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Matrix metalloproteinase-9 deletion rescues auditory evoked potential habituation deficit in a mouse model of Fragile X Syndrome

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Abstract

Sensory processing deficits are common in autism spectrum disorders, but the underlying mechanisms are unclear. Fragile X Syndrome (FXS) is a leading genetic cause of intellectual disability and autism. Electrophysiological responses in humans with FXS show reduced habituation with sound repetition and this deficit may underlie auditory hypersensitivity in FXS. Our previous study in *Fmr1* knockout (KO) mice revealed an unusually long state of increased sound-driven excitability in auditory cortical neurons suggesting that cortical responses to repeated sounds may exhibit abnormal habituation as in humans with FXS. Here, we tested this prediction by comparing cortical event related potentials (ERP) recorded from wildtype (WT) and *Fmr1* KO mice. We report a repetition-rate dependent reduction in habituation of N1 amplitude in *Fmr1* KO mice and show that matrix metalloproteinase −9 (MMP-9), one of the known FMRP targets, contributes to the reduced ERP habituation. Our studies demonstrate a significant up-regulation of MMP-9 levels in the auditory cortex of adult *Fmr1* KO mice, whereas a genetic deletion of *Mmp-9* reverses ERP habituation deficits in *Fmr1* KO mice. Although the N1 amplitude of *Mmp-9*/*Fmr1* DKO recordings was larger than WT and KO recordings, the habituation of ERPs in *Mmp-9/Fmr1* DKO mice is similar to WT mice implicating MMP-9 as a potential target for reversing sensory processing deficits in FXS. Together these data establish ERP habituation as a translation relevant, physiological pre-clinical marker of auditory processing deficits in FXS and suggest that abnormal MMP-9 regulation is a mechanism underlying auditory hypersensitivity in FXS.

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Keywords

Fragile X Syndrome; autism; auditory cortex; sensory hypersensitivity; cortical event related potentials; matrix metalloproteinase-9

Introduction

Autism spectrum disorders (ASD) affects ~1% of children globally (Lai et al., 2014). Sensory processing deficits are commonly observed in ASD (Green et al., 2015), but the underlying cellular mechanisms remain unclear. Fragile X syndrome (FXS) is a leading known inherited form of ASD affecting ~1 in 4000 males and 1 in 8000 females (Sherman 2002). FXS results from expansion and hyper-methylation of CGG trinucleotide repeats in the un-translated 5' region of the *Fragile X Mental Retardation (FMR1)* gene, leading to reduced FMR1 protein (FMRP) (Bailey et al., 1998; O'Donnell and Warren, 2002). FMRP regulates translation, and its absence causes abnormal protein synthesis resulting in a wide array of symptoms including intellectual disabilities, sensory hypersensitivity, hyperarousal, hyperactivity, repetitive behaviors and social communication deficits (Hagerman et al., 1986; Hagerman et al., 1991; Roberts et al., 2007; Hagerman and Hagerman, 2013; Yu and Berry-Kravis, 2014).

Behavioral and functional studies indicate abnormal auditory sensitivity in humans with FXS (St Clair et al., 1987; Castren et al., 2003; Van der Molen et al., 2012a,b; Schneider et al., 2013; Rotschafer and Razak, 2014). A consistent observation in FXS is that the amplitude of the N1 component of auditory event related potentials (ERP) shows reduced habituation with sound repetition compared to the control group, suggesting neural correlates of auditory hypersensitivity. However, the mechanisms underlying ERP habituation deficits are not known.

Fmr1 knockout (KO) mouse, a pre-clinical model of FXS, also exhibits auditory hypersensitivity including audiogenic seizures (Chen and Toth, 2001; Musumeci et al., 2007) and enhanced cortical responses to sounds (Rotschafer and Razak, 2013). The similarities in sensory phenotypes in humans and the mouse model provide an opportunity to develop translation-relevant biomarkers for therapeutic approaches. The circuit mechanisms of sensory processing may be more tractable and conserved across species compared to complex social behaviors and cognitive impairments. Therefore, a model of auditory processing deficits in FXS will enable discovery of candidate therapies and promote a mechanistic understanding of sensory processing deficits in FXS.

A previous electrophysiology study revealed an abnormally sustained response to sounds in auditory cortical neurons in *Fmr1* KO mice (Rotschafer and Razak, 2013). This is consistent with increased UP states observed in the somatosensory cortex of *Fmr1* KO mice (Gibson et al., 2008). Sustained sound-driven increase in excitability may impact how the auditory system habituates to repeated stimuli in a repetition rate dependent manner. To test this prediction, we recorded ERPs from the auditory cortex of WT and *Fmr1* KO mice in response to repeated sounds and compared N1 amplitude habituation profiles. We report a significant repetition rate-dependent habituation in WT ERPs, which was impaired in *Fmr1*

KO mice. The impaired ERP habituation in mice is similar to the deficits observed in humans with FXS.

The ERP habituation deficits in humans with FXS are reversed by minocycline treatment but the underlying mechanisms are not known (Schneider et al. 2013). Beside its antibiotic and anti-inflammatory activity, minocycline can reduce the levels and activity of matrix metalloproteinase-9 (MMP-9) (Lee et al., 2006; Bilousova et al., 2009; Dziembowska et al 2013). MMP-9 is an endopeptidase that can cleave extracellular matrix (Ethell and Ethell, 2007) and its translation is negatively regulated by FMRP (Janusz et al., 2013). MMP-9 is elevated in the cortex and hippocampus of both humans with FXS and *Fmr1* KO mice (Sidhu et al., 2014; Gkogkas et al., 2014). Genetic deletion of *Mmp-9* in *Fmr1* KO mice reverses some molecular and physiological hippocampal deficits and FXS-associated behaviors (Sidhu et al., 2014). Therefore, we hypothesized that enhanced MMP-9 activity may also contribute to auditory cortical ERP habituation deficits in *Fmr1* KO mice and can be reversed by *Mmp-9* deletion. We found that MMP-9 levels were significantly higher in the auditory cortex of adult *Fmr1* KO mice compared to WT mice and that the ERPs habituated in *Mmp-9/Fmr1* double KO (DKO) mice similar to WT mice, implicating MMP-9 as a target for reversing sensory processing deficits in FXS.

Methods

Mouse handling and genotype

The FVB.Cg-*Mmp-9*^{tm1Tvu}/J and FVB.129P2-*Fmr1*^{tm1Cgr}/J (*Fmr1* KO) and FVB.129P2-Pde6b^{+}Tyr$^{c\text{-}ch}$/AntJ controls (WT) were obtained from The Jackson Laboratory. The FVB.Cg-*Mmp-9*^{tm1Tvu}/J mice were backcrossed, in-house, with the *Fmr1* KO mice or WT mice to generate *Fmr1*/*Mmp-9* DKO or *Mmp-9* KO mice, respectively. All genotypes were confirmed by PCR analysis of genomic DNA isolated from mouse tails. Mice were maintained in an AAALAC accredited facility under 12 hour light/dark cycles, and fed standard mouse chow. All mouse studies were done according to NIH and Institutional Animal Care and Use Committee guidelines. Food and water was provided *ad libitum*. Auditory ERPs were obtained from 6 WT, 6 *Fmr1* KO, 5 *Mmp-9* KO, and 4 *Mmp-9/Fmr1* DKO mice, all males between 1.5 – 2 months old. In some mice, recordings were obtained from two different cortical sites separated >200 μm resulting in 7 recording sites in WT mice, 9 recording sites in *Fmr1* KO mice, 10 recording sites in *Mmp-9* KO mice and 7 recording sites in *Mmp-9/Fmr1* DKO mice.

Surgery for auditory ERP studies

Mice were anesthetized with isoflurane inhalation (0.2–0.5%), secured with a bite bar, and placed on a stereotaxic apparatus (model 930; Kopf, CA). Toe pinch reflex was used to measure anesthetic state every 20–30 minutes throughout the experiment, and isoflurane levels were adjusted as needed. Once the animal was anesthetized, a midline sagittal incision was made along the scalp to expose the skull, and the right temporalis muscle was displaced. A dental drill was used to remove the skull overlying the right auditory cortex identified using previously published methods (Martin del Campo et al. 2012). Location of core auditory cortex was identified using vasculature, and confirmed with extracellular

recordings in response to sounds. At the end of the experiment, mice were euthanized with sodium pentobarbital (i.p. 125 mg/kg).

Acoustic Stimulation for ERP studies

All experiments were conducted in a sound-attenuated chamber lined with anechoic foam (Gretch-Ken Industries, Oregon). Acoustic stimuli were generated using custom software driving a Microstar digital signal processing board or through a TDT RZ6 system and presented through a free-field speaker (LCY-K100 ribbon tweeters; Madisound, WI) located 6 inches away at a 45° angle from the animal's left ear. Sound intensity was modified using programmable attenuators (PA5; Tucker-Davis Technologies, Florida). The speaker output fluctuation was within +/− 3 dB for frequencies between 5 and 35 kHz as measured using a ¼ inch B&K microphone.

ERP Habituation Paradigm

All recordings obtained in this study were from isoflurane-anesthetized mice. The habituation paradigm consisted of trains of repeating broadband noise (BBN, 100 msec duration, 5 msec rise/fall time, 70 dB SPL) presented at varying repetition rates. After 5 minutes of baseline recordings with no auditory stimulation, at least 20 repetitions of the BBN was presented at 0.25, 0.50, 1, 2, 3 or 4 Hz repetition rate. The order in which BBN stimulus trains with different repetition rates were presented was selected at random. It is important to note that the phrase 'habituation' is simply used to describe the reduction in response amplitude within a stimulus train and is not intended to implicate any specific mechanism.

Deviant Sound Paradigm

Each stimulus train consisted of nine stimuli presented at 5 Hz. The train was repeated 10 times at 0.25 Hz. The first five were tone pips (12 kHz, 50 ms duration, 5 msec rise/fall time). The sixth sound (deviant) was BBN (50 ms duration). The seventh through ninth stimuli were the 12 kHz tone. The intensity of each stimulus was 60 dB SPL. A 12 kHz tone was used for these experiments because this frequency was sufficient to elicit robust auditory cortex responses and is a sensitive frequency based on mouse audiograms. Responses were normalized to the 1^{st} response within each of the 10 trains. N1 amplitudes for each position were then individually averaged across all 10 trains so that each recording location contributed 1 sample for each position in the train for genotype comparisons. Deviant sound response indices were calculated for differences between the response just before and after the oddball noise. The following equations were used for comparing differences in response before the deviant sound (5^{th}response − 6^{th}response) / (5^{th} response + 6^{th} response) and after the deviant sound (6^{th} response − 7^{th} response) / (6^{th} response + 7^{th} response). The values of these indices range between −1 and +1 with 0 indicating no difference in N1 amplitude and more positive values indicating a higher response to the first of the two sounds compared. These indices were calculated for each of the 10 trains, and then averaged for each spot.

Auditory ERP recordings

Auditory ERP recordings were obtained using the BioPac system (BIOPAC Systems, Inc.) from mice anesthetized with isoflurane. Concentric bipolar stainless steel electrodes (FHC, Inc.) were advanced into the brain using a Kopf direct drive 2660 micropositioner. An un-insulated stainless steel wire (California Fine Wire company) was attached to the neck muscle for grounding purposes. At each recording location, habituation and oddball testing began only after sound-driven ERPs with an N1 component with ~45 ms latency could be reliably produced.

The BioPac MP150 acquisition system was connected to an EEG 100C amplifier unit to which the bipolar concentric recording electrode and ground were attached. The hardware was set to high-pass (0.5Hz) and low-pass (35Hz) filters. Normal EEG output data was collected, alpha detection mode was not used and gain was kept the same (10,000×) between all recordings. Data were sampled at a rate of either 5 or 20 kHz using Acqknowledge software, and sound delivery was synchronized with EEG recording using a 5 ms digital TTL pulse to mark the onset of each sound in a train. To detect N1 amplitudes and latencies, a search window of 25–75ms after sound onset was used to find the minimum peak as well as latency of the auditory ERP.

Statistical Analyses

Data were extracted from Acqknowledge and statistics were calculated using Microsoft Excel and SPSS (IBM Inc). Curve fitting was done using OriginPro8 package. Habituation was characterized for each recording location using a single coefficient exponential decay function ($y = y0 + Ae^{(-x/t)}$, where $y0$ = asymptote, A = amplitude, t = decay constant). To assist in curve fitting, direct weighting was used. The weight of the first point was set to 100, while the remaining 19 were set to 1. This method essentially forces the fit curve to include the first point, while all subsequent points are treated equally to each other. Doing this allowed for more recording locations to be successfully fit and allow for statistical genotype analysis of fit parameters. In all cases where genotype means are reported, SEM was used, and is also reflected in all error bars in figures. If during sound train presentation, non-stimulus driven artifacts were observed, the entire train was removed from subsequent analysis. Other recordings from the same location were used if no artifacts were present. In all cases, p values < 0.05 were considered significant for ANOVA and Student's t-tests. Where t-tests were performed, r was calculated as an effect size. When 3 genotypes were used for ANOVA comparisons, Post-hoc Bonferroni tests were used to determine individual pair wise differences.

Gelatin Zymography

Postnatal day (P) 40 mice (n=5 mice per group) were euthanized with isoflurane and the auditory cortex was dissected based on coordinates (Paxinos and Franklin, 2004) and previous electrophysiological and dye-placement studies (Martin del Campo et al., 2012). The tissues were flash-frozen on dry ice and stored at −80° C. Gelatin gel zymography was performed as previously described with minor modifications (Sidhu et al., 2014). Briefly, auditory cortical tissues were resuspended in 100 μL of 100 mM Tris-HCl (pH=7.6) buffer containing 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij35, 0.02% Na_3N, 1% Triton X-100, 100

μM PMSF and PI cocktail (Sigma). The gelatinases, MMP-2 and MMP-9, were pulled down with gelatin agarose beads (Sigma, G5384) and separated on 10% polyacrylamide Tris-Glycine gel with 0.1% gelatin as the substrate (Life Technologies). Following separation, gels were soaked in renaturing buffer (Life Technologies) to remove all traces of SDS and allow the MMPs to regain most of their enzymatic activity. In addition to active forms, inactive pro-forms of MMP-9 and MMP-2 are also detected by gelatin zymography. SDS polyacrylamide gel electrophoresis (PAGE) allows for unfolding of MMPs displacing pro-domain from the active site, resulting in the activation of the pro-form that is usually inactive. Therefore, gel zymography shows total levels of both MMP-9 and MMP-2 on the same gel. Following renaturing, gels were incubated in developing buffer (Life Technologies) for 96 hours, which allowed the gelatinases (MMP-2 and MMP-9) to degrade gelatin in the gel. Gels were then stained with coomassie blue overnight to uniformly stain the gels after which de-staining revealed areas of MMP protein as unstained bands. Gelatin degradation levels were proportional to total levels of MMP-2 and MMP-9 proteins and were quantified by densitometry using CS3 Adobe Photoshop. Statistical analysis was performed using Student's t-test for comparisons between two groups.

Results

The first aim of this study was to determine whether properties of auditory ERPs are different between WT and *Fmr1* KO mice. The second aim was to test the hypothesis that enhanced MMP-9 activity may contribute to auditory cortical ERP habituation deficits in *Fmr1* KO mice and can be reversed by *Mmp-9* deletion. The focus was on the amplitudes and latencies of the N1 component in response to trains of sounds with different repetition rates.

MMP-9 levels are increased in the auditory cortex of KO mice

We found an up-regulation in MMP-9 levels in the auditory cortex of *Fmr1* KO mice at P40. Gel zymography revealed a significant increase in levels of MMP-9 in *Fmr1* KO (mean intensity, $M = 45.47$), compared to WT ($M = 22.39$) mice (Figure 1). This difference is statistically significant ($t(8) = 4.65$, $p = 0.0016$). In contrast, MMP-2 levels are not different between *Fmr1* KO ($M = 35.97$) and WT ($M = 35.16$) mice ($t(8) = 0.13$, $p = 0.8983$). Increased MMP-9 levels suggest enhanced endogenous activity of MMP-9 in the auditory cortex of *Fmr1* KO mice providing a justification for examining the effects of *Mmp-9* deletion on auditory electrophysiological responses in *Fmr1* KO mice.

Average N1 amplitude is similar in WT and Fmr1 KO mice, but larger in *Mmp-9/Fmr1* DKO mice

To determine whether *Fmr1* KO mice show analogous deficits in N1 habituation as seen in humans, we presented 20 repeats of broadband noise at various repetition rates (0.25, 0.5, 1, 2, 3 and 4 Hz) and recorded cortical ERPs. Figure 2 shows representative examples of auditory ERP traces from individual WT (Figure 2A), *Fmr1* KO (Figure 2B) and *Mmp-9/Fmr1* DKO (Figure 2C) mice at different repetition rates. Each trace was obtained by averaging 20 responses. Genotype averages of N1 amplitudes (Figure 2D) were analyzed using a two-way ANOVA of genotype and rate, which showed a main effect of repetition

rate on N1 amplitude ($F(5,119) = 13.194$, $p < 0.000001$), an effect of genotype ($F(2,119) = 16.344$, $p < 0.000001$), but no genotype x rate interaction ($F(10,119) = 1.013$, $p = 0.437$). Post-hoc Bonferroni tests show that WT and *Fmr1* KO are not different in N1 amplitudes ($p = 0.723$), but that *Mmp-9/Fmr1* DKO is different from both WT ($p = 0.0002$) and *Fmr1* KO ($p < 0.000001$).

Pairwise comparisons of WT and *Mmp-9/Fmr1* DKO mice show higher amplitudes in *Mmp-9/Fmr1* DKO mice for the 3 and 4 Hz rates (Figure 2D). Additionally, raw amplitudes of *Mmp-9* KO mice showed no difference when compared to WT mice using independent samples t-test (all $p > 0.5$), except at the lowest rate of 0.25Hz, which was significantly lower than WT, $p = 0.046$ (data not shown). These data indicate that the average N1 amplitude across 20 repetitions was dependent on the repetition rate in all groups of mice tested. There are no differences in average N1 amplitudes between WT and *Fmr1* KO, but the *Mmp-9/Fmr1* DKO mice showed larger N1 amplitudes from WT mice at the fastest rates repetition rates tested.

The N1 amplitudes have to be normalized in order to compare habituation dynamics across animals and genotypes. Normalizing each response in the train to the first response renders the data susceptible to idiosyncrasies of a single response. In order to produce reliable normalized measures, N1 amplitudes were averaged across a 0.25Hz train of sound presentations, a rate at which there was minimal habituation. All other N1 amplitudes at each recording site for the 0.25, 0.5, 1, 2, 3 and 4 Hz repetition rates were then normalized to the average N1 amplitude of 0.25 Hz responses. This approach allows for a comparison to a stable average for each observation. Two-way ANOVA of genotype and repetition rate reveals no differences for the normalized 1^{st} response between genotype ($F(2,123) = 1.482$, $p = 0.231$), repetition rate ($F(5,123) = 0.593$, $p = 0.216$), or genotype x repetition rate interaction ($F(10,123) = 0.593$, $p = 0.817$) (Figure 2E).

Early N1 amplitude habituation is not different between genotypes

Habituation of N1 amplitudes in the auditory cortex to repeated stimulation occurs rapidly, with a large percentage of total habituation occurring by the second stimulation (Figure 3, 4). A two-way ANOVA of genotype and repetition rate reveals no differences for the 2^{nd} response between genotype ($F(2,123) = 2.500$, $p = 0.086$) or a genotype x repetition rate interaction ($F(10,123) = 0.746$, $p = 0.680$). However, 2^{nd} responses are different depending on the repetition rate ($F(5,123) = 13.898$, $p < 0.000001$), with more habituation occurring at faster rates for all genotypes (Figure 2F). This indicates that ERPs in all three genotypes of mice habituate to auditory stimuli by the second sound in the train and there is no statistical difference between the genotypes.

Steady state habituation is decreased in *Fmr1* KO mice and rescued in *Mmp-9/Fmr1* DKO mice

In order to characterize habituation over the entire train, individual data were fit to an exponential decay function, $y = y0 + Ae^{(-x/t)}$, where y0 = asymptote, A = amplitude, t = decay constant. Several individual observations from all genotypes at 0.25Hz repetition rate failed to fit the exponential decay functions (consistent with a lack of habituation at 0.25 Hz)

and thus 0.25 Hz rate was not analyzed further using those fitting parameters (Figure 3A, 4A). At higher rates (>0.5 Hz), differences were found in maximum habituation levels (*y0*) across the entire sound presentation train (Figure 3, 4).

A two-way ANOVA on *y0* of genotype and repetition rate reveals a main effect of genotype ($F(2,99) = 12.561$, $p < 0.00002$) and repetition rate ($F(4,99) = 46.394$, $p < 0.000001$), but not a genotype x repetition rate interaction $F(8,99) = 0.471$, $p = 0.874$. Post-hoc pairwise Bonferroni tests reveal differences between WT and *Fmr1* KO mice ($p = 00001$) (Figure 3), *Mmp-9/Fmr1* DKO and *Fmr1* KO ($p = 0.048$), but not *Mmp-9/Fmr1* DKO and WT mice ($p > 0.05$) (Figure 4). This indicates that for repetition rates between 0.5 and 4 Hz, *Fmr1* KO mice show consistent N1 amplitude habituation deficits compared with WT and *Mmp-9/Fmr1* DKO mice.

Also, *Mmp-9* KO mice were compared with WT at each rate for *y0*, with no difference seen at any repetition rate (all $p > 0.05$). Taken together, these results indicate that at steady state, normalized N1 amplitudes are higher in the *Fmr1* KO mice compared to WT mice and a genetic deletion of *Mmp-9* in *Fmr1* KO mice reverses this steady state habituation deficit.

N1 latency and variability was similar in WT and *Fmr1* KO mice

Latencies to peak N1 responses were measured and plotted as a function of stimulus # for each train (Figure 5). Average latencies of N1 responses for each individual train were measured and then averaged across genotypes. Once this single value was collected for each train, genotype averages were calculated. Two-way ANOVA on N1 latency of genotype and rate shows a main effect of genotype ($F(2,118) = 6.369$, $p = 0.002$), but not of rate ($F(5,118) = 2.247$, $p = 0.054$), or genotype x rate interaction ($F(10,118) = 0.742$, $p = 0.683$). Post-hoc Bonferroni analysis reveals a difference between WT and *Mmp-9/Fmr1* DKO ($p = 0.002$), but not between WT and *Fmr1* KO ($p = 0.171$), or *Mmp-9/Fmr1* DKO and *Fmr1* KO mice ($p = 0.204$). *Mmp-9* KO also showed no difference in latency at any rate tested when compared to WT animals (all $p > 0.05$, data not shown). To determine which rates produced different latencies between WT and *Mmp-9/Fmr1* DKO, average N1 latencies were compared for each rate using independent sample t-tests. The only rates to reach significance were the lowest rates of 0.25Hz ($t(12) = 2.577$, $p = 0.024$, $r = 0.60$), and 0.5Hz ($t(12) = 3.558$, $p = 0.004$, $r = 0.72$), with *Mmp-9/Fmr1* DKO having consistently shorter N1 latencies (Figure 5A, B).

When N1 is averaged across a train, variability in latency can produce a reduction in average amplitude even if the individual components themselves are not different in amplitude (Mouraus and Ionnetti, 2008). To determine if the habituation differences between genotypes can be explained by latency variability, the Fano factor of N1 latency (Variance of N1 latency / Mean N1 latency) was measured for each recording site and each rate and compared between genotypes. In all genotypes, as repetition rate increased, variability in N1 latencies across the train also increased. For each repetition rate, Fano factors were averaged for each genotype for statistical analysis. Differences in Fano factors were analyzed using a two-way ANOVA of genotype and repetition rate. The analysis showed that there was an effect of genotype ($F(2,117) = 6.953$, $p = 0.001$), and an effect of repetition rate ($F(5,117) = 13.152$, $p < 0.000001$), with no genotype x repetition rate interaction ($F(10,117) = 0.845$, $p =$

0.587). Post-hoc Bonferroni analysis shows that differences in Fano Factor existed in *Mmp-9/Fmr1* DKO mice when compared to WT or *Fmr1* KO mice ($p = 0.009$), with no differences between WT and *Fmr1* KO mice ($p = 0.9$). *Mmp-9* KO showed no difference in Fano factor when compared to WT at any rate using independent samples t-tests, (all $p > 0.05$, data not shown). At 0.25 Hz N1 latencies were extremely stable throughout the entire stimulus train for WT (Fano Factor: 0.00019 ± 0.00008, $n = 7$), *Fmr1* KO (Fano Factor: 0.00052 ± 0.00046, $n = 9$), and *Mmp-9/Fmr1* DKO mice (Fano Factor: 0.00028 ± 0.00024) with no differences observed between genotypes ($F(2,20) = 0.308$, $p = 0.738$) (Figure 5A). No differences were found between genotypes at any other individual rate as well ($p > 0.05$), so differences in *Mmp-9/Fmr1* DKO genotype only emerge when all rates are pooled together. Differences in the amplitude of ERPs in *Mmp-9/Fmr1* DKO mice (Figure 2C, F) could be due to generally lower variability of latency, leading to more synchronous activity, and possibly contributing to larger N1 amplitudes. However, the deficit in steady state habituation in *Fmr1* KO mice compared to WT mice is unlikely related to differences in variability of N1 peak latency, which were not observed between those genotypes.

Deviant sound evoked ERP amplitudes are not different between groups

Deviant sound stimulation paradigms are used to determine if neural responses change when characteristics of a repeated sound change during an acoustic train. Differences in response to the deviant sound may contribute to saliency detection. Therefore, we tested if a deficit in habituation also resulted in a deficit in processing stimulus contrast. The hypothesis tested is that the ERP generated by the deviant BBN stimulus in a train of 12 kHz tones in the WT mice will be larger in amplitude compared to the habituated response, whereas in *Fmr1* KO mice, the habituated responses and the deviant sound response will be similar thus providing less information on stimulus contrast.

A 5 Hz train of 12 kHz tone pips was delivered with a deviant BBN sound presentation in the 6^{th} position followed by three more 12 kHz tone pips (Figure 6). Each train was presented 10 times. Responses throughout the train were normalized to the N1 amplitude of the first response in each of the 10 trains, and averages were calculated for each position (Figure 6A). The normalization was not done for the 0.25 Hz train used for the habituation data because of the differences in the main stimuli used in the two experiments (BBN noise *versus* tone). Two-way ANOVA on normalized N1 amplitude of genotype and position in the train revealed a main effect of position ($F(8,162) = 14.289$, $p < 0.000001$), indicating that habituation across the train occurs for all genotypes. Additionally, a main effect of genotype was found ($F(2,162) = 30.631$, $p < 0.000001$), with no genotype x position interaction ($F(16,162) = 0.725$, $p = 0.766$). Post-hoc analysis showed that the effect of genotype is due to the differences between *Fmr1* KO mice and WT or *Mmp-9/Fmr1* DKO mice ($p < 0.000001$), but no differences between WT and *Mmp-9/Fmr1* DKO mice ($p = 0.9$). Comparison of the responses to each stimulus in the train in isolation using independent samples t-tests revealed some differences between WT and *Fmr1* KO mice and between *Mmp-9/Fmr1* DKO and Fmr1 KO mice (Figure 6A), but no difference between WT and *Mmp-9/Fmr1* DKO at any position ($p>0.05$). It should also be noted that no differences are seen early in the train between any genotypes, which is consistent with earlier results

showing similar early habituation between genotypes, but impaired responses only in *Fmr1* KO genotype during later, steady state habituation.

Discrimination indices were calculated for responses between the tone pip preceding the deviant sound (position 5), and the tone pip directly after (position 7). No differences were observed for either 5^{th} -6^{th} transition ($F(2,18) = 0.725$, $p = 0.498$) or from 6^{th}-7^{th} transition ($F(2,18) = 0.255$, $p = 0.778$) (Figure 6B). These results indicate that while *Fmr1* KO mice have a deficit in habituation across repeated sounds for a 5 Hz repetition rate, no differences in response to the deviant noise or the tone following the deviant noise were observed. Habituation responses in *Mmp-9/Fmr1* DKO mice were similar to WT, indicating that a genetic deletion of *Mmp-9* reversed the deficits in *Fmr1* KO mice at a stimulus repetition rate of 5 Hz.

Discussion

The main findings of this study are as follows: 1) There was a significant repetition rate-dependent reduction in steady state habituation of N1 amplitudes in *Fmr1* KO mice compared to WT mice; 2) MMP-9 levels were higher in the adult auditory cortex of *Fmr1* KO mice compared to WT mice and N1 habituation in *Mmp-9/Fmr1* DKO mice was similar to WT mice; 3) there were no significant differences in N1 amplitude or latency between the WT and *Fmr1* KO mice, but at some rates, the N1 amplitudes were larger, and latencies shorter and less variable, in the *Mmp-9/Fmr1* DKO mice. *Mmp-9* KO and WT mice ERPs were similar in all regards tested except for the small N1 amplitude difference at 0.25 Hz rate. 4) The deviant stimulus response did not show differences across genotypes that were detectable with N1 amplitudes. These data suggest that enhanced steady state cortical activation in the presence of repeated acoustic stimulation may contribute to auditory hypersensitivity in FXS and suggest that abnormal MMP-9 levels may lead to the auditory hypersensitivity in FXS.

Habituation deficits: implications for FXS

Sound evoked cortical N1 amplitudes showed a rapid and repetition rate-dependent reduction across all three genotypes. In response to a stimulus train, the amplitude of the second response in the train is a measure of early habituation, a process related to sensory gating (Rosburg et al., 2004). The response amplitude to the second sound of a pair is normally reduced compared to the first. This was seen in all three genotypes with no significant difference between them suggesting normal sensory gating in *Fmr1* KO mice. The reduction in response to subsequent stimuli in the train over the steady state is thought to reflect refractory/recovery dynamics of neurons and circuits of the auditory system (Fruhstorfer et al., 1970; Karhu et al., 1997). We observed an impaired reduction in the steady state N1 amplitude across the stimulus train in *Fmr1* KO mice, suggesting a deficit in the refractory/recovery dynamics in FXS. The responses asymptote to a stable level after the first two to three repetitions, but the asymptote amplitude was significantly higher in *Fmr1* KO than WT mice. This indicates a higher steady state response in the auditory cortex of *Fmr1* KO mice with repeated stimulation. These results suggest that auditory

hypersensitivity observed in humans with FXS may be due to constantly elevated response in a repetitive acoustic environment.

Habituation to repeated stimuli is also thought to contribute to a build-up of an auditory memory trace, which generates the ERP component known as mismatch negativity when a deviant sound is present. The amplitude of mismatch negativity is lower in humans with FXS (Van der Molen et al., 2012b) that may also reflect a reduced habituation of steady state response. The deviant sound paradigm in this study, however, did not reveal differences between genotypes except for abnormal habituation for the 5 Hz rate in *Fmr1* KO mice. Additional studies in which the deviant stimulus position is randomized within a train are required to gain further insight into sound contrast detection in *Fmr1* KO mice.

The reduced habituation of N1 amplitude in *Fmr1* KO mice is similar to observations in humans with FXS providing, for the first time, an analogous functional sensory outcome measure in mice and humans (Castren et al., 2003; Van der Molen et al., 2012; Schneider et al., 2013). Likewise, the lack of differences in N1 latency between *Fmr1* KO and WT recordings is consistent with observations in humans (Van der Molen et al., 2012a,b). However, unlike data from humans with FXS, the N1 amplitude was not higher in the *Fmr1* KO compared to WT mice. Factors such as differences in repetition rates, anesthesia and scalp *versus* cortical recordings may contribute to these differences. The higher average N1 amplitude in human FXS may be related to the reduced habituation when responses are averaged over multiple repetitions and variability of N1 latency suggesting future studies to evaluate Fano factors for N1 latency.

Mechanisms of habituation deficits in FXS

A previous *in vivo* study of the auditory cortex revealed hyper-responsive neurons in *Fmr1* KO mice (Rotschafer and Razak, 2013). The sustained component of the response, which was mostly absent in WT neurons, was significant in *Fmr1* KO neurons. *Fmr1* KO neurons also showed broader frequency tuning curves. The phasic onset response and the lack of sustained response in cortical neurons depend on an inhibitory input that is delayed relative to the excitatory input (Wehr and Zador, 2003, Wu et al., 2008). The recovery dynamics of cortical neurons to repeated stimuli would at least partly depend on this delayed inhibition in addition to factors such as synaptic depression. Weaker inhibition following an excitatory response to a sound is predicted to result in reduced habituation. The delayed synaptic inhibition is generated by fast spiking interneurons, putatively expressing parvalbumin (PV, Wu et al., 2008). In the somatosensory cortex of *Fmr1* KO mice, PV neurons receive reduced excitatory drive resulting in reduced inhibition in the network and increased UP states (Selby et al., 2007; Gibson et al., 2008). Thus reduced PV-dependent inhibition in the auditory cortex may result in the sustained responses in *Fmr1* KO neurons. Reduced cortical GABA-A mediated inhibition observed in Fmr1 KO cortex may also contribute to broader tuning curves in the *Fmr1* KO mice (Wang et al., 2000). Recovery windows during repeated stimulation in the auditory cortex may also depend on intrinsic excitability and deficits in presynaptic BK channels in *Fmr1* KO mice (Deng et al., 2013) contributing to the habituation phenotype. Thus when a sound is repeated, more neurons across the cortex may

be activated in a sustained fashion in *Fmr1* KO mice due to a combination of reduced synaptic inhibition and enhanced intrinsic excitability resulting in hyperexcitability in FXS.

Role of MMP-9 in FXS habituation deficits

MMP-9 levels were abnormally high in the auditory cortex of *Fmr1* KO mice and the ERP habituation deficits were reversed in *Mmp-9/Fmr1* DKO mice suggesting that abnormally high levels of MMP-9 may contribute to auditory processing deficits in FXS. Although MMP-9 is involved in sensory system development (reviewed in Reinhard et al., 2015), we found that auditory ERP N1 amplitude and habituation are mostly unaffected by the removal of MMP-9 (Mmp9 KO mice). However, removal of MMP-9 in the *Fmr1* KO mice reverses the ERP habituation deficit seen in the *Fmr1* KO mice.

It must be noted that not all ERP properties were similar between the *Mmp-9/Fmr1* DKO and WT mice. *Mmp-9/Fmr1* DKO mice had shorter N1 latencies at the slowest (0.25 and 0.5 Hz) repetition rates, smaller variance in latency and higher N1 amplitude than the WT and *Fmr1* KO mice for some repetition rates. The smaller latency variance may contribute to the larger N1 amplitude because of increased synchrony. Averaging of 100s of trials is typically performed to obtain high signal to noise ratios in ERP studies. Using an averaging approach, differences in average amplitudes could be either because the individual responses have different amplitudes or because of increased latency jitter of individual responses, which then “smear” the average (Mouraux and Ionnetti, 2008). The smaller N1 latency Fano factor of the *Mmp-9/Fmr1* DKO recordings suggest the latter may be the reason here. It remains unclear why the DKO recordings show the latency differences, as this is the first report of auditory responses in this genotype. However, even with elevated N1 amplitudes, *Mmp-9/Fmr1* DKO mouse ERPs show habituation at all rates in a manner similar to WT mice. MMP-9 regulates normal development of sensory cortex (Kaliszewska et al., 2012) and abnormally high or low levels may result in auditory circuit differences.

MMP-9 is a member of a large family of endopeptidases that cleaves extracellular matrix and cell surface receptors at synapses allowing for synaptic and circuit level reorganization. MMP-9 levels are high in the cortex and hippocampus of humans with FXS and *Fmr1* KO mice (Sidhu et al., 2014; Gkogkas et al., 2014). Deletion of MMP-9 from *Fmr1* KO mice results in reversal of molecular and synaptic deficits in the hippocampus of *Fmr1* KO mice as well as some FXS-associated behaviors (Sidhu et al., 2014). Treatment with minocycline, which reduces MMP-9 activity and levels, reduces ERP habituation deficits humans with FXS (Schneider et al., 2013). The reversal of habituation deficits in *Mmp-9/Fmr1* DKO mice in the present study suggest that minocycline may indeed act by reducing MMP-9 levels in the auditory system of humans with FXS.

MMP-9 targets components of the extracellular matrix known as perineuronal nets (PNNs), which preferentially surround PV-expressing inhibitory interneurons in the cortex. High MMP-9 levels may result in reduced PNN formation around PV interneurons affecting their activity (Cabungcal et al., 2013) and leading to impaired inhibition (Selby et al., 2007; Gibson et al., 2008), and enhanced excitability in *Fmr1* KO auditory cortex. Stabilization of PNN around PV interneurons due to a genetic deletion of *Mmp-9* may be responsible for the reversal of habituation deficits in *Mmp-9/Fmr1* DKO mice. Future studies are needed to

characterize PV/PNN expression in *Mmp-9/Fmr1* DKO mice and the effects of PNN disruption on auditory ERPs.

Conclusions

Auditory cortical ERP habituation is reduced in *Fmr1* KO mice in a manner analogous to that seen in humans with FXS. The abnormal levels of MMP-9 in the auditory cortex of the *Fmr1* KO mice suggest that the ERP habituation deficit may have a local cortical origin. However, FMRP is expressed across the auditory system and the observed cortical habituation deficits may have multiple origins (Strumbos et al., 2010; Wang et al., 2014; Rotschafer et al., 2015). Future studies will address this question by examining ERPs in forebrain-specific *Fmr1* KO mice. Although ERPs have low spatial resolution to address mechanisms at the circuit, cortical depth or single neuron level, the similarity of the habituation deficits between humans and mice provides a functional biomarker that is translation relevant. High throughput auditory ERP measurements can aid development of therapeutic strategies using *in vivo* physiological measure to complement studies at the molecular and behavioral levels. Given that similar sensory processing deficits are seen in FXS and ASD, auditory ERP habituation may serve more broadly as a pre-clinical model for drug discovery in autism.

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Significance

Fragile X Syndrome (FXS) is the leading known genetic cause of autism spectrum disorders. Individuals with FXS show symptoms of auditory hypersensitivity. These symptoms may arise due to sustained neural responses to repeated sounds, but the underlying mechanisms remain unclear. For the first time, this study shows deficits in habituation of neural responses to repeated sounds in the *Fmr1* KO mice as seen in humans with FXS. We also report an abnormally high level of matrix metalloprotease-9 (MMP-9) in the auditory cortex of *Fmr1* KO mice and that deletion of Mmp-9 from *Fmr1* KO mice reverses habituation deficits. These data provide a translation relevant electrophysiological biomarker for sensory deficits in FXS and implicate MMP-9 as a target for drug discovery.

Highlights

- *Fmr1* KO mice were deficient in habituation of sound evoked responses, similar to humans.
- *Fmr1* KO mice had abnormally high levels of Mmp-9 in auditory cortex.
- Habituation in *Fmr1* KO was rescued with the additional knock out of *Mmp-9*.
- These data provide a translational relevant functional biomarker for Fragile X Syndrome.

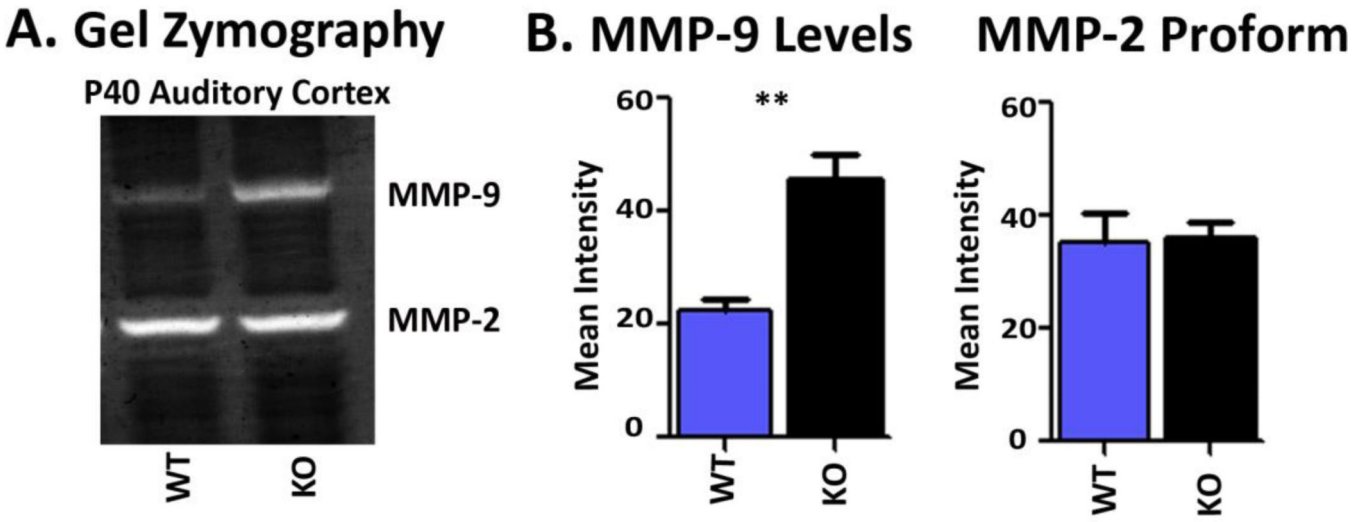

Figure 1. Mmp-9 levels are upregulated in the auditory cortex of adult *Fmr1* KO mice

(A) Detection of the levels of MMP-9 and MMP-2 in P40 auditory cortex of WT and *Fmr1* KO mice. Levels of the gelatinases were detected by gelatin zymography and quantified by densitometry. (B) Bar graphs show average levels of MMP-9 (left) and MMP-2 (right) and the error bars indicate SEM (n=5 mice per group). MMP-9 levels were higher in *Fmr1* KO mice (KO) than in WT mice, $t(8) = 4.65$, $p = 0.0016$ (**); however there were no differences in the levels of MMP-2 between *Fmr1* KO mice (KO) and WT mice, $t(8) = 0.13$, $p = 0.8983$.

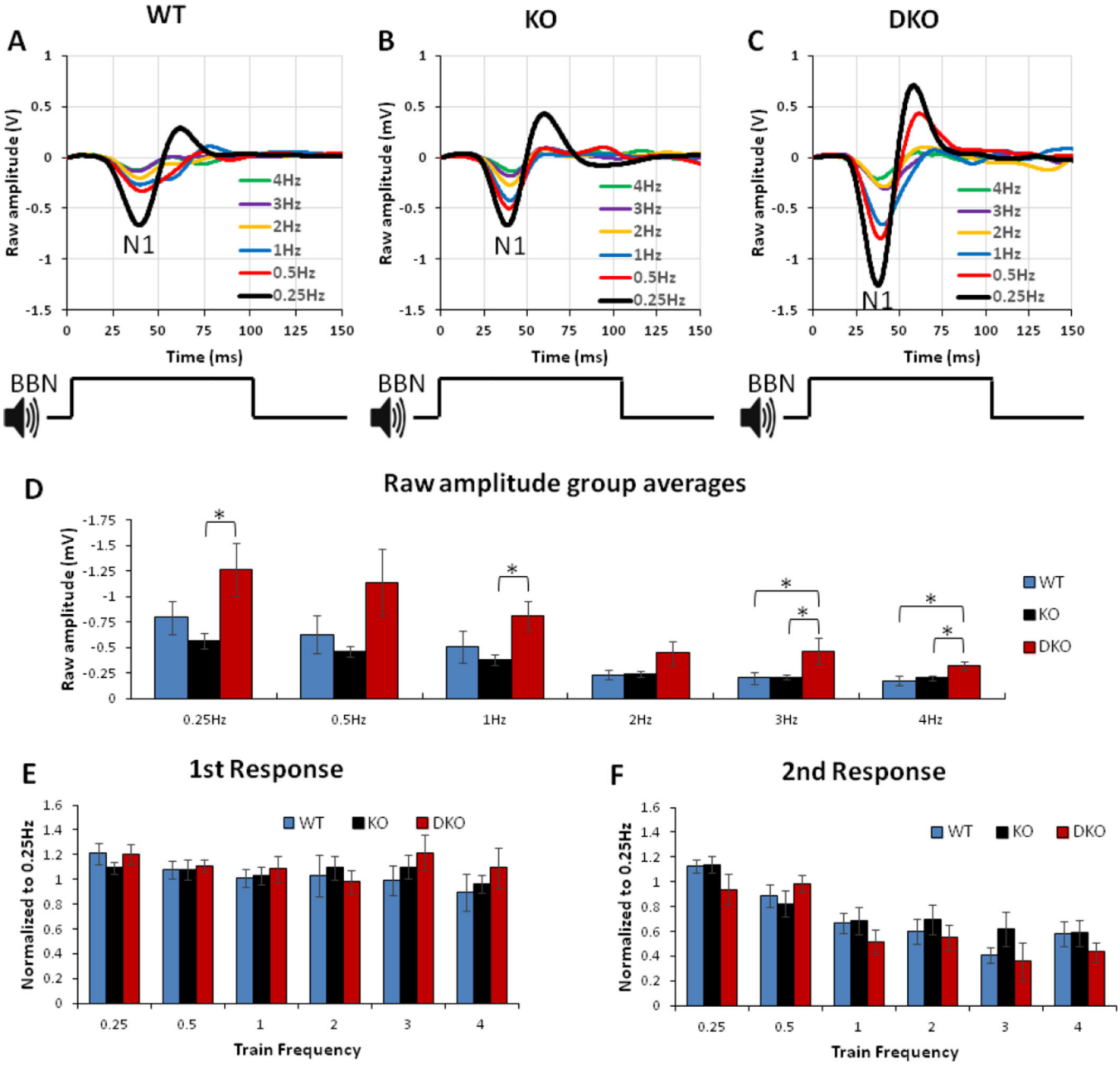

Figure 2. Sound evoked ERPs in auditory cortex and early habituation

(A–C) Graphs show representative average ERPs across 20-sound presentation trains at different repetition rates plotted for individual examples from WT (A), *Fmr1* KO (B), and *Mmp-9/Fmr1* double KO (DKO) (C) mice. The N1 component is marked for all genotypes. The 100ms broadband noise (BBN) used as the stimulus is represented below the ERPs with sound onset at 0 msec. Habituation to broadband noise was calculated by normalizing each response in an individual train, to the average amplitude of 20 responses at the same recording location during a 0.25 Hz train of BBN.

(D) Graph shows raw average amplitudes for each rate for WT (n=7 recording sites), *Fmr1* KO (n=9 recording sites), and *Mmp-9/Fmr1* DKO (n=7 recording sites) for each rate.

(E) Graph shows average normalized N1 amplitudes during the first sound in each train and error bars indicate SEM.

(F) Graph shows average normalized N1 amplitudes for the second sound in each train and error bars indicate SEM. The lack of differences in the normalized amplitudes during the first sound indicate stable responses throughout the recording session while no difference in the second response indicate that all genotypes habituate similarly early in the sound trains. Statistical analysis was performed using two-way ANOVA followed by Bonferonni Post-hoc comparisons showing significant differences between genotypes at specific rates (*$p<0.05$).

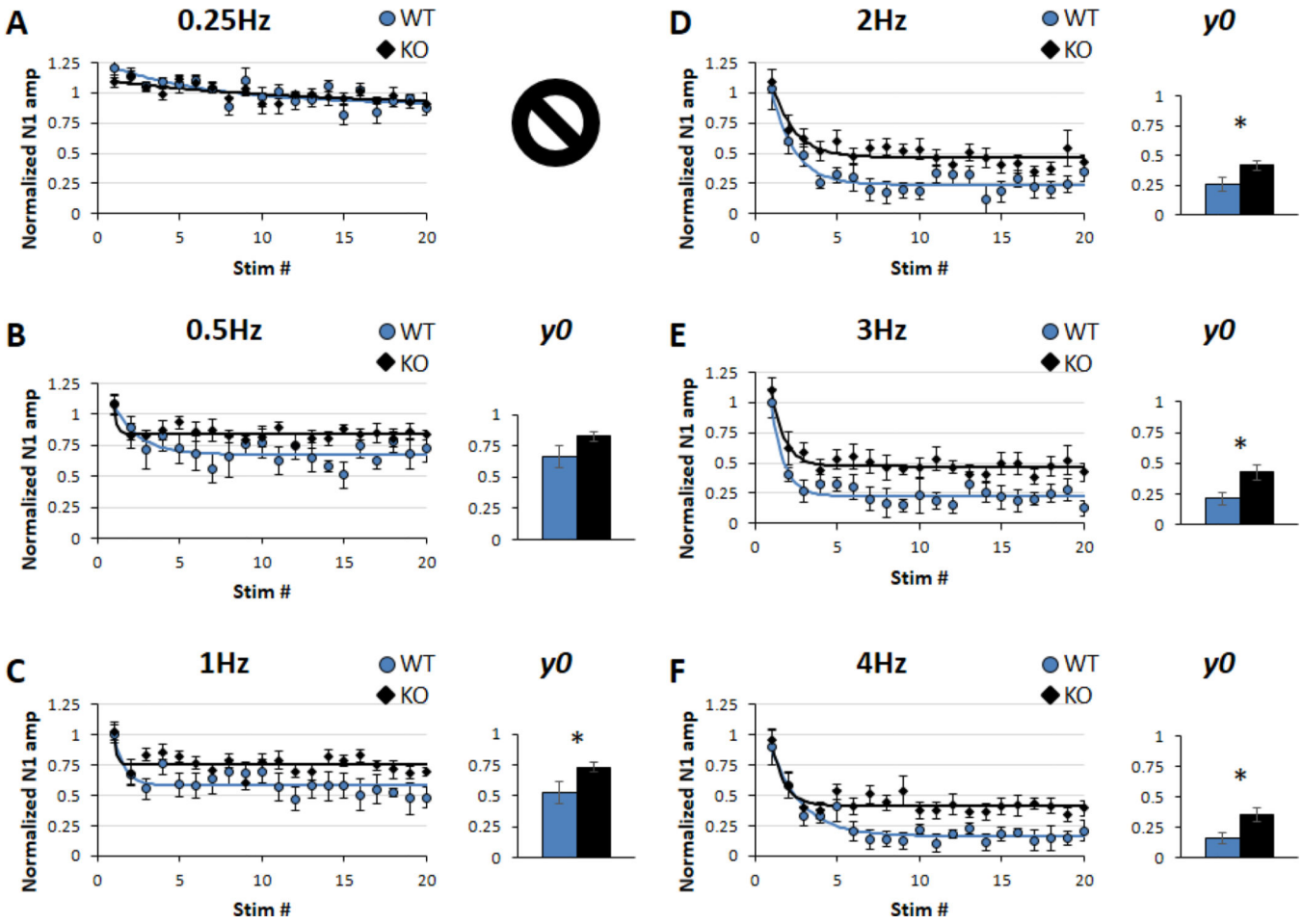

Figure 3. *Fmr1* KO mice show a deficit in steady state habituation to sounds

Individual BBN trains were fit to an exponential decay function $y = y0 + Ae^{(-x/t)}$. The parameter *y0*, which represents the y value at which a limit is reached, was calculated for each train and averages between genotypes were compared for each repetition rate. Each line plotted in this figure was fit to the averaged group data points, conversely the histograms shown next to the plots are genotype averages of individually fitted curves. (A–F) Graphs show representative average ERPs in WT and *Fmr1* KO mice across 20-sound presentation trains for 0.25Hz (A), 0.5 Hz (B), 1Hz (C), 2Hz (D), 3Hz (E) and 4Hz (F) repetition rates. At 0.25Hz repetition rate, multiple individual observation were unsuccessfully fit using the decay function, therefore statistical analysis of *y0* could not be performed. (B) Student's t-tests revealed no significant difference at 0.5Hz ($p = 0.09$). (C–F) Rates between 1Hz-4Hz had consistent differences ($p < 0.05$), with KO recordings being consistently higher than WT at steady state.

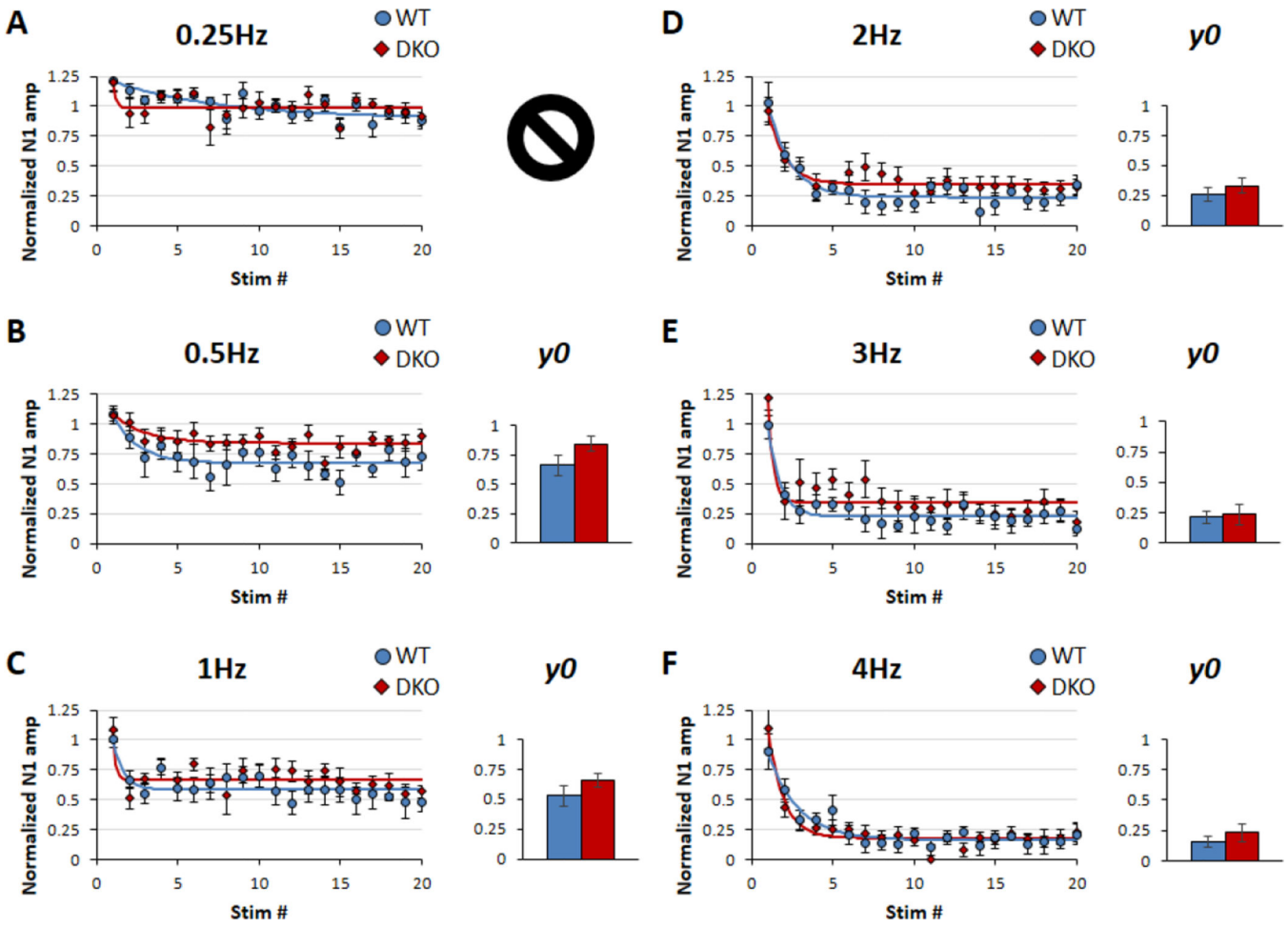

Figure 4. ***Mmp-9/Fmr1*** **DKO mice show rescue of deficit in steady state habituation of N1 amplitude**

(A–F) Graphs show representative average ERPs in WT and *Mmp-9/Fmr1* DKO mice across 20-sound presentation trains for 0.25Hz (A), 0.5 Hz (B), 1Hz (C), 2Hz (D), 3Hz (E) and 4Hz (F) repetition rates. The same procedures and comparisons were made here as in Figure 2. (A) At 0.25Hz repetition rate, multiple individual observation were unsuccessfully fit using the decay function, therefore statistical analysis of *y0* could not be performed. (B–F) Student's t-tests revealed no significant difference between WT and DKO recordings at any rate tested ($p > 0.05$).

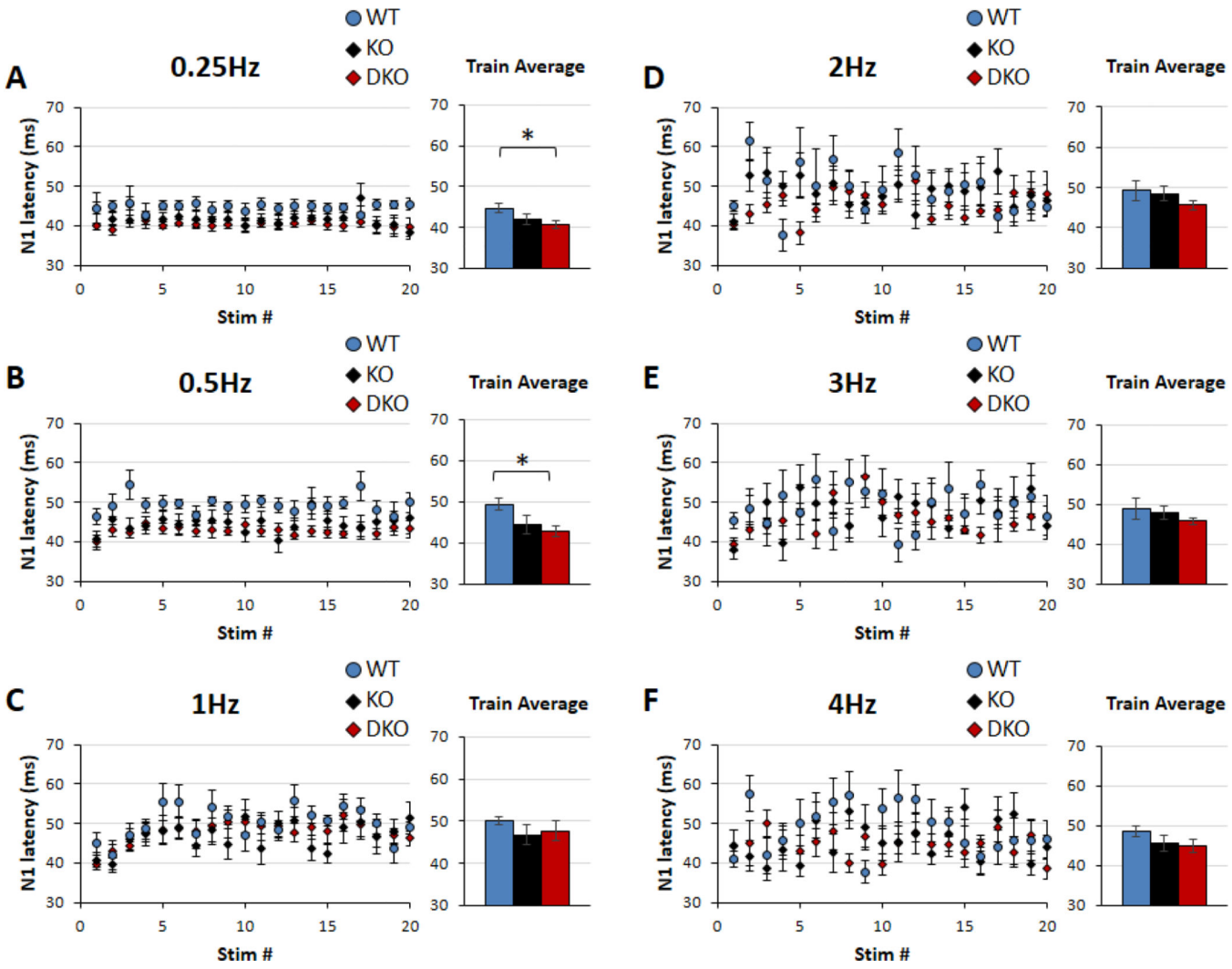

Figure 5. N1 Latencies are not different between WT and *Fmr1* KO mice

(A–F) Graphs show average N1 latencies and error bars indicate SEM. (A) Averaged N1 latencies at 0.25Hz were very consistent across the entire stimulus train for all genotypes. Statistical analysis was performed using two-way ANOVA followed by Bonferonni Post-hoc comparisons showing significant differences in average N1 latencies between WT and DKO genotypes at 0.25Hz (A, *p<0.05) and 0.5Hz (B, *p<0.05). (C–F) All other rates were tested in the same manner; no differences were seen between genotypes for latency at theses fast rates.

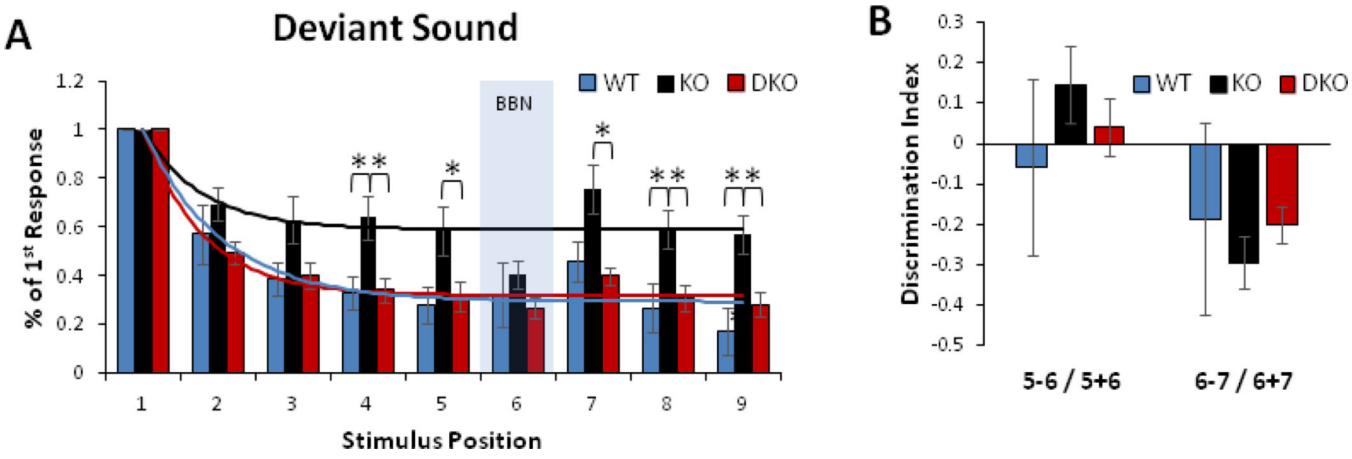

Figure 6. Deviant sound paradigm at 5Hz reveals habituation deficit in *Fmr1* KO but not *Mmp-9/Fmr1* DKO

(A) All sounds in the train consisted of 12 kHz tone pips and a deviant BBN sound presentation in the 6th position (highlighted area). Lines were fit to a decay function using genotype averages. After normalization to the 1st response in the train there was an effect of genotype and position in the train. WT (blue, n = 5 recording sites); KO (black, n = 9 recording sites); DKO (red, n = 7 recording sites). (B) Discrimination indexes were calculated for differences in response to the deviant noise showing no difference between genotypes. In general, a specific deficit in steady state levels of habituation was restricted to KO animals using tone pips at 5Hz, similar to the KO deficit to BBN at high rates, with the DKO mice showing responses similar to WT mice. Statistical analysis was performed using two-way ANOVA followed by Bonferonni Post-hoc comparisons showing significant differences between genotypes at specific positions in the train (*p<0.05).