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Reversal of ultrasonic vocalization deficits in a mouse model of Fragile X Syndrome with minocycline treatment or genetic reduction of MMP-9

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ABSTRACT

Fragile X Syndrome (FXS) is a leading genetic cause of autism and intellectual disabilities. The *Fmr1* knockout (KO) mouse is a commonly studied pre-clinical model of FXS. Adult male *Fmr1* KO mice produce fewer ultrasonic vocalizations (USVs) during mating, suggestive of abnormal social communication. Minocycline treatment for 2 months from birth alleviates a number of FXS phenotypes in mice, including USV call rate deficits. In the current study, we investigated if treatment initiated past the early developmental period would be effective, given that in many cases, individuals with FXS are treated during later developmental periods. Wildtype (WT) and *Fmr1* KO mice were treated with minocycline between postnatal day (P) 30 and P58. Mating-related USVs were then recorded from these mice between P75 and P90 and analyzed for call rate, duration, bandwidth, and peak frequency. Untreated *Fmr1* KO mice call at a significantly reduced rate compared to untreated WT mice. After minocycline treatment from 1 to 2 months of age, WT and *Fmr1* KO mice exhibited similar call rates, due to an increase in calling in the latter group. Minocycline is thought to be effective in reducing FXS symptoms by lowering matrix-metalloproteinase-9 (MMP-9) levels. To determine whether abnormal MMP-9 levels underlie USV deficits, we characterized USVs in *Fmr1* KO mice which were heterozygous for MMP-9 (MMP-9$^{+/-}$/*Fmr1* KO). The MMP-9$^{+/-}$/*Fmr1* KO mice were between P75 and P90 at the time of recording. MMP-9$^{+/-}$/*Fmr1* KO mice exhibited significantly increased USV call rates, at times even exceeding WT rates. Taken together, these results suggest that minocycline may reverse USV call rate deficits in *Fmr1* KO mice through attenuation of MMP-9 levels. These data suggest targeting MMP-9, even in late development, may reduce FXS symptoms.

1. Introduction

Fragile X Syndrome (FXS) is a leading genetic cause of intellectual and social communication disabilities and autism in humans, affecting 1 in 8000 females and 1 in 4000 males [1,2]. FXS is caused by an expansion of CGG trinucleotide repeats in the 5′ untranslated region of the *fragile X mental retardation (Fmr1)* gene. This leads to a reduction or loss of fragile X mental retardation protein (FMRP, [3]), which is involved in normal synaptic development and plasticity [4,5]. FXS phenotypes in humans include a wide range of learning disabilities, cognitive impairment, hyperactivity, anxiety, and language deficits such as repetition of sounds and words and articulation difficulties [6–8]. The *Fmr1* knockout (KO) mouse recapitulates various FXS-like phenotypes including learning deficits, anxiety, and sensory hypersensitivity [9,10]. *Fmr1* KO mice also show abnormal social interactions including reduced ultrasonic vocalizations (USV) during mating interactions [11].

Minocycline is an FDA-approved antibiotic that has recently been tested as a potential treatment for FXS [12–14]. Eight weeks of oral administration of minocycline in FXS patients is associated with significant improvements in Aberrant Behavior Checklist-Community Edition Irritability Subscale scores. These subscales measure various problem behaviors within 5 domains, including lethargy, hyperactivity, stereotypy, and inappropriate speech [12]. Minocycline also improved language and behavioral functions in children with FXS based on caretaker reports [13]. A randomized, double blind, placebo-controlled trial of children and adolescents (age 3.5–16 years) with up to 3 months of minocycline treatment showed improvement in the Clinical Global Impression Scale [15]. At the physiological level, minocycline treatment in humans with FXS reverses habituation deficits in sound evoked electrophysiological responses [14]. There is also evidence for reversal

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of various behavioral phenotypes in the *Fmr1* KO mouse with minocycline treatment [16]. Minocycline treatment from birth to 2 months restores USV call rates of *Fmr1* KO mice to WT levels [11]. In addition, KO mice treated for a month beginning a week after birth show less anxiety in the elevated plus maze and more exploratory behavior than untreated KO mice [17].

FXS is a neurodevelopmental disorder, but current clinical trials of drugs are conducted in adolescents and young adults. Few studies have compared treatments across different developmental ages in terms of efficacy in reversing symptoms. Dansie et al., [16] showed that minocycline treatment for 4 or 8 weeks reverses hyperactivity and anxiety-like behaviors in *Fmr1* KO mice, and beneficial effects were long lasting when treatment occurred during early development [16]. In our previous study, *Fmr1* KO mice treated with minocycline from birth to 2 months restored USV call rate deficits to WT levels [11]. However, it remains unclear if the effectiveness was due to modification of vocalization-related circuits during a critical developmental period, or if late developmental treatment is sufficient to reverse USV call rates. Therefore, the first aim of this study was to determine if minocycline treatment in 1–2 month old mice would reverse USV call rate deficits. In order to test this, we analyzed mating related USVs produced by *Fmr1* KO mice that were administered minocycline between P30 and P58 (1–2 months of age). The mice were tested between P75 and P90 (2.5–3 months of age). In addition to call rates, we characterized properties of individual calls including duration, spectral bandwidth, and peak frequency to gain insight into whether syllable structure is abnormal in the *Fmr1* KO mice.

Minocycline acts on multiple mechanisms in the brain including microglia function and apoptosis pathways [18]. In addition, minocycline may reduce FXS symptoms through inhibition of matrix metalloproteinase-9 (MMP-9), an enzyme involved in cleavage of extracellular matrix components [19], including specialized assemblies found around inhibitory interneurons called perineuronal nets [20,21]. MMP-9 translation is regulated by FMRP and MMP-9 mRNA is a known FMRP target [22]. Indeed, MMP-9 levels are elevated in FXS [23–26], and minocycline administration lowers plasma and brain MMP-9 levels in both humans and mice [17,23]. Genetic loss or reduction of MMP-9 in *Fmr1* KO mice restores both structural and functional deficits associated with FXS, including dendritic spine abnormalities in adult hippocampus [25], impaired perineuronal net (PNN) formation around parvalbumin interneurons in the auditory cortex [20], and auditory evoked potential habituation deficits in KO mice [26]. As minocycline may restore FXS-related deficits through attenuation of MMP-9 levels [27], the second aim of this study was to determine if genetic reduction of MMP-9 in *Fmr1* KO mice would restore USV call rates to WT levels. In order to test this, we analyzed USVs in in 2.5–3 month old *Fmr1* KO mice which were heterozygous for MMP-9 (MMP-9 $^{+/-}$*/Fmr1* KO).

2. Methods

2.1. Mice

Breeding pairs of FVB.Cg−*Mmp*−*9tm1Tvu*/J, FVB.129P2-Pde6b^{+}Tyr^{c-ch}Fmr1^{tm1Cgr}/J (Jax 004624; Fmr1 KO) and their congenic controls FVB.129P2-Pde6b^{+}Tyr^{c-ch}/AntJ controls (Jax 002848; WT) obtained from Jackson laboratories were housed in an accredited vivarium with 12 h light/dark cycle. The FVB.Cg−*Mmp*−*9tm1Tvu*/J mice were backcrossed, in-house, with *Fmr1* KO or WT mice for at least five generations. Genetic reduction in MMP-9 levels was achieved by deleting only 1 allele of the *Mmp9* gene in *Mmp9* $^{+/-}$*/Fmr1* KO mice [20]. The MMP-9 $^{+/-}$*/Fmr1* KO mice (henceforth, 'HET' mice) were housed in the same vivarium room as all other mice used in this study. Neither the minocycline treated nor the *Mmp9* $^{+/-}$*/Fmr1* KO mice show any overt deficits in behavior or appearance. At the time of USV recording, all mice were between 2.5–3 months of age. As minocycline treatment did not need to be administered in the HET mice, these mice were weaned into separate cages until they reached 2.5–3 months of age for USV recordings. All studies were performed in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee.

2.2. Minocycline administration

WT and *Fmr1* KO mice were housed in separate cages. Minocycline (30 mg/kg) was dissolved in drinking water of WT and *Fmr1* KO mice and provided every day for 28 days starting at P30. Water consumption was tracked daily by determining water levels with a graduated cylinder prior to adding fresh water. Previous studies have shown that this method of minocycline administration leads to detectable concentrations of minocycline in mouse blood [28]. After minocycline treatment, USVs were recorded from male WT and *Fmr1* KO mice between P75 and P90. None of the female partner mice received minocycline treatment. One of the major purposes of the present study was to compare our findings with data from Rotschafer et al., [11] in which WT and *Fmr1* KO mice were treated with minocycline from birth to 2 months of age, after which USVs were recorded ˜3 months of age. To stay consistent with that protocol, and to identify if treatment between just 1 and 2 months of age (young adult) would have the same effect, we recorded USVs at 2.5 to 3 months age in this study.

2.3. Recording mouse vocalizations

The main goal of this study was to record USVs from mice in a social interaction task. We chose courtship behavior because the USVs generated in this context have been well characterized [29] and are likely less influenced by developmental experience [30]. Male mice produce stereotyped USVs when placed with a receptive female [31]. We did not use female urine as a stimulus [32,33] or record vocalizations after the female was removed [34] because these do not constitute the same dyadic social interactions as observed in courtship. In addition, because one of the goals of the study was to compare with our previous results in terms of optimal treatment windows with minocycline, we used similar methods with dyadic mixed-sex pairing. Females can produce acoustically similar calls as males in complex social interactions (e.g., 2 males, 2 females). In dyadic mixed-sex interactions, Warburton et al. [35] concluded based on laryngeal nerve damage that males were the primary emitters of USVs. Nevertheless, to reduce the potential impact of genotype on female vocalizations in our study, we only used female *Fmr1* KO mice as the partner mouse in all tests.

To record USV calls, virgin male WT or *Fmr1* KO mice were placed in a 50.8 × 40.6 x 20.3 cm plastic recording chamber within a soundproof room (Gretch-Ken Industries Inc.) and allowed to habituate for 5 min. The female mouse was not in the chamber during this habituation period; it was only the test mouse for the first 5 min. Estrus was induced in virgin female *Fmr1* KO mice 36 h before pairing with a male, and then placed inside the recording chamber with WT or *Fmr1* KO male mice for 10 min. USVs were recorded during this 10 min of pairing. Estrus was induced by i.p. injection of 0.06 mL of 0.6 mg/mL estradiol benzoate solution 36 h prior to mating and 0.02 mL of 6 mg/mL progesterone 4 h prior to mating [36]. USVs were recorded with a full spectrum Petterssen D1000x (Pettersson Elektronik AB, Upsala, Sweden) bat detector (250 kHz sampling rate) placed 22 cm above the enclosure. This detector has been mostly used to record bat echolocation calls, with a few studies using it to record vocalizations of other mammals including genetic mouse models [37–39]. The detector gain was consistent across pairings. USV calls were recorded when the following virgin male mice were paired with virgin female untreated *Fmr1* KO mice: untreated WT (WT, n = 7), untreated *Fmr1* KO (KO, n = 6), minocycline-treated WT (MTWT, n = 6), minocycline-treated *Fmr1* KO (MTKO, n = 8), and MMP-9$^{+/-}$*/Fmr1* KO (HET, n = 10). Once mice were paired one time for USV recording, they were not used again in this study.

2.4. Vocalization analysis

USVs were analyzed using Avisoft SASLab Pro software. Audio recordings were first cut to 1 min segments. A bandpass filter from 30 to 100 kHz was used to remove all background noise outside the frequency range of interest. Thus only USVs were analyzed. A pulse train analysis was applied to capture and tag the calls that crossed a particular intensity threshold. The tag associated with each call was its respective chronological order number in the set of calls per minute. The pulse train analysis refers to the software function used to automatically extract the duration, bandwidth, and peak frequency information from each call that met the threshold for tagging. A threshold was individually set for each 1-minute segment of the sound file and placed in such a way that it captured all of the calls recorded in that time frame. Calls were then tagged and manually inspected. Tags were kept for calls which matched the known shapes of mouse USVs. Tags were removed if sounds that were not calls (background noise) were detected. These procedures resulted in the deletion of < 3% of all tagged sounds. Due to the unique intensity threshold placed on each segment and subsequent manual inspection to delete background noise, it is likely that all USV calls recorded were detected as such.

Calls were then analyzed for call rate (USVs/minute), individual call duration (measured as the time between the beginning and end of each individual USV), bandwidth (the range of frequencies within each USV), and peak frequency (highest frequency value reached by each particular USV). Statistical analyses were conducted using SigmaPlot software and the tests used are mentioned alongside description of results below.

3. Results

3.1. Minocycline treatment from 1 to 2 months reverses USV call rate deficit in Fmr1 KO mice

Mating-elicited USVs were recorded from adult WT and *Fmr1* KO mice between 2.5 and 3 months of age. Four groups of mice were compared in a 2 genotype (WT, *Fmr1* KO) x 2 treatment (regular water, MT (minocycline-treated) water) design (WT, *Fmr1* KO, MTWT and MTKO). The major goals of this analysis were to determine whether the vocalization properties of adult *Fmr1* KO mice were different than WT mice, and to determine if minocycline treatment between 1–2 months of age corrected such deficits.

Fig. 1 shows example USVs recorded from a WT (A), *Fmr1* KO (B), and HET (C) mouse. These snippets are illustrative of the main finding that *Fmr1* KO mice call at a reduced rate compared to WT mice, similar to our previous findings [11]. Fig. 2A shows the average minute-by-minute changes in call rate across the 4 groups of mice over the course of a 10-minute courtship period. All groups showed a decline in call rate between approximately the first and second half of the mating window. During the first 5 min, where most calling occurred, *Fmr1* KO mice produced significantly lower call rates compared to WT mice. When the average call rate over the first 5 min was compared (Fig. 2B), a 2-way ANOVA showed an effect of genotype ($F(1,23) = 5.02$, $p = 0.035$), but no effect of treatment ($F(1,23) = 0.20$, $p = 0.70$) and no significant treatment x genotype interactions ($F(1,23) = 2.25$, $p = 0.15$). Tukey post-hoc pairwise comparison for the untreated groups revealed a WT vs. *Fmr1* KO difference ($p = 0.016$), but no difference between MT treated WT and *Fmr1* KO groups ($p = 0.60$).

To determine if the untreated *Fmr1* KO mice called at a significantly lower rate than the MT treated *Fmr1* KO mice, we performed a 2-way ANOVA (time in minutes and treatment as factors) and found a significant effect of treatment ($F(1,60) = 9.13$, $p = 0.004$), but no significant effect of time ($F(4,60) = 1.95$, $p = 0.11$) or treatment x time interactions ($F(4,60) = 0.17$, $p = 0.96$). A similar analysis of WT and MT treated WT mice showed no difference for treatment ($F(1,55 = 1.37$, $p = 0.25$), time ($F(4,55) = 1.88$, $p = 0.13$) or treatment x time interactions ($F(4,55) = 0.06$, $p = 0.99$). This indicates that during the first 5 min of dyadic pairing the untreated *Fmr1* KO mice called at a significantly lower rate than untreated WT mice, and minocycline treatment in the 1–2 month age range is sufficient to increase USV call rate in *Fmr1* KO mice to levels similar to WT mice. In addition, minocycline treatment did not affect call rate in WT mice demonstrating that the effects of minocycline are specific to the *Fmr1* KO mice. There were no differences in average duration and bandwidth of individual calls in the first five minutes (2-way ANOVA: average duration: genotype effect: $F(1,23) = 0.12$, $p = 0.74$; treatment effect: $F(1,23) = 1.59$, $p = 0.22$; interaction: $F(1,23) = 2.74$, $p = 0.11$; average bandwidth: genotype effect: $F(1,23) = 3.54$, $p = 0.073$; treatment effect: $F(1,23) = 0.019$, $p = 0.89$; interaction: $F(1,23) = 0.0005$, $p = 0.98$), but there was a trending genotype difference in average peak frequency (2-way ANOVA: average peak frequency: genotype effect: $F(1,23) = 4.41$, $p = 0.05$; treatment effect: $F(1,23) = 0.34$, $p = 0.57$; interaction: $F(1,23) = 0.03$, $p = 0.86$). Bonferroni post-hoc pairwise comparisons for genotype revealed a WT vs. *Fmr1* KO difference ($p = 0.05$), but no difference between MT treated WT and *Fmr1* KO groups ($p = 0.18$) (Fig. 3A-C). With the exception of a trend for decreased average call peak frequency in KO mice, the call properties were mostly similar between genotypes and treatments.

Although the second 5 min of dyadic interactions produced significantly reduced calling, we examined potential genotype or treatment effects during this time window. The average call rate over the second 5 min was compared using a 2-way ANOVA (Fig. 2C), but no significant effects were observed for genotype ($F(1,23) = 0.63$, $p = 0.44$), treatment ($F(1,23) = 0.16$, $p = 0.69$), or treatment x genotype interaction ($F(1,23) = 0.18$, $p = 0.67$). No differences were observed in any of the average spectrotemporal properties of the calls during the second five minutes (2-way ANOVA: average duration: genotype effect: $F(1,22) = 3.47$, $p = 0.08$; treatment effect: $F(1,22) = 0.65$, $p = 0.43$; interaction: $F(1,22) = 0.68$, $p = 0.42$; average bandwidth: genotype effect: $F(1,22) = 0.31$, $p = 0.58$; treatment effect: $F(1,22) = 3.86$, $p = 0.06$; interaction: $F(1,22) = 0.35$, $p = 0.56$; average peak frequency: genotype effect: $F(1,22) = 3.58$, $p = 0.072$; treatment effect: $F(1,22) = 0.31$, $p = 0.59$; interaction: $F(1,22) = 0.36$, $p = 0.55$) (Fig. 3D–F).

3.2. Genetic reduction of MMP-9 in Fmr1 KO mice restores USV call rates to WT levels

To determine if abnormal MMP-9 levels in the *Fmr1* KO mouse may be a mechanism leading to reduced USV call rate, we recorded calls from MMP-9 $^{+/-}$*/Fmr1* KO mice, which are *Fmr1* KO mice heterozygous for MMP-9 (henceforth referred to as HET mice). Fig. 4A shows the average minute-by-minute dynamics of call rates across the 3 groups of mice (WT, *Fmr1* KO, and HET). The WT and *Fmr1* KO mice data are the same as shown in Fig. 2. A qualitative examination of Fig. 4A shows that HET mice called at a rate similar to the WT mice and higher than the *Fmr1* KO mice over the first 5 min of the mating window. Interestingly, the HET mice appear to sustain the calling rate even during the second half of the mating window. These observations were confirmed by a 2-way ANOVA (time in minutes and genotype as factors) which showed a main effect of genotype ($F(2,200) = 21.64$, $p < 0.001$), an effect of time ($F(9,200) = 5.60$, $p < 0.001$) but no time x genotype interactions ($F(18,200) = 1.45$, $p = 0.11$). Tukey post-hoc tests showed significant differences in call rates between WT and KO mice ($p < 0.01$), KO and HET mice ($P < 0.001$) and WT and HET mice ($p < 0.01$). This suggests that the genetic reduction of MMP-9 in *Fmr1* KO mice restored call rates to WT levels. The observed difference in call rate between WT and HET mice is attributed to enhanced calling in the latter group throughout the mating window.

Reduced call rates in KO mice and restoration of calls with MMP-9 reduction is also evident from analysis of average call rates during the first (Fig. 4B) and second half of the mating window (Fig. 4C). During

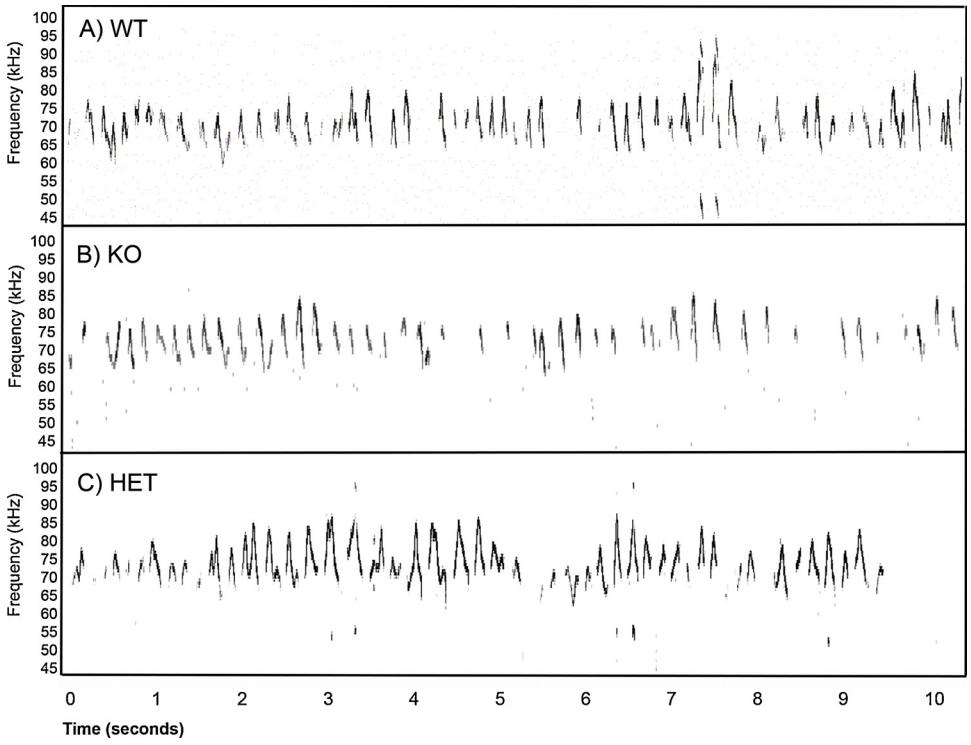

Fig. 1. Example spectrograms of ultrasonic vocalizations recorded in a mating context from a male WT (A), a male *Fmr1* KO (B), and a male HET (C) mouse. In all three examples, the females were untreated, virgin and estrous induced *Fmr1* KO mice. These example 10 s snippets were chosen to illustrate the point that *Fmr1* KO mice produce calls at a reduced rate.

the first 5 min of mating, KO mice exhibited fewer calls than WT or HET mice (One-way ANOVA: $F(2,20) = 3.74$, $p = 0.04$; Tukey test of main effects WT vs. KO $p = 0.07$, HET vs. KO $p = 0.08$, WT vs. HET $p = 1.00$). In contrast, during the second five 5 min of mating, WT and KO call rates were comparably low, while HET mice maintained a high call rate (One-way ANOVA: $F(2,20) = 5.89$, $p = 0.01$: Tukey test of main effects WT vs. KO $p = 1.00$, HET vs. KO $p = 0.033$, HET vs. WT $p = 0.02$.

There were no differences in the spectrotemporal properties of the calls in the first 5 min of courtship interactions (One-way ANOVA: average duration: $F(2,20) = 1.25$, $p = 0.31$; average bandwidth: $F(2,20) = 0.75$, $p = 0.49$; average peak frequency: $F(2,20) = 0.98$, $p = 0.39$) (Fig. 5). In the second five minutes, there was a significant increase in average call duration between WT and HET mice (1-way ANOVA: $F(2,20) = 4.32$, $p = 0.03$, Tukey test of main effects HET vs. WT $p = 0.03$, KO vs. HET $p = 0.15$, KO vs. WT $p = 0.80$), but no difference in bandwidth ($F(2,20) = 2.09$, $p = 0.15$) or peak frequency ($F(2,20) = 0.68$, $p = 0.52$). The average difference in call duration during the second 5 min may stem from the fact the WT and *Fmr1* KO mice produce far fewer calls compared to HET mice during that time. Taken together, these data indicate that genetic reduction of MMP-9 in *Fmr1* KO mice increases call rates. These findings suggest that enhanced MMP-9 may contribute to reduced calling rates in *Fmr1* KO mice.

4. Discussion

In this study, we found that adult *Fmr1* KO mice produce USVs at a significantly reduced rate compared to WT mice during dyadic mixed-sex courtship interactions. Minocycline treatment between 1 and 2 months of age results in an increase in USV call rate in the *Fmr1* KO mice, without affecting WT mice. These data indicate that treatment with minocycline during young adult ages is sufficient to increase call rates in the ˜3 month old *Fmr1* KO mice, similar to the treatment from birth [11]. The third major and novel finding of this study is that genetic reduction of MMP-9 in *Fmr1* KO mice results in increased USV call rates. Aside from an increase in average duration of calls in the HET

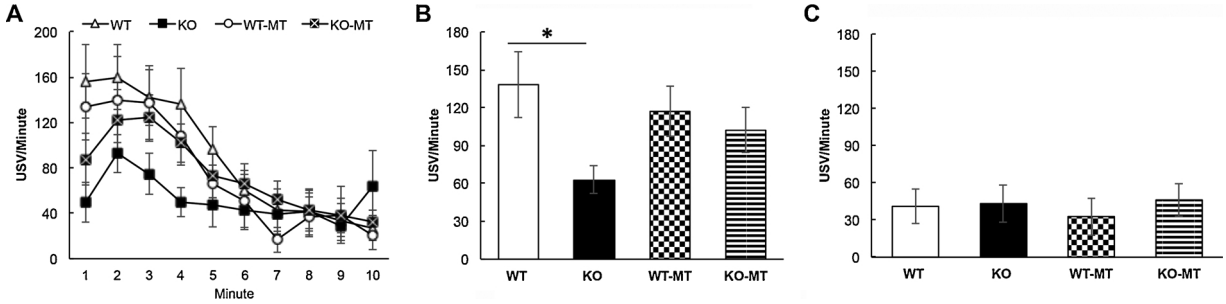

Fig. 2. Minocycline treatment (MT) between P30-P58 increases calling rate in *Fmr1* KO mice to WT levels. A) Minute-by-minute mean (+/- s.e.) USV call rate during the course of 10 min courtship interactions in a 2 genotypes (WT, *Fmr1* KO) x 2 treatments (regular water, MT (minocycline-treated) water) design. The graph shows that the calling rates tend to decrease over time in all 4 groups of mice. Over the first 5 min, WT mice call at a higher rate (calls/minute) than the *Fmr1* KO mice. The MT mice (both genotypes) tend to call at intermediate rates. B) To quantify genotype and treatment effects, the mean calling rates during the first 5 min were compared in the 2 genotypes x 2 treatments design. For the first 5 min, a 2-way ANOVA showed a genotype effect $F(1,23) = 5.02$, $p = 0.035$, but no significant effect of treatment ($p = 0.70$) and no significant treatment x genotype interaction ($p = 0.10$). Tukey post-hoc pairwise comparison revealed a WT vs. *Fmr1* KO difference in the untreated group (*$p = 0.016$), but not a difference in the MT treated group ($p = 0.60$). C) For the second 5 min of pairing, a 2-way ANOVA showed no genotype effect $F(1,23) = 0.63$, $p = 0.44$, no effect of treatment ($p = 0.69$), and no significant treatment x genotype interaction ($p = 0.67$).

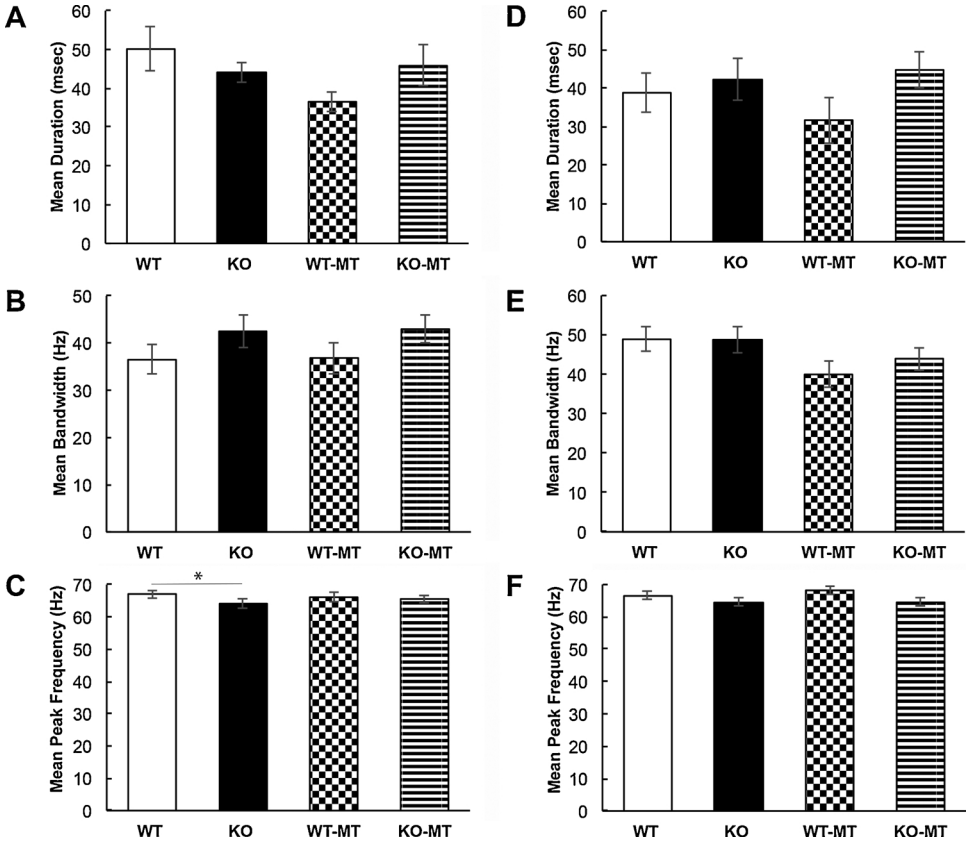

Fig. 3. Spectrotemporal properties of USV calls are not grossly affected by minocycline treatment during the first 5 min of dyadic interactions. A) Average duration of individual USV calls during the first 5 min showed no significant difference between the groups (2-way ANOVA, $p = 0.74$). B) Average USV bandwidth during the first 5 min showed no significant difference between the groups (2-way ANOVA, $p = 0.073$). C) Average USV peak frequency during the first 5 min showed a trending decrease in *Fmr1* KO mice (2-way ANOVA, $p = 0.05$). (D–F) Same data as in (A–C) during the second half (5 min.) of recording window when the mice called at a reduced rate.

mice and a trending difference in the average peak frequency of calls between the untreated WT and *Fmr1* KO mice, there were no major differences in spectrotemporal properties of calls across groups. Together these data show that *Fmr1* KO mice produce social vocalizations at a reduced rate, which is reversed with minocycline treatment, even if administered late in development. Reduction of MMP-9 in the *Fmr1* KO mice causes an overall increase in calling, sometimes even more than WT levels, generating motivation to more closely examine function of MMP-9 in USV generation in WT mice in the future. The similar effects of minocycline treatment and MMP-9 manipulation on call rates suggest that one pathway of minocycline action may be through reduction of MMP-9 activity.

Minocycline has beneficial effects in both humans with FXS and in animal models. Schneider et al. [14] showed that ~3 month minocycline treatment (placebo controlled) of young (mean age 10.5 years) children with FXS caused a reduction in amplitude of auditory event-related potentials (ERP), as well as improved ERP amplitude habituation in response to repeated sound presentation. The enhanced ERP amplitude and reduced habituation may reflect increased synchronization of neural signals in the cortex (Goncalves et al., 2013), and possible neural correlates of hypersensitivity to sounds in FXS. An open label study of minocycline in older FXS individuals (ages 13–32) indicated improvement in the aberrant behavior checklist-community edition (ABC-C) irritability subscale, the clinical global improvement scale (CGI), and the visual analog scale for behavior (VAS) [12]. In the drosophila model of FXS, Siller and Broadie [27] demonstrated that minocycline

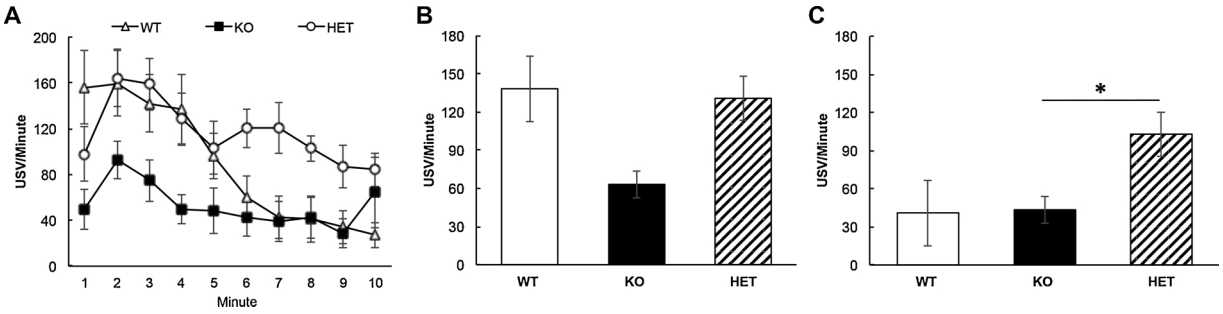

Fig. 4. Genetic reduction of MMP-9 in *Fmr1* KO (HET) mice increases USV calling rate to WT levels. A) Minute by minute changes in average USV call rate during a 10 min courtship window. The WT and *Fmr1* KO data are the same as shown in Fig. 2. Over the first 5 min, the HET mice showed call rates similar to the WT mice, and generally more calls than the *Fmr1* KO mice. Interestingly, the HET mice continued to call at a relatively higher rate even during the last 5 min compared to the other genotypes. A 2-way ANOVA (genotype and time) ($F(2,200) = 21.64$, $p < 0.001$) shows that the *Fmr1* KO mice exhibit significantly lower call rates compared to WT mice (Tukey test of main effects WT vs. KO $^{**}p < 0.01$) and genetic reduction of MMP-9 in HET mice restored call rates to WT levels (KO vs. HET $^{***}p < 0.001$; WT vs. HET $^{*}p < 0.05$). The WT vs. HET difference is likely carried by the enhanced calling in the latter group throughout the recording. B) Analysis of just the first 5 min shows a genotype difference, likely carried by reduced call rate in the *Fmr1* KO mice (1-way ANOVA: $F(2,20) = 3.74$, $p = 0.042$; Tukey test of main effects WT vs. KO $p = 0.07$, HET vs. KO $p = 0.08$, WT vs. HET $p = 1.00$). C) Analysis of the second 5 min shows a genotype difference (One Way ANOVA: $F(2,20) = 5.89$, $p = 0.01$: Tukey test of main effects HET vs. KO $p = 0.033$).

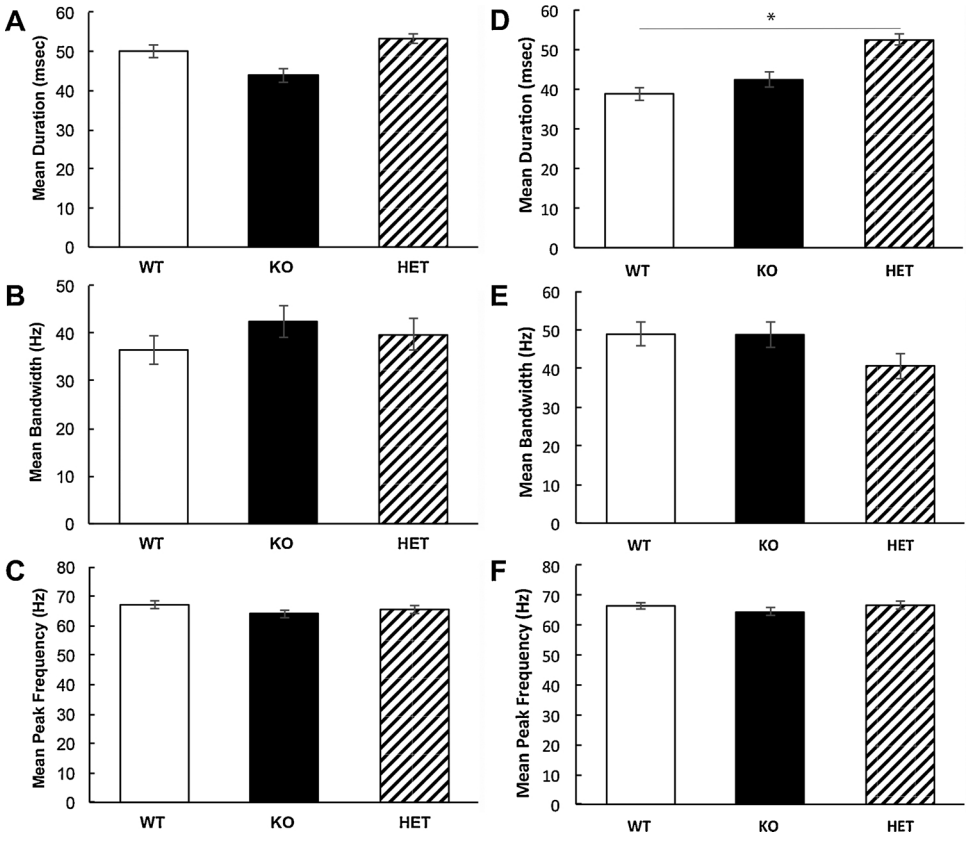

Fig. 5. Call properties were not different in HET mice compared to WT and *Fmr1* KO mice during the first 5 min of dyadic interactions. A) Average duration of USV calls during the first 5 min was comparable across genotypes (1-way ANOVA, $p = 0.31$). B) Average USV bandwidth during the first 5 min was comparable across genotypes (1-way ANOVA, $p = 0.49$). C) Average USV call peak frequency during the first 5 min was comparable across genotypes (1-way ANOVA, $p = 0.39$). Same data as in (A–C) during the second half (5 min.) of recording window.

normalized synaptic structure in multiple brain regions. In *Fmr1* KO mice, Bilousova et al. [17] showed that minocycline treatment promoted dendritic spine maturation in hippocampal neurons and reduced anxiety-like behaviors. Chronic treatment with minocycline reversed deficits in novel-mouse social interaction and novel-object recognition tests in *Fmr1* KO mice [40,41]. Minocycline treatment of *Fmr1* KO mice during early development caused long lasting amelioration of anxiety-like behaviors [16]. Minocycline administration also reduced the number and severity of audiogenic seizures in *Fmr1* KO mice. Interestingly, Dansie et al., [16] showed that treatment in adult mice also caused behavioral changes, but these benefits were transient and were lost as soon as treatment was stopped. Our data show that minocycline administered during adolescent and young adult ages (P30-P58) in mice is sufficient to reverse USV call rate deficits in *Fmr1* KO mice, even when these mice were tested 15–30 days after cessation of minocycline administration. Thus outcomes on the elevated plus maze and open field behavioral tests show long-term benefits only with early developmental minocycline treatment. But, adult treatment is sufficient to reverse USV calling deficits in a sustained manner (at least 15–30 days past the last injection). This indicates that different outcome measures are affected differently by early *versus* late treatments and should be taken into account during drug treatment design and outcome measure evaluation for effective preclinical and clinical tests. Currently, however there is very little published data on the longevity of drug effects in FXS, or any autism spectrum disorder, to compare young *versus* adult treatments.

The beneficial effects of minocycline may occur through inhibition of apoptotic cell death, reduced activation and proliferation of microglia and reduced inflammation [18,42,43]. Another well-characterized effect of minocycline is reduction of MMP-9 levels and activity [44]. MMP-9 is an endopeptidase involved in cleavage of extracellular matrix, and is a target of FMRP-based translational control [22]. Indeed, MMP-9 levels are increased in FXS, in both humans and in mouse models [24,25]. Minocycline treatment causes reduction of MMP-9 levels in FXS [23]. We have previously shown that reduction or removal of MMP-9 in the *Fmr1* KO mice alleviates auditory cortex hyperexcitability, and corrects abnormal ERP habituation [20,26]. The suggestion that minocycline acts via MMP inhibition is supported by Siller and Broadie [27] who showed that removal of MMP-1 from the drosophila FXS model normalized synaptic architecture in a manner similar to minocycline treatment. Our current data show that genetic reduction of MMP-9 in the *Fmr1* KO mice reverses USV call rate deficits similar to minocycline treatment. Although these data do not eliminate any other potential targets of minocycline action, they do suggest that the recovery of USV call rate with minocycline may occur through reduction of MMP-9. Indeed, in WT mice, MMP-9 levels are typically higher in the brain during early development, and decrease into adulthood. In contrast, in the few regions of the *Fmr1* KO mice that have been examined, MMP-9 levels continue to stay high into adulthood [17,25,20,24,26]. Therefore, the specific effect of minocycline on *Fmr1* KO, but not WT, mice may occur through reduction of excessive MMP-9 levels without affecting normal MMP-9 levels in WT mice.

USVs produced by males in a courtship context and by pups when separated from their mothers have stereotypical elements that make them useful as potential biomarkers of social communication deficits in pre-clinical models of genetic disorders [45]. Indeed, a number of studies have shown altered USV call rates and/or properties in models of autism spectrum disorders. USVs are also sensitive to treatments, another property that makes them useful as biomarkers. In the *Fmr1* KO mouse, a number of studies have examined USV properties in both adults and pups. Roy et al. [46] recorded vocalizations from P8 pups for 3 min after isolation from their mothers. They found no differences in the number of calls, but found that a specific type of call ('flat') was emitted with increased carrier frequency in the *Fmr1* KO pups. They also found a decrease in the percentage of downward calls relative to total calls in these mice and an increase in the average bandwidth of calls in the Fmr1 KO pups. There were no genotype differences in the average duration of calls. Lai et al. [47] analyzed pup calls at three time

points in development (P4, P7 and P10) and found a transient increase in the number of calls made by *Fmr1* KO pups at P7. Although these studies are not directly comparable to the adult courtship context recordings of our study, these data nevertheless point to significant differences in USVs in *Fmr1* KO mice compared to WT from a very early age. Whether these differences in calls result in different maternal care remain unknown. Belagodu et al., [34] recorded from adult mice and in contrast to our data, they did not find a difference in call rate, but found that the *Fmr1* KO mice produced a higher proportion of specific syllables compared to WT mice. While strain differences (FVB vs. C57bl/6) may partly explain the alternate findings, the main difference between the two studies is that Belagodu et al., [34] recorded vocalizations after the female mouse was removed. Therefore, the mice are likely calling under different motivational contexts across the two studies.

One potential drawback of our approach is that females may make some of the calls recorded. While female mice can produce USVs when presented with another female [48,49], female mice do not readily vocalize during dyadic courtship sessions [35]. When presented with laryngeal-nerve transected males, no calls were detected [35], leading to the conclusion that males are the primary producers of USVs in adult courtship mixed-sex pairs. More recently, Neunuebel et al. [50], used microphone array based localization to show that females and males produce acoustically similar USVs in complex social interactions (2 males and 2 females). Taken together, these studies suggest that USV calling depends critically on the social context, with females potentially contributing to the calls recorded with increasingly more complex social grouping. We minimized the possible influence of genotype differences in female song production by using only *Fmr1* KO females in dyadic courtship interactions. However, we cannot exclude the possibility that female mice produced vocalizations and their call rate changed depending on the male genotype. Future studies will test *Fmr1* KO mice using the localization system similar to that developed by Neunuebel et al., [50].

Genetic reduction of MMP-9 in the *Fmr1* KO mice results in an increase in calling USV calling rate. This suggests that MMP-9 may be involved in the development and/or functioning of vocalization circuitry in mice. Future studies should determine if genetically reducing MMP-9 in WT mice would also affect USV calling rate. It remains unclear where along the vocalization pathway MMP-9 reduction may increase vocal output in *Fmr1* KO mice. Regions in the vocal production pathway in the mouse include the anterior cingulate cortex, motor cortex, periaqueductal gray and pontine reticular formation [51]. Future studies will examine MMP-9 levels in these regions in *Fmr1* KO mice to identify specific circuit deficits. Comparisons of studies across ages in the *Fmr1* KO mice indicate that USV calls are different compared to WT mice. In adult mice engaged in dyadic mixed-sex courtship interactions, *Fmr1* KO mice call at a reduced rate with no differences in basic spectrotemporal call properties. Minocycline treatment between 1–2 months of age is sufficient to reverse the call rate deficits suggesting, 1) that USVs have the potential to serve as outcome measures in pre-clinical models of autism and 2) treatment in late development is sufficient to reverse USV deficits. Future studies with specific inhibitors of MMP-9 that do not impact apoptosis or microglia function are warranted to develop treatments that specifically impact pathways affected by reduction of FMRP.

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