required for the establishment of the startle response during the critical period of auditory development. Brain Res 1110:159–165.

Zorio DAR, Jackson CM, Liu Y, Rubel EW, Wang Y (2017) Cellular distribution of the fragile X mental retardation protein in the mouse brain: Fmrp distribution in the mouse brain. J Comp Neurol 525:818–849.

Fig 1. Increased auditory ABR threshold in *Fmr1* mice at 16kHz compared to B6 mice. Tonal ABR measurements were made on mice at 3 frequencies, 4000, 8000, and 16000Hz. There were no differences in ABR auditory thresholds at 4000 and 8000Hz between *Fmr1* and B6 mice. However, at 16kHz *Fmr1* mice had increased thresholds compared to B6. ** p<0.01

Fig 2. No difference in startle amplitude or latency between B6 and *Fmr1* mice, decreased force in *Fmr1* mice. A, illustration of the experimental setup. Animals were placed in the center of the speaker array in the presence of 70dB SPL broadband noise playing from the central speaker at 0° (blue speaker). Startle eliciting stimuli were presented by an additional speaker placed directly over the mouse's head (not shown). B, the animal's startle amplitude increased with stimulus level (dB SPL) for both B6 (red) and *Fmr1* mice (teal). A total of 22 B6 and 14 *Fmr1* mice were tested in this task, with a subset (6 B6 and 8 *Fmr1*) tested below 80 dB SPL. C, Force of response to the startle stimuli was smaller for *Fmr1* mice compared to B6. D, latency to respond (in ms) to the startle stimulus decreased with increasing stimulus intensity (dB SPL) for both B6 and *Fmr1* mice. * p<0.05

Fig 3. *Fmr1* mice have decreased PPI and increased latency to startle during a gap detection task. 42 mice (20 B6 and 22 *Fmr1*) were tested in a gap detection task, where a 20 ms quiet gap was introduced into 70dB SPL broadband noise (blue speaker at the center of the array), which was presented at varying ISIs before the startle eliciting stimulus presentation (A). *Fmr1* mice (teal) showed less PPI at 10 ms and 20 ms ISI compared to B6 (red; B). *Fmr1* mice startle responses latencies were longer to 1, 2, 5, 40, 80, 160 and 240 ms ISIs compared to wildtype (C). Boxes show conditions where PPI is not different from zero for both genotypes. * p<0.05, ** p<0.01, *** p<0.001.

Fig 4. *Fmr1* mice have decreased PPI and increased startle latency in response to a 90° speaker swap compared to B6. 26 mice (12 B6, 14 *Fmr1*) were tested in a 90° angle speaker swap task with varying ISI (A). *Fmr1* mice (teal) have lower PPI at 20 ms and 30 ms ISIs than B6 mice (red; B). *Fmr1* mice had longer latencies to startle at all ISIs except 1 ms and 2 ms compared to B6 mice. Boxes show conditions where PPI is not different from zero for both genotypes.* p<0.05, ** p<0.01, *** p<0.001.

Fig 5. *Fmr1* mice had a reduction in PPI compared to B6 at 90° speaker swap and longer latencies to startle in all conditions. 25 mice (11 B6 and 14 *Fmr1*) were tested with varying angles of a speaker swap, both left and right directions across the midline, and constant 20 ms ISI (A, example leftward swap is shown). *Fmr1* mice (blue) show less PPI at 90° than B6 mice (red; B). *Fmr1* mice had longer latencies to startle at all angle conditions compared to B6 (C). Boxes show conditions where PPI is not different from zero for both genotypes. * p<0.05, *** p<0.001.

Fig 6. *Fmr1* mice showed less PPI of their startle, and longer latencies in some conditions compared to B6 mice. A signal noise was played at varying sound levels from a

loudspeaker offset by 7.5° from a 70 dB SPL masker noise at 0° (A). *Fmr1* mice (teal) had reduced PPI of their startle at 9, 12, and 15 dBs attenuation compared to B6 mice (red; B). *Fmr1* mice also had increased latency to startle at 9 and 24 dBs attenuation compared to B6 (C). Boxes show conditions where PPI is not different from zero for both genotypes. * p<0.05, ** p<0.01, *** p<0.001.

Fig 7. *Fmr1* mice had reduced PPI at 90° and 15 dB attenuation with increased latency to startle in several conditions compared to B6. The signal was varied at 2 levels of attenuation (15 and 24 dB) and varied at several angles from the 70 dB SPL masker at 0° to the left or right (A). *Fmr1* mice (blue) had decreased PPI of their startle response at 90° from the masker only at 15dB attenuation compared to B6 mice (red; B, C). *Fmr1* mice had increased latency to startle at 7.5 and 30° at 15 dB attenuation (D) and 7.5, 22.5, 30, 45, 90° at 24 dB attenuation (E) compared to B6 mice. Boxes show conditions where PPI is not different from zero for both genotypes. * p<0.05, ** p<0.01, *** p<0.001.

Table 1: Summary statistics for ABR measurements

Experiment		*B6*			*Fmr1*			
		Mean ± SEM	Median	IQR Q1-Q3	Mean ± SEM	Median	IQR Q1-Q3	p-value
ABR audiogram (dB SPL)	4000	59.3 ± 3.7	55.0	55.0-65.0	61.7 ± 2.1	65.0	57.5-65.0	0.6278
	8000	39.3 ± 36.1	35.0	35.0-45.0	45.0 ± 3.7	45.0	37.5-52.5	0.2503
	16000	37.9 ± 3.6	35.0	35.0-40.0	53.3 ± 3.1	55.0	47.5-55.0	0.0040

Table 2: Summary statistics for startle threshold and PPI experiments

Experiment		*B6*			*Fmr1*			
		Mean ± SEM	Median	IQR Q1-Q3	Mean ± SEM	Median	IQR Q1-Q3	p-value
Threshold (dB SPL)	60	329.0 ± 13.7	289.0	267-369	380.1 ± 36.8	312.5	272-412	0.2903
	70	387.0 ± 36.1	320.0	276-363	349.0 ± 22.3	306.0	273-360	0.0706
	80	391.0 ± 17.6	326.0	273-489	457.3 ± 37.8	320.5	266-539	0.8195
	90	633.8 ± 33.9	586.0	397-758	731.3 ± 69.2	502.5	380-869	0.5794
	100	887.0 ± 47.9	815.5	543-1187	748.4 ± 50.5	687.5	450-946	0.0583
	110	1001.9 ± 58.7	954.0	564-1297	879.0 ± 54.6	813.0	546-1099	0.0828
	120	805.2 ± 53.2	608.0	400-1220	880.7 ± 72.7	706.0	447-1146	0.7422
Gap Detection (ISI)	1	0.02 ± 0.03	-0.0008	-0.22-0.26	0.03 ± 0.03	0.06	-0.11-0.27	0.5314
	2	0.09 ± 0.03	0.08	-0.13-0.32	0.08 ± 0.02	0.11	-0.04-0.27	0.4421
	5	0.24 ± 0.02	0.26	0.05-0.43	0.19 ± 0.02	0.23	0.03-0.38	0.1026
	10	0.44 ± 0.02	0.49	0.35-0.62	0.33 ± 0.02	0.37	0.21-0.49	0.0153*

	20	0.54 ± 0.02	0.59	0.41-0.69	0.38 ± 0.02	0.42	0.26-0.56	0.0011**
	40	0.23 ± 0.02	0.24	0.10-0.41	0.18 ± 0.03	0.27	0.03-0.42	0.3753
	80	0.10 ± 0.02	0.15	-0.04-0.35	0.14 ± 0.02	0.21	0.04-0.34	0.3789
	160	0.15 ± 0.02	0.17	0.01-0.38	0.13 ± 0.02	0.19	-0.01-0.36	0.6436
	240	0.15 ± 0.02	0.06	-0.03-0.35	0.13 ± 0.02	0.19	-0.02-0.34	0.7266
90° Speaker Swap (ISI)	1	-0.05 ± 0.05	-0.04	-0.24-0.21	-0.10 ± 0.04	-0.05	-0.35-0.15	0.5659
	2	0.04 ± 0.04	0.10	-0.16-0.27	-0.04 ± 0.05	0.04	-0.28-0.27	0.3390
	5	0.12 ± 0.05	0.19	-0.14-0.43	0.07 ± 0.05	0.12	-0.13-0.31	0.5434
	10	0.27 ± 0.03	0.26	0.13-0.44	0.11 ± 0.04	0.15	-0.02-0.33	0.0823
	20	0.35 ± 0.03	0.39	0.18-0.56	0.13 ± 0.05	0.19	-0.05-0.42	0.0136*
	30	0.31 ± 0.04	0.37	0.12-0.54	0.12 ± 0.05	0.16	-0.13-0.41	0.0388*
	40	0.26 ± 0.03	0.28	0.10-0.46	0.12 ± 0.05	0.18	-0.09-0.38	0.1329
	80	0.32 ± 0.03	0.36	0.23-0.51	0.16 ± 0.04	0.24	-0.06-0.41	0.0809
	100	0.27 ± 0.04	0.32	0.09-0.48	0.20 ± 0.03	0.19	0.03-0.43	0.4565
	150	0.37 ± 0.03	0.42	0.19-0.56	0.23 ± 0.03	0.27	0.06-0.45	0.1400
	300	0.25 ± 0.03	0.25	0.11-0.43	0.12 ± 0.04	0.15	-0.06-0.35	0.1571
Speaker Swap (Angle)	7.5°	-0.01 ± 0.02	-0.008	-0.21-0.22	-0.04 ± 0.03	0.06	-0.12-0.21	0.4647
	15°	0.04 ± 0.02	0.03	-0.14-0.26	0.06 ± 0.02	0.10	-0.07-0.29	0.6043
	30°	0.005 ± 0.03	0.04	-0.18-0.23	0.02 ± 0.02	0.08	-0.12-0.26	0.6542
	45°	0.10 ± 0.03	0.17	-0.14-0.36	0.13 ± 0.02	0.17	0.010-0.37	0.3635
	90°	0.22 ± 0.02	0.24	0.08-0.40	0.12 ± 0.02	0.18	-0.01-0.37	0.0228*
Threshold SRM (dB attenuation)	9	0.40 ± 0.02	0.42	0.25-0.56	0.27 ± 0.03	0.30	0.19-0.44	0.0309*
	12	0.48 ± 0.02	0.55	0.33-0.65	0.31 ± 0.03	0.34	0.23-0.47	0.0067**
	15	0.49 ± 0.02	0.56	0.37-0.65	0.34 ± 0.02	0.36	0.23-0.51	0.0153*
	18	0.32 ± 0.03	0.32	0.21-0.47	0.29 ± 0.02	0.32	0.18-0.46	0.6152
	21	0.18 ± 0.03	0.21	0.07-0.38	0.17 ± 0.04	0.27	-0.03-0.39	0.7820
	24	0.05 ± 0.03	0.10	-0.09-0.25	0.09 ± 0.03	0.08	-0.11-0.33	0.5292
	27	-0.04 ± 0.04	0.04	-0.23-0.19	0.05 ± 0.04	0.12	-0.11-0.32	0.1145
SRM 15 dB attenuation (Angle)	7.5°	0.42 ± 0.03	0.46	0.23-0.61	0.34 ± 0.03	0.40	0.23-0.48	0.2315
	15°	0.38 ± 0.03	0.35	0.20-0.61	0.30 ± 0.03	0.33	0.17-0.49	0.2133
	22.5°	0.36 ± 0.03	0.37	0.22-0.58	0.33 ± 0.03	0.40	0.17-0.49	0.6620
	30°	0.34 ± 0.03	0.40	0.19-0.57	0.31 ± 0.02	0.35	0.21-0.47	0.6156
	45°	0.41 ± 0.03	0.45	0.23-0.61	0.31 ± 0.03	0.35	0.15-0.47	0.1220
	90°	0.42 ± 0.03	0.49	0.27-0.64	0.21 ± 0.04	0.31	0.04-0.43	0.0030**
SRM 24 dB attenuation (Angle)	7.5°	-0.02 ± 0.04	-0.002	-0.24-0.19	0.07 ± 0.04	0.11	-0.09-0.35	0.1804
	15°	-0.05 ± 0.03	0.07	-0.16-0.28	0.06 ± 0.04	0.13	-0.07-0.28	0.8865
	22.5°	-0.006 ± 0.03	0.009	-0.19-0.18	-0.02 ± 0.05	0.11	-0.16-0.30	0.8580
	30°	0.02 ± 0.04	0.07	-0.20-0.28	0.04 ± 0.04	0.08	-0.11-0.31	0.7420
	45°	0.03 ± 0.04	0.13	-0.20-0.28	0.04 ± 0.04	0.09	-0.11-0.32	0.8239
	90°	0.18 ± 0.03	0.24	0.06-0.38	0.18 ± 0.03	0.23	-0.03-0.43	0.9639

Values in red indicate a PPI not significantly different than zero

Table 3: Summary statistics for latency (ms)

Experiment		*B6*			*Fmr1*			
		Mean ± SEM	Median	IQR Q1-Q3	Mean ± SEM	Median	IQR Q1-Q3	p-value
Threshold (dB SPL)	60	47.2 ± 5.87	47.0	21.5-75.5	45.2 ± 7.6	47.0	18.0-63.0	0.8331
	70	41.9 ± 5.18	35.0	22.0-73.0	34.1 ± 6.3	26.0	15.3-41.5	0.2136
	80	36.6 ± 2.88	32.0	20.0-48.5	35.4 ± 3.5	32.0	18.3-43.5	0.7538
	90	25.4 ± 1.47	24.0	19.3-32.8	26.0 ± 1.9	24.0	18.0-33.0	0.8117
	100	25.1 ± 1.41	22.0	20.0-30.3	26.6 ± 2.0	24.5	21.0-32.0	0.6117
	110	25.0 ± 1.27	22.0	20.0-27.5	26.5 ± 1.7	24.0	20.0-31.0	0.5933
	120	26.9 ± 1.89	22.0	17.0-33.0	27.8 ± 2.4	23.0	18.0-34.5	0.7275
Gap Detection (ISI)	1	26.6 ± 1.34	23.0	20.0-27.0	29.5 ± 1.48	26.0	19.8-33.0	0.0260*
	2	26.6 ± 1.38	23.0	17.0-29.0	30.9 ± 1.43	28.0	20.0-35.0	0.0057**
	5	28.9 ± 1.30	25.0	21.8-32.0	29.9 ± 1.35	27.0	18.0-36.0	0.1541
	10	34.2 ± 1.47	31.0	23.0-42.0	34.3 ± 1.74	28.5	20.0-45.0	0.3521
	20	35.7 ± 1.84	31.0	20.0-48.0	36.1 ± 1.88	30.0	20.0-51.5	0.3142
	40	28.2 ± 1.33	24.0	21.0-31.0	33.7 ± 1.61	29.0	20.0-43.5	0.0006***
	80	22.5 ± 0.53	23.0	21.0-24.3	30.4 ± 1.58	28.0	22.0-34.3	0.0002***
	160	21.7 ± 0.74	23.0	20.3-26.0	28.4 ± 1.51	28.0	20.8-33.0	0.0012**
	240	21.3 ± 0.84	22.5	19.0-25.0	32.0 ± 1.76	28.0	22.0-34.3	<0.0001***
90° Speaker Swap (ISI)	1	23.2 ± 1.52	22.0	21.0-24.0	26.1 ± 1.68	25.0	22.0-32.0	0.2625
	2	21.6 ± 0.70	22.0	20.0-24.0	28.8 ± 2.15	26.0	21.0-33.0	0.0098
	5	22.3 ± 1.17	22.0	20.0-24.3	29.5 ± 2.06	25.5	22.0-36.8	0.0107*
	10	23.5 ± 1.46	24.0	20.0-29.0	30.9 ± 2.74	29.0	16.8-39.3	0.0100*
	20	23.0 ± 1.23	24.0	20.8-26.0	32.7 ± 2.78	29.0	23.0-39.0	0.0008***
	30	24.2 ± 1.06	24.0	21.0-30.3	34.5 ± 2.92	28.0	20.3-47.0	0.0005***
	40	24.6 ± 1.70	24.0	22.0-31.5	32.3 ± 2.40	30.0	22.0-40.0	0.0062**
	80	23.8 ± 1.23	24.0	20.8-30.3	33.2 ± 2.58	30.0	22.0-39.0	0.0013**
	100	22.3 ± 1.38	23.0	18.5-28.3	30.7 ± 2.87	28.0	14.0-41.0	0.0044**
	150	26.7 ± 1.68	26.0	22.0-31.5	32.2 ± 2.36	31.0	25.5-39.5	0.0489*
	300	23.4 ± 1.29	24.0	21.0-28.0	32.1 ± 2.25	29.0	29.0-23.0	0.0020**
Speaker Swap (Angle)	7.5°	21.5 ± 0.70	22.0	21.0-23.8	30.1 ± 1.30	27.0	22.0-34.0	<0.0001***
	15°	21.8 ± 0.79	21.0	20.0-23.8	29.4 ± 1.44	25.0	20.0-33.0	<0.0001***
	30°	22.0 ± 0.76	22.0	20.0-24.0	28.0 ± 1.22	25.0	21.0-33.0	<0.0001***
	45°	21.5 ± 0.69	22.0	20.0-24.0	31.1 ± 1.30	28.0	22.0-35.0	<0.0001***
	90°	24.9 ± 0.95	23.0	21.0-27.0	31.7 ± 1.56	27.0	20.8-35.3	<0.0001***
Threshold SRM (dB	9	18.0 ± 2.42	10.0	5.0-22.5	36.1 ± 3.32	37.0	14.0-52.0	<0.0001***
	12	23.9 ± 2.78	15.0	10.0-31.0	34.0 ± 3.40	29.0	13.0-48.0	0.0231
	15	35.6 ± 2.50	31.0	19.5-53.5	36.6 ± 3.98	29.0	15.0-57.0	0.8374
	18	26.0 ± 1.87	24.0	21.0-29.8	35.8 ± 2.91	33.0	19.0-49.0	0.0224*

	21	25.4 ± 1.49	24.0	21.0-28.5	32.2 ± 2.92	28.5	17.5-42.0	0.0994
	24	23.4 ± 1.30	22.0	21.0-28.0	35.3 ± 2.44	31.0	24.0-45.0	0.0042**
	27	24.7 ± 1.67	22.0	21.0-25.0	30.3 ± 2.43	25.0	21.0-35.0	0.1750
SRM 15 dB attenuation (Angle)	7.5°	25.4 ± 1.82	23.5	13.3-32.0	38.3 ± 3.49	32.0	18.0-57.5	0.0008***
	15°	27.8 ± 2.13	24.0	16.5-33.0	33.0 ± 3.06	31.0	12.5-43.5	0.1969
	22.5°	24.8 ± 1.97	23.0	14.0-30.0	29.6 ± 2.66	25.5	14.0-44.0	0.2396
	30°	23.5 ± 1.88	23.0	13.0-31.0	32.0 ± 3.45	26.0	11.0-48.0	0.0288*
	45°	25.5 ± 2.02	23.0	14.0-30.8	31.8 ± 3.29	24.0	14.0-41.0	0.1131
	90°	22.5 ± 2.52	13.0	8.5-29.5	29.0 ± 2.97	18.0	11.0-47.0	0.0803
SRM 24 dB attenuation (Angle)	7.5°	23.6 ± 1.71	22.0	20.0-24.0	29.8 ± 2.33	25.0	22.0-34.0	0.0074**
	15°	25.3 ± 1.61	22.0	21.0-27.0	25.7 ± 1.98	23.5	14.0-32.0	0.5322
	22.5°	23.6 ± 1.51	22.0	21.0-24.0	32.0 ± 2.06	28.0	22.5-33.5	0.0004***
	30°	22.4 ± 1.46	22.0	21.0-24.0	30.1 ± 1.96	28.0	21.0-38.0	0.0014**
	45°	25.1 ± 1.62	22.0	20.0-27.0	30.8 ± 1.94	27.0	23.0-36.0	0.0098**
	90°	26.0 ± 1.61	24.0	24.0-29.0	26.3 ± 2.45	23.5	11.3-32.0	0.5437