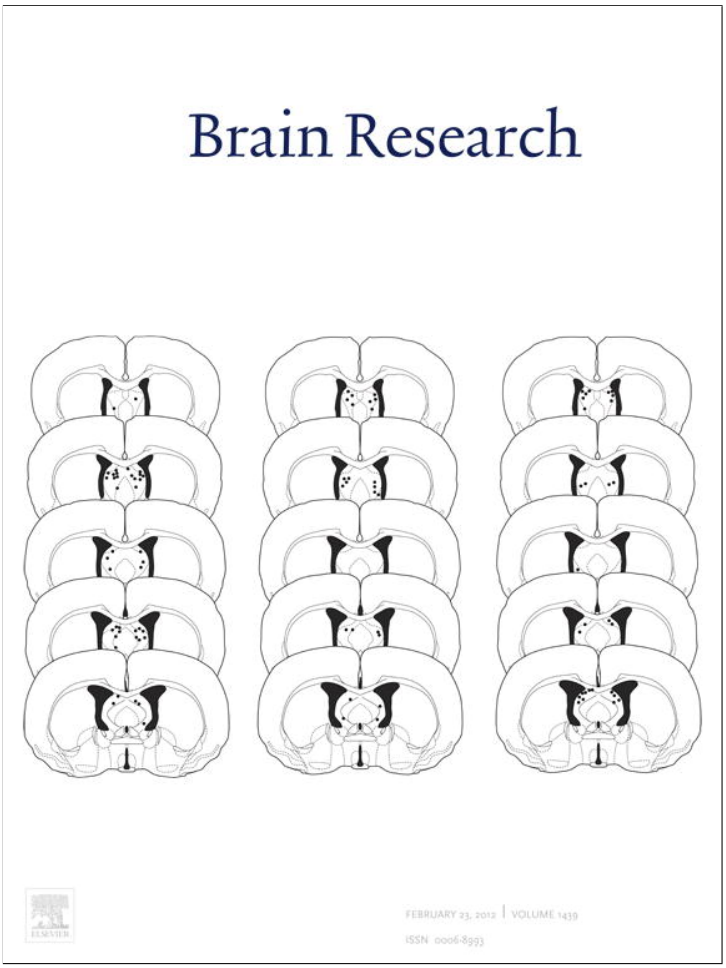

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Research Report

Minocycline treatment reverses ultrasonic vocalization production deficit in a mouse model of Fragile X Syndrome

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ABSTRACT

Fragile X Syndrome (FXS) is the most common inherited form of intellectual disability, with behaviors characteristic of autism. Symptoms include abnormal social behavior, repetitive behavior, communication disorders, and seizures. Many symptoms of FXS have been replicated in the *Fmr1* knockout (KO) mice. Whether *Fmr1* KO mice exhibit vocal communication deficits is not known. By recording ultrasonic vocalizations (USV) produced by adult male mice during mating, we show that USV calling rate (number of calls/second) is reduced in *Fmr1* KO mice compared to WT controls. The WT control and *Fmr1* KO groups did not differ in other aspects of mating behavior such as time spent sniffing, mounting, rooting and without contact. Acoustic properties of calls such as mean frequency (in kHz), duration and dynamic range of frequencies were not different. This indicates a specific deficit in USV calling rate in *Fmr1* KO mice. Previous studies have shown that treatment of *Fmr1* KO mice with minocycline for 4 weeks from birth can alleviate some behavioral symptoms. Here we tested if minocycline also reversed vocalization deficits in these mice. Calling rate increased and was similar to WT controls in adult *Fmr1* KO mice treated with minocycline for four weeks from birth (P0–P28). All acoustic properties measured were similar in treated and untreated WT control mice indicating minocycline effects were specific to vocalizations in the *Fmr1* KO mice. These data suggest that mating-related USVs are robust and relevant biomarkers of FXS, and that minocycline treatment is a promising avenue for treatment of FXS symptoms.

1. Introduction

Fragile X Syndrome (FXS) is the most common single gene inherited form of intellectual disability, affecting 1 in 4000 males and 1 in 8000 females (McNaughton et al., 2008). FXS is the result of an expansion of the CGG trinucleotide repeats in the 5′ untranslated region of the *fragile X mental retardation* (*FMR1*) gene, leading to transcriptional silencing and a failure to produce fragile X mental retardation protein (FMRP, McNaughton et al., 2008). In the resulting syndrome, patients experience an array of symptoms, including intellectual disability, anxiety, executive and social impairments, hyperactivity, seizures and visuomotor impairments (Hagerman et al., 2009; McNaughton et al., 2008; Rudelli et al., 1985). Addi-

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tionally, FXS patients show abnormalities in language output including production of shorter and less complex utterances and sentence structures (Price et al., 2008) and fluctuating rate of speech, and repetitions of sounds, words or phrases (Hanson et al., 1986). There are delays in linguistic development, articulation difficulties, poor co-articulation, substitutions and omissions of words, fewer intelligible words produced, and difficulty with sound sequencing (Barnes et al., 2009; Fidler et al., 2007; Largo and Schinzel, 1985; Roberts et al., 2001, 2002).

The *Fmr1* knockout (*Fmr1* KO) mouse displays a wide range of symptoms and pathological features typical of FXS, making it a useful model system (Bernardet and Crusio, 2006; Chen and Toth, 2001; Mineur et al., 2006; Spencer et al., 2005). Although many of the behavioral symptoms have been characterized in these mice, possible deficits in vocal output based communication have not been addressed. Consequently, it remains unclear if the *Fmr1* KO mice are suited to study communication deficits seen in FXS. Sexually mature male mice produce complex sequences of ultrasonic vocalizations (USV) when paired with a receptive female. These calls may be used to evaluate mouse social communication (Holy and Guo, 2005; Nyby et al., 1977; Portfors, 2007; Sales, 1972). The first aim of this study was to compare mating-related USV between WT control and *Fmr1* KO mice. We present evidence that *Fmr1* KO mice exhibit a reduced rate of calling (number of calls/second) without a significant change in acoustic properties. Furthermore, to ensure that any differences in USV production seen in *Fmr1* KO mice were not the result of abnormal mating behavior, we scored mating sessions for time spent performing specific behaviors associated with mating (according to McGill, 1962). We found that the time spent performing various behaviors associated with mating did not significantly differ between *Fmr1* KO and WT control mice.

Recently, minocycline has garnered interest as a possible treatment for humans with FXS (Paribello et al., 2010; Utari et al., 2010). Though the mechanisms through which minocycline influences behavior in FXS are only beginning to be understood, preliminary studies of minocycline as a treatment for FXS are promising. For instance, Bilousova et al. (2009) demonstrated that minocycline treatment from birth rescues dendritic spine morphology in *Fmr1* KO mice. Minocycline treatment also increased exploratory behavior in an elevated plus maze, suggesting a decrease in anxiety-like behavior in *Fmr1* KO mice (Bilousova et al., 2009). Moreover, in humans with FXS who had participated in an open label minocycline trial for two weeks, 54% of treated patients showed improvements in language use, as reported by the patients' caregivers. Improvements included more intelligible language produced, more 'expressive' language used, and an overall increase in the amount of language used (Utari et al., 2010). Additionally, minocycline treatment improved attention span in 50% and social communication skills in 44% of patients (Utari et al., 2010). The second aim of this study was to evaluate the therapeutic potential of minocycline in mating-related vocal behavior in the *Fmr1* KO mice. We present evidence that the rate of calling in *Fmr1* KO mice is restored to that of control animals with minocycline treatment without affecting acoustic properties. Thus social vocalizations serve as a useful biomarker in FXS to study potential therapies such as minocycline treatment for communication deficits in FXS.

2. Results

The major aims of this study were to determine if mating-related USV differed between the WT control and *Fmr1* KO mice, whether *Fmr1* KO mice displayed an altered array of mating behaviors, and if minocycline treatment affected vocalization production. The four experimental groups were untreated WT control (WT), minocycline treated WT control (MTWT), untreated knockout (KO) and minocycline treated knockout (MTKO). All mating pairs were genotype and treatment matched.

2.1. *Minocycline treatment restores the rate of ultrasonic production in Fmr1 KO mice*

As reported previously in other strains of mice (Holy and Guo, 2005; Pomerantz et al., 1983), WT and *Fmr1* KO mice, which are generated on FVB background with restored *pde6b* allele to prevent retinal degeneration, produce USV when paired with a female mouse (e.g. spectrograms in Fig. 1). We compared USV production across WT, KO, MTWT, and MTKO groups during the first 10 min after pairing a male and a female mouse. First, we analyzed calling rate (calls/second). KO mice called at a reduced rate compared to WT mice (Fig. 2A). A two-way ANOVA for each group (WT, KO, MTWT, and MTKO) by minute in the mating session revealed a significant main effect of the treatment ($F(3,400)=42.1$, $p<0.001$; $\eta^2=0.178$). No effect was observed for time. A Tukey hsd post-hoc test revealed that the KO group produced significantly fewer vocalizations per second as compared to the WT group ($p<0.01$). No significant difference was observed between WT, MTWT, and MTKO groups. The calling rate collapsed across the first 10 min of a mating session shows the difference between the KO and the other three groups (Fig. 2B). These results indicate that KO mice vocalize at a reduced rate, and minocycline treatment reversed this deficit. In contrast, there were no differences in the calling rates between WT and MTWT suggesting the specificity of minocycline actions on the vocalizations of *Fmr1* KO mice and that the treatment did not have adverse side effects on vocalizations in the WT mice.

2.2. *Acoustic properties of calls are not different between the four groups*

No significant differences were found in the duration of individual USVs (one-way ANOVA $F(3,36)=1.99$, $p=0.381$), the average frequency (here 'frequency' is used analogous to pitch) of calls (one-way ANOVA $F(3,36)=2.59$, $p=0.164$), or the dynamic range of frequency (one-way ANOVA $F(3,36)=0.953$, $p=0.655$) in the WT, KO, or minocycline treated groups (Fig. 3). To assess variations in USV properties within each trial, we calculated coefficient of variation for USV duration, average frequency, and dynamic range, and then compared treatment groups. We did not find a significant difference in coefficient of variation for USV duration (one-way ANOVA

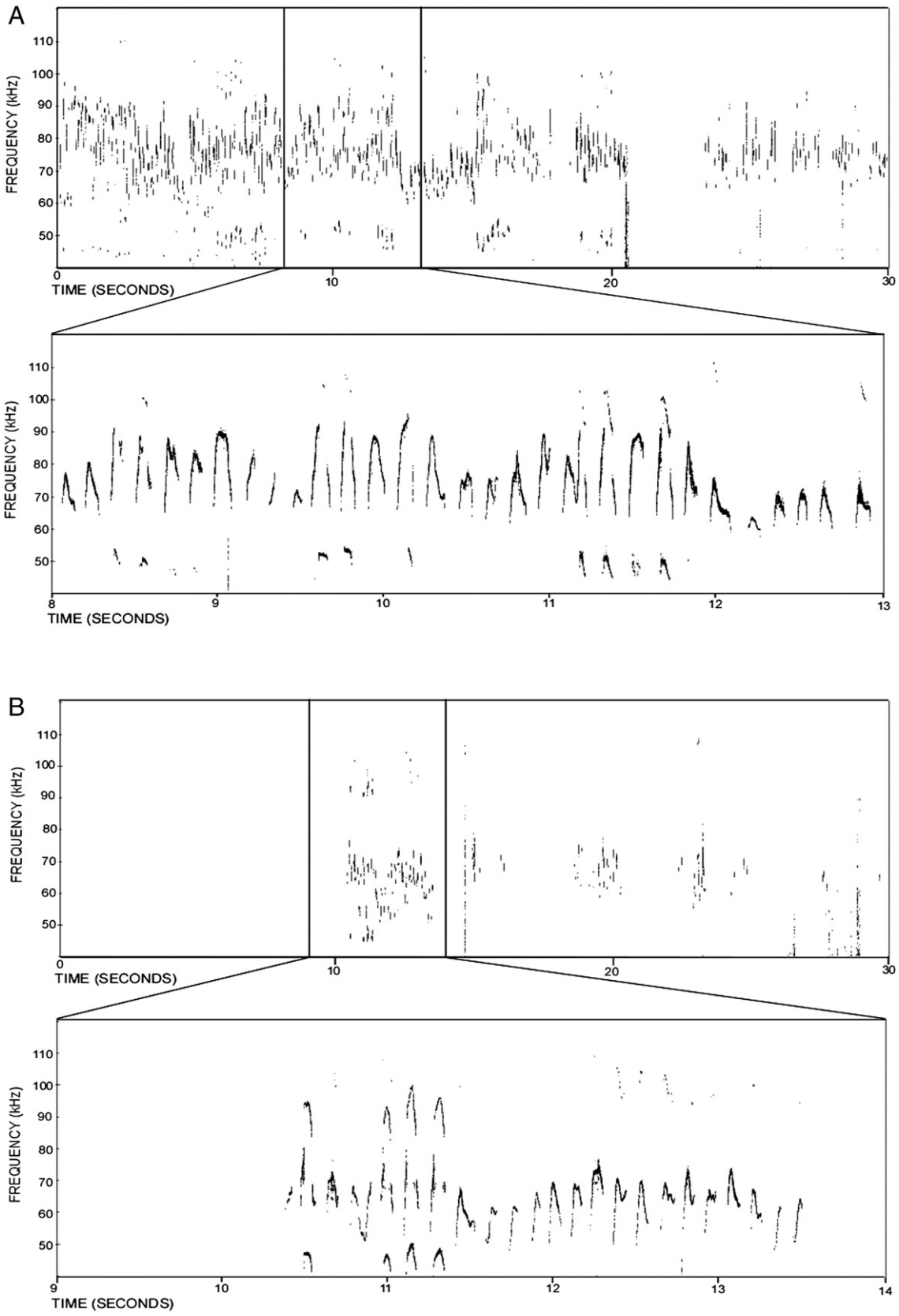

Fig. 1 – Example ultrasonic vocalizations recorded from WT (A) and *Fmr1* KO mouse (B) during mating.

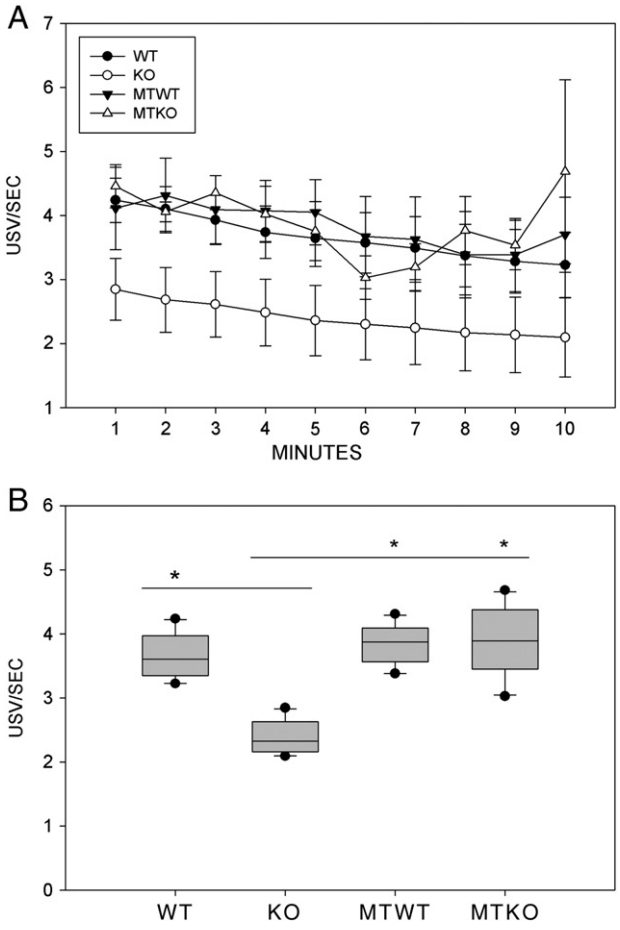

Fig. 2 – Fmr1 KO mice vocalize less and minocycline reverses this deficit (A) Mean number of USVs per second in WT, KO, MTWT, and MTKO groups during each minute of the first 10 min after introducing a male and female mouse. (B) Calls/second collapsed across 10 min. The KO (n=14) group produced significantly fewer USV/s than WT (n=17), MTWT (n=9), and MTKO (n=6) groups. WT, MTWT, and MTKO groups do not display significant differences.

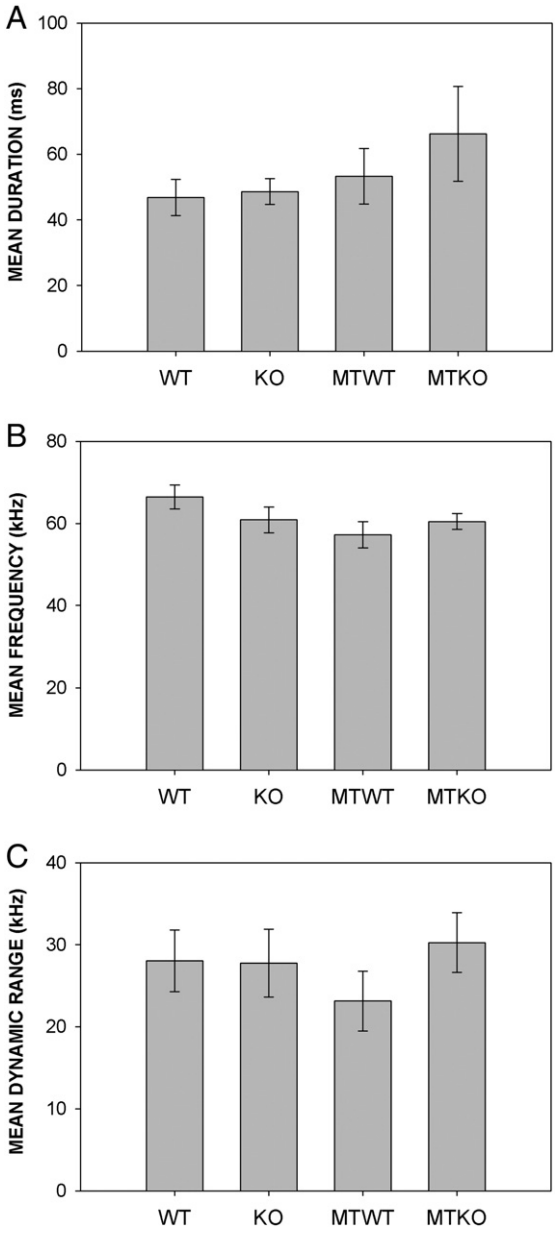

Fig. 3 – Acoustic properties of USVs are not different across groups. Mean duration (A), frequency (B), and dynamic range (C) of USVs produced by WT, KO, MTWT, MTKO mice during mating. No significant differences were found in any category between groups.

F(3,36)=3.207, p=0.361), average frequency (one-way ANOVA F(3,36)=0.2.549, p=0.070), or dynamic range (one-way ANOVA F(3,36)=0.278, p=0.841). These findings demonstrate that the main difference in control and *Fmr1* KO mouse calling lies in vocalization production rate rather than the acoustic properties of calls.

2.3. *Additional controls*

2.3.1. *Mating behavior was not altered in Fmr1 KO mice*

The altered production of USVs could be reflective of an overall change in mating behavior in the KO mice. Different aspects of mating behaviors were determined according to McGill (1962). A one-way ANOVA revealed that there was no significant difference in the amount of time WT and KO mice spent grooming (F(1,7)=0.422, p=0.083), rooting (F(1,7)=0.422, p=0.254), sniffing (F(1,7)=0.422, p=0.746), or not in contact (F(1,7)=0.422, p=0.792) with the female (Fig. 4A). This suggests that the differences seen in USV calling rate between WT and KO groups stems specifically from a reduced tendency to vocalize rather than an altered array of mating behaviors that might affect calling rate.

2.3.2. *Estrus state of females*

In our initial studies, the estrus state of females was not controlled. It is possible the estrus state of females provides a signal back to the male to alter USV calling. To determine if the receptivity of females influenced calling rate, we induced estrus in a small number of WT (n=10) and KO (n=6) mice and compared their calling rate with those of un-induced mice (Fig. 4B). There was no significant differences in the number of USVs produced per second between the estrus induced

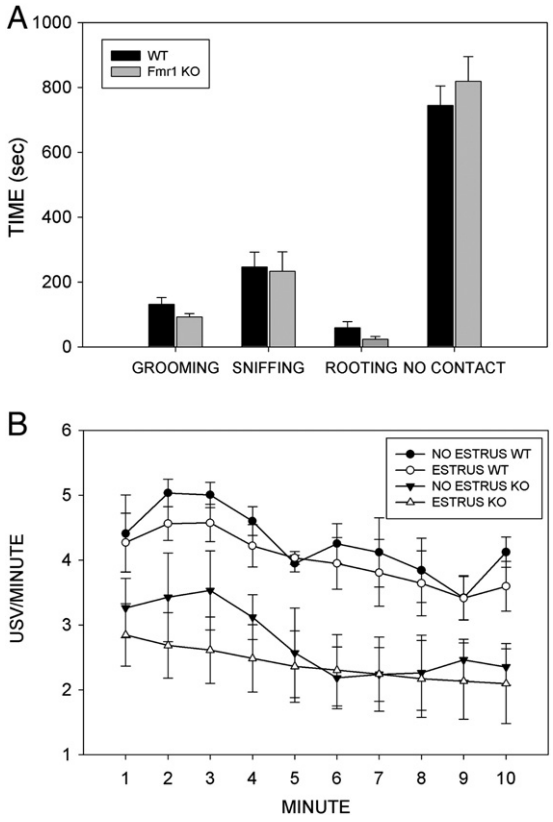

Fig. 4 – (A) Time mating control and knockout pairs spent grooming, sniffing, rooting, or in no contact during the first 20 min of mating. There was no significant difference in time spent on each behavior between the two groups. (B) Calls per second in control and *Fmr1* KO mice in which estrus was induced, and in which estrus was not induced. No significant difference was observed in either group.

pairs and the un-induced pairs of WT mice (one-way ANOVA $F(1,18)=1.73$, $p>0.05$; $\eta^2=0.088$) or estrus induced pairs and the un-induced pairs of KO mice (one-way ANOVA $F(1,18)=3.47$, $p>0.05$; $\eta^2=0.162$). Because estrus state of females did not affect male USV calling rate, data from the estrus induced and un-induced groups were pooled in the previous figures.

3. Discussion

The first main result of this study is that *Fmr1* KO mice vocalize at a slower rate than WT controls in a mating context. There was no overlap between the calling rate of WT and KO mice (Fig. 2B) indicating that this parameter can be used as a robust and sensitive biomarker of vocal output. Given that language production deficit is a symptom of FXS, USV calling rate is a biomarker relevant to communication disorders associated with FXS and possibly other autistic spectrum disorders. Moreover, the reduction in USV production seen in *Fmr1* KO mice was not associated with altered mating behavior, suggesting decreased vocalization production is a specific deficit within the mating context. The functional significance of reduced USV rate is unclear. While it has been established that USV production is required to attract female mice (Pomerantz et al., 1983), the importance of USV calling rate in sexual selection is not known. Future studies will address if WT females chose males based on calling rate. The second major finding is that four weeks of minocycline treatment from birth reverses the vocalization deficit. This suggests that mating-related vocalizations can be used as outcome measures of efficacy of potential treatments in preclinical animal models of FXS, and potentially other mouse models of autism. The treated WT control mice did not show any difference with the untreated WT controls indicating the specificity of minocycline to the *Fmr1* KO mice.

These results add to the recent studies demonstrating the therapeutic effects of minocycline treatment in both *Fmr1* KO mice and human subjects with FXS. In KO mice, 28 days of minocycline treatment starting at birth resulted in a restoration of mature dendritic spines (Bilousova et al., 2009). Future studies are required to determine if there are associations between minocycline treatment and structural features in brain regions involved in rodent vocalizations. Human subjects who received minocycline treatment for at least 2 weeks showed improvements in language use, attention, and social communication (Utari et al., 2010). The parents of the subjects that had undergone the treatment were asked to score changes in behavior according to Likert Scale. The most significant gains were seen in range of language used, tendency to engage in verbal communication and an amelioration of irritable behavior (Utari et al., 2010). Most recent results of an open-label clinical trial conducted in Canada also showed significant functional benefits of an 8 week course of minocycline treatment to human subjects with FXS as measured with the Aberrant Behavior Checklist-Community Edition Irritability Subscale (Paribello et al., 2010). While these initial results are encouraging, there is a need for placebo controlled minocycline studies in human FXS patients. The side effects associated with minocycline treatment were generally mild. Subjects most commonly reported dizziness and diarrhea upon minocycline treatment, as well as instances of sleepiness, headache, fatigue, nausea, and pruritus (Paribello et al., 2010). Minocycline treatment is also associated with tooth discoloration (Antonini and Luder, 2010). Gastrointestinal problems and decreased appetite were also reported with minocycline use (Utari et al., 2010). More serious side effects of minocycline are rare, but include drug-induced lupus and elevated ANA levels (Schlienger et al., 2000).

How minocycline influences symptoms of FXS is only beginning to be understood. Though the changes to neuronal circuitry which underlie the symptoms of FXS and minocycline action are unknown, the ability of minocycline to induce mature dendritic spines in *Fmr1* KO hippocampal neurons has been previously reported both *in vitro* and *in vivo* (Bilousova et al., 2009). Previous studies have shown that manipulations, which rescue dendritic spine morphology, are also associated with improvements in behavioral performance typical of FXS (Dansie et al., in preparation; Hayashi et al., 2007). Specifically, inhibition of p21-activated kinase (PAK) restores dendritic spine morphology in *Fmr1* KO mice, and is associated with improved performance in open field and fear conditioning tasks. Matrix metalloproteinase 9 (MMP9) has been suggested as a possible locus of action of minocycline in these mice

(Bilousova et al., 2009). MMPs influence dendritic spine development and hence synaptic stability (Bilousova et al., 2009). MMP-9 activity is up-regulated in the hippocampus of *Fmr1* KO mice and may be partially responsible for abnormal dendritic spine development (Ethell and Ethell, 2007), whereas minocycline treatment reduces MMP-9 levels and activity. Dendritic spine enlargement associated with long term potentiation is facilitated by MMP-9 mediated proteolysis of the extracellular matrix (Wang et al., 2008). However, excessive levels of most abundant MMPs within the brain, MMP-2, MMP-3, and MMP-9, have all been implicated in several pathological conditions (Yong et al., 2004). The abnormal dendritic spine development associated with FXS, may in part result from excessive MMP-9 activity as well. Minocycline treatment acts to inhibit MMP-9 activity, and restores dendritic spine morphology as well as a host of FXS typical behaviors (Bilousova et al., 2009). Beside the ability to regulate MMP-9 activity, several other mechanisms of minocycline action have been also reported, including its inhibitory effects on microglia proliferation, anti-apoptotic effects and even its ability to directly influence the functions of glutamate receptors (Elewa et al., 2006; Imbesi et al., 2008; Kim and Suh, 2009). Minocycline has been also shown to be beneficial in animal models of several neurodegenerative diseases and these effects of minocycline were shown to be mediated through the regulation of the MAP kinase pathway and caspase activity (Kim and Suh, 2009).

Communication disorders have been observed in other mouse models of ASDs. In a tuberous sclerosis (TSC) model, mouse pups heterozygous for the TSC2 gene were shown to vocalize less than their wild-type counterparts (Young et al., 2010). Behavioral abnormalities in TSC are thought to be derived from unchecked cell proliferation within the brain following TSC1 or TSC2 mutation. Additionally, pups of Angelman syndrome model mice display increased USV production (Jiang et al., 2010). Autism-like characteristics have also been modeled in neuroligin-4 (NL-4) knockout mice. NL-4 knockout mice lack the murine ortholog of human NL-4, which codes for the synaptic cell adhesion protein neuroligin-4 (Jamain et al., 2008). NL-4 knockout mice were placed in contact with a female mouse and the USVs the male produced were recorded. NL-4 knockout males demonstrated a longer latency to calling and an overall reduction in the number of USVs produced (Jamain et al., 2008). Therefore, social vocalizations may serve as a useful biomarker to study potential therapies for these communication disorders as well. While some studies use isolated pup USVs to test mouse communication, we focused on the mating paradigm for two reasons. First, there is some evidence suggesting that rodent pup vocalizations are associated with a physiological response to under-developed thermoregulation abilities, and may be a reflexive behavior (Blumberg and Alberts, 1990; Blumberg and Stolba, 1996). Second, mating-related USVs are more complex and provide a larger repertoire of calls to perform vocal analysis and associated social behaviors.

In this study, minocycline treatment began at birth and continued to P28. Although mating occurred after minocycline treatment had ceased at least a month earlier, the calling rate increased to WT levels. This suggests developmental normalization that persists in the *Fmr1* KO mice. One caveat is that mothers were treated in this study and that may have altered maternal behaviors and indirectly affected adult behaviors of their progeny. In addition, it is unclear how effective minocycline treatment is and how long the benefits will last when treatment is begun at a later age. Future studies will address direct administration of minocycline to pups and the optimal dosage (concentration, age and length) to improve mating-related calling rate in the KO mice. Studies will be especially concerned with the efficacy of minocycline treatment on adult animals. These studies will be informative for clinical studies since most candidates for minocycline treatment are adolescents and adults.

4. Experimental procedures

4.1. Mice

FVB.129P2-*Fmr1*^{tm4Cgr} (*Fmr1* KO) and FVB.129P2-*Pde6b*$^{+}$*Tyr*$^{c\text{-}ch}$/AntJ control mice were obtained from Jackson Laboratories and housed in an accredited vivarium with 12 h light/dark cycle. All studies were performed in accordance with the National Institutes of Health and with Institutional Animal Care and Use Committee guidelines.

4.2. Minocycline administration

Minocycline (30 mg/kg) was added to the mother's drinking water every day for 28 days. This method of minocycline administration has been previously shown to yield detectable concentrations of minocycline in the blood of adult mice (Lee et al., 2006) and in the breast milk of lactating dams (Lin et al., 2005; Luzi et al., 2009). Newborn *Fmr1* KO mice were therefore exposed to minocycline through suckling, which was previously shown to reduce MMP-9 levels in the brains of these mice and anxiety-like behaviors (Bilousova et al., 2009; Lee et al., 2006). After the initial 28 days of treatment, mice did not receive any additional minocycline prior to mating. Virgin males and females were paired when they were between 2 and 3 months old to record mating-related vocalizations.

4.3. Recording mouse vocalizations

USV produced during mating behavior were recorded using a full spectrum Petterssen D1000x bat detector (250 kHz sampling rate) maintained 5 cm above the enclosure. Recordings were performed between 10:00 and 15:00. Video recording for behavior analysis was done using a Sony HDR-CX350V camcorder. Male mice were introduced to female mice in a 28.8×21.6×28.8 cm enclosure. Recording continued until mating occurred or until 20 min had passed. Four groups of mice were studied: control (WT), *Fmr1* KO (KO), minocycline treated controls (MTWT) and minocycline treated *Fmr1* KO (MTKO). All pairings of males and females were within group. USV and behavioral data were obtained by mating 17 pairs of WT mice and 14 pairs of KO mice. Estrus was induced in 10 of the WT pairs and 6 of the KO pairs. In the remaining pairs, estrus was not induced, but mating did occur. Estrus was induced in female mice by injecting 0.075 mL of 0.04 mg/mL

estradiol benzoate solution 36 to 48 h prior to mating and 0.018 mL of 0.60 mg/mL progesterone solution 4 h prior to mating (McGill, 1962). Vocal recordings were also obtained for nine MTWT pairs and six MTKO pairs, in which estrus was not induced but mating did occur. There was no difference between the estrus induced and un-induced conditions in the WT and KO mice (see results). Therefore, data within each group were collapsed across the induced and un-induced conditions. While female mice can produce social vocalizations when presented with another female (Portfors, 2007), female mice do not readily vocalize during mating sessions (Warburton et al., 1989). When presented with laryngeal-nerve transected males, female mice failed to produce any vocalizations (Warburton et al., 1989), suggesting that vocalizations in the mating context are mostly produced by the males.

4.4. *Vocalization analysis*

Acoustical waveforms were stored on a personal computer and processed in MATLAB in a method similar to that described in Holy and Guo (2005). A 1.6 ms window was moved along the waveform and the power spectrum was determined for that window. The beginning of a vocalization was determined to be the window in which the spectral purity was at least 25% of total power concentrated into a single frequency bin. The end of the vocalization was defined as the window preceding the window in which less than 25% of the total power was concentrated into a single frequency. Additional criteria for a sound to be considered a vocalization were mean frequency (between 40 and 120 kHz) and duration (between 5 and 210 ms). Vocalizations in which less than 3 ms separated two successive calls were merged and considered a single vocalization. Based on these criteria the beginning and end of each call can be distinguished above the background noise and used to determine the number of calls per second.

4.5. *Properties of vocalizations*

A 9th order bandpass Butterworth filter was used to filter out frequencies below 30 kHz and above 120 kHz. Each vocalization was sectioned into 2 ms half overlap segments. A high-temporal-resolution power spectral density estimation was used to determine the frequency at which the maximal power occurred at each time bin and the resulting frequencies concatenated into a frequency array (see Supplemental Fig. 1). From the frequency array, mean frequency and dynamic range, defined as the difference between the high and low frequencies within a call, were calculated. Additionally, the duration of each call was analyzed.

Each mating session was segmented into portions representing the first 10-min of the mating session. Call properties were then compared across the four groups (WT, KO, MTWT and MTKO) over the first 10 min of each mating trial. A two-way ANOVA for treatment group × ime was used to assess differences in call rate and acoustic properties. ANOVAs for acoustic properties were adjusted for the random disturbance that can occur from large datasets as each measure consisted of more than 48,000 total vocalizations.

4.6. *Behavioral analysis*

To determine if USV calling was specifically affected or if there was an overall change in mating behaviors, each mating session was scored for time spent performing specific behaviors associated with mouse mating. Rodent mating behavior has been well characterized (Bialy et al., 2000; McGill 1962; Nyby, 1972; White et al., 1998) allowing us to isolate and identify specific mating behaviors. JWatcher software was used to mark the time points at which each behavior occurred and the amount of time spent performing each behavior. Two raters scored the behavior and were blind to the genotype of the mouse pairs. Behaviors scored were rooting, grooming, ano-genital sniffing, and no contact. Instances of mounting and mounting-with-intromission were also noted, but did not occur within the first 20 min of all trials and therefore were omitted from analysis. During rooting, the male pushed his snout, head, or shoulders underneath the female (McGill, 1962). Grooming was scored as one mouse licking the fur of its conspecific. Ano-genital sniffing was identified as the male mouse sniffing the ano-genital region of the female. Mounting and mounting-with-intromission were distinguished by speed and depth of thrusting performed by the male. Mounting was characterized by fast, shallow thrusts, while mounting-with-intromission was distinguished by slower, deeper thrusting. During periods of no contact, the mice were not in physical contact with one another nor were they engaged in any of the otherwise listed behaviors. After each rater scored a trial, time spent performing each behavior was totaled and compared using paired *t*-test. There was no difference in rater scoring of any of the behaviors (rooting paired *t*-test $p=0.655$, grooming paired *t*-test $p=0.314$, ano-genital sniffing paired *t*-test $p=0.541$, no contact paired *t*-test $p=0.158$).

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