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Auditory Processing and Ultrasonic Vocalization Production in a Mouse Model of Fragile X Syndrome

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Sarah Elizabeth Rotschafer

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Dissertation Committee:
Dr. Khaleel Razak, Chairperson
Dr. Iryna Ethell
Dr. Peter Hickmott

The Dissertation of Sarah Elizabeth Rotschafer is approved:

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Committee Chairperson

University of California, Riverside

ABSTRACT OF THE DISSERTATION

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Sarah Elizabeth Rotschafer

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Dr. Khaleel A. Razak, Chairperson

Fragile X syndrome (FXS) is the most common cause of inherited intellectual impairment and best understood single cause autism. Symptoms of FXS include social anxiety, repetitive behavior, communication disorders, hyperactivity, and seizures. Additionally, event related potentials studies using sound stimuli indicate that auditory processing is abnormal in FXS. Many symptoms of FXS have been replicated in the *Fmr1* knockout *(*KO) mice, which serve as a useful model for studying sensory processing abnormalities and symptoms associated with FXS. *Fmr1* KO mice exhibit acoustic hypersensitivity and propensity for audiogenic seizures, which suggests altered auditory processing, though the nature of such changes are unknown. Although evidence of unusual social interaction and repetitive behaviors has been found in *Fmr1* KO mice, communication anomalies have not yet been modeled. In order to establish the *Fmr1* KO mouse as a model for the auditory processing and communication disorders associated with FXS, we developed an assay for assessing vocalization production and performed single unit

extracellular electrophysiology in the auditory cortex of *Fmr1* KO mice. To quantify possible communication abnormalities in *Fmr1* KO mice, we elicited mouse ultrasonic vocalizations (USV) by placing male mice in contact with female mice. This technique revealed that *Fmr1* KO mice produce USVs at a decreased rate when compared to wild type mice. Minocycline is a tetracycline analog that has been shown to rescue some symptoms of FXS in open label human trials. Using our USV assay, we demonstrate that minocycline treatment, USV production rate was restored to wild types levels in *Fmr1* KO mice. To investigate auditory processing anomalies in *Fmr1* KO mice, we performed extracellular single unit recordings in the auditory cortex of anesthetized mice. Presenting single frequency tones revealed expanded frequency tuning, enhanced response magnitude, and greater variability in first spike latency. Frequency modulated sweep stimuli revealed altered sensitivity to FM sweep rate.

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Chapter 1: Auditory Processing in Fragile X Syndrome

1.1 Introduction

Fragile X syndrome (FXS) is a genetic disorder that effects 1 in 4000 males and 1 in 8000 females born in the United States every year (Hagerman, 2008). FXS results from expansion and hypermethylation of CGG trinucleotide repeats in the promoter region of the FMR1 gene, which leads to a failure to produce fragile x mental retardation protein (FMRP) (Bailey et al., 1998; O'Donnell and Warren, 2002). FMRP inhibits translation of synaptic mRNAs in response to mGluR stimulation, and loss of FMRP typically results in an over-production of associated synaptic proteins (Bassell and Warren, 2008). Individuals with FXS experience a wide array of symptoms, such as language abnormalities, hyperactivity, intellectual impairment, macroorchidism, and unusual dendritic spine morphology (Hagerman et al., 1986; Berry-Kravis et al., 2007; Roberts et al., 2007a; Hagerman, 2008; Barnes et al., 2009). A 1991 study by Hagerman et al established a 13 item diagnostic list of FXS symptoms, which included: pervasive speech, large/prominent ears, macroorchidim, tactile defensiveness, mental retardation, hyperactivity, short attention span, hand flapping, hand biting, poor eye contact, hyperextendable joints, simian crease, and a familial history (Hagerman et al., 1991). Additionally, 10-20% of FXS patients experience seizure (Incorpora et al., 2002; Hagerman and Stafstrom, 2009).

FXS is the most commonly inherited form of autism. There are three criteria that individuals must meet in order to be diagnosed with autism. Individuals must show 1) aberrant social behavior, 2) repetitive behavior, and 3) abnormalities in communication.

Approximately 15-33% of individuals with FXS are diagnosed with autism, and approximately 5% of all cases of autism can be attributed to FXS (Bailey et al., 1998; Cohen et al., 2005). Individuals with FXS display several deficits in social behavior. Children with FXS often display a 'pervasive lack of responsiveness to others' in early childhood, are unwilling to engage in peer play or co-operative play, and generally avoid making eye contact during social interactions (Hagerman et al., 1986). When interactions with peers do occur, children with FXS often display dysfunctional social play (Reiss and Freund, 1992). FXS patients also avoid nonverbal social interactions, and frequently demonstrate gaze aversion, or lack of social eye contact (Cohen et al., 1988; Hessl et al., 2006). Gaze aversion is often accompanied by also turning away of the body when interacting with others, especially when confronted with unfamiliar people or environments (Feinstein and Reiss, 1998; Roberts et al., 2007b). Gaze avoidance likely stems from heightened social anxiety in FXS, as gaze avoidance is associated with heightened limbic-hypothalamic-pituitary-adrenal (L-HPA) activity (Hessl et al., 2006). Consistent with increased L-HPA activity, individuals with FXS show elevated levels of cortisol and anxiety which may impact their willingness to communicate and the quality of social interactions they experience (Wisbeck et al., 2000; Tsiouris and Brown, 2004).

Additionally, individuals diagnosed with FXS demonstrate a variety of repetitive behaviors, including rocking, hand-flapping, echolalia, repetitive body movements, and self-injurious behavior (Cohen et al., 1988; Baumgardner et al., 1995; Feinstein and Reiss, 1998; Belser and Sudhalter, 2001; Baranek et al., 2005). FXS patients also display a strong preference for routine. Often, individuals with FXS over-focus on a constricted

range of interests or a specific stimuli and will become preoccupied with arranging objects (Gillberg et al., 1986; Steinhausen et al., 2002).

FXS patients also consistently show communication abnormalities. Generally, aberrant communication manifests through delays in language development and alterations in language usage (Fidler et al., 2007). Using the Reynell Developmental Language Scales, Roberts et al demonstrated delays in communication development in FXS patients manifesting as poor expressive and receptive language skills (Roberts et al., 2001). Receptive language focused on verbal comprehension ability and was assessed through FXS patients' ability to recognize sound and word patterns. Expressive language was gauged through the breadth of patients' vocabulary and ability to verbalize ideas (Roberts et al., 2001). In particular, individuals with FXS experience difficulty articulating words, poor co-articulation, substitutions and omissions of words, reduction in the number of intelligible syllables produced, echolalia, and difficulty sequencing sounds (Largo and Schinzel, 1985; Hanson et al., 1986; Belser and Sudhalter, 2001; Roberts et al., 2001; Roberts et al., 2007a; Barnes et al., 2009).

Examinations of FXS patients have revealed a variety of neuroanatomical aberrations. The neocortex of individuals with FXS consistently display a profusion of abnormally long, thin dendritic spines with a reduction in the number of short, mushroom-shaped spines (Rudelli et al., 1985; Hinton et al., 1991; Irwin et al., 2002). Moreover, voxel-based morphometry has found unusually large caudate nucleus in FXS patients and a reduction in superior temporal gyrus, amygdala, anterior ventral cerebral

gray matter, and anterior midinferior cerebral gray matter (Gothelf et al., 2008). Interestingly, superior temporal gyrus size was correlated with patients' IQ, while caudate nucleus size was correlated with the severity of symptoms seen (Gothelf et al., 2008). While the size of the superior temporal gyrus decreases with age, hippocampal size is increases in FXS patients, (Hessl et al., 2004; Gothelf et al., 2008). Diffuser tensor imaging found alterations in the frontal-caudate and parietal sensory-motor white matter tracts in FXS patients (Barnea-Goraly et al., 2003). Fundamentally however, many FXS symptoms may be attributed to over-arching arousal modulation problems, which may underlie the tendency in FXS to avoid sensory experience (Belser and Sudhalter, 1995; Cohen, 1995; Baranek, 2002; Baranek et al., 2002). In a test of electrodermal responses to olfactory, auditory, visual, tactile, and vestibular stimuli, children with FXS showed greater peak amplitude, more peaks, and a failure to habituate to stimuli, suggesting a general over-arousal to sensory stimuli (Miller et al., 1999).

FXS patients demonstrate unusual responses to auditory stimuli specifically, as measured by prepulse inhibition (PPI) and auditory startle response (ASR) tests (Frankland et al., 2004; Hessl et al., 2009; Yuhas et al., 2011; Schneider et al., 2012). In PPI tests, subjects are typically presented with a less intense (quieter) prepulse stimulus followed by a more intense (louder) startle stimulus. The prepulse stimulus acts to suppress the response to the startle stimulus, as most often measured using ocular electromyogram recordings (Frankland et al., 2004; Hessl et al., 2009). Reduced prepulse inhibition has been found in individuals with the FXS permutation condition (fragile X-associated tremor/ataxia, FXTAS), individuals with FXS but not autism, and

individuals with FXS and autism (Frankland et al., 2004; Hessl et al., 2009; Yuhas et al., 2011; Schneider et al., 2012). Interestingly, patients with autism but not FXS did not show altered prepulse inhibition (Yuhas et al., 2011). The PPI paradigm is an especially good metric as the magnitude of the PPI response in FXS patients was associated with the severity of their FXS-related symptoms (as measured through IQ, attention span, autism, and adaptive behaviors)(Frankland et al., 2004), and the number of CGG repeats patients possess (Schneider et al., 2012). The protocol for ASR testing is similar to that for PPI testing, with subjects monitored for their response to the startle stimulus alone. The FXTAS group showed a decrease in the degree of ASR (Schneider et al., 2012). Overall, impaired performance on auditory tests is thought to reflect an underlying problem with sensory gating in FXS.

1.2 Auditory Processing in FXS

To assess cognitive processing in humans with FXS, various event-related brain potential (ERP) techniques were employed. ERPs reflect the responses of neuronal populations in response to specific cognitive processes and can be detected using electroencephalograms (EEG) and magnetoencephalograms (MEG) (Luck, 2005). Auditory ERP sensory responses are comprised of N1and P2 components, as well as families of N2 and P3 components (Luck, 2005). The N1 and P2 components (vertex-potential complex) are often studied together and can be elicited by simple and complex auditory stimuli, such as pure tones or musical notes (Naatanen and Picton, 1987).

Typically, the N1-P2 complex is found within the 80-200ms following auditory stimulation (Crowley and Colrain, 2004).

Further investigation of the individual components suggests the N1 component is generated by structures within the frontal and temporal lobes (Hari et al., 1982; Naatanen and Picton, 1987). In particular, three basic components have been identified which give rise to a composite N1. The fronto-caudally predominant component is seen at 100ms and thought to be generated along the supratempoal plane (Naatanen and Picton, 1987). The biphasic component is thought to be generated by the lateral superior temporal gyrus, with a positive peak at 100ms and a negative peak at 150ms (Naatanen and Picton, 1987). The last basic component identified is the vertex-negative wave, which is seen at 100ms, though the structures responsible for generating this component are less well understood; putatively, the vertex-negative wave is attributed to transient activation of arousal networks (Naatanen and Picton, 1987). There is also MEG and EEG evidence that the auditory cortex is a prime contributor to the N1 component (Zouridakis et al., 1998). N1 itself has been shown to be modulated by the pitch and intensity of auditory stimuli (Beagley and Knight, 1967; Butler, 1968; Pantev et al., 1988; Alain et al., 1997; Butler and Trainor, 2012), and is sensitive to attention effects (Naatanen and Picton, 1987; Luck, 2005; Naatanen et al., 2011a). Specifically, as the intensity of auditory stimulus is increased, the N1 and P2 amplitude increases (Beagley and Knight, 1967; Picton et al., 1970).

Recordings using electrophysiological techniques taken while individuals with FXS performed oddball or deviant tone detection tasks uncovered a host of abnormal activity. In five studies using EEG, the N1 component was enlarged in FXS participants (St Clair et al., 1987; Rojas et al., 2001; Castren et al., 2003; Van der Molen et al., 2012a, b). Moreover, Castren et al., 2003 observed a lack of N1 habituation in FXS individuals when presented with repeating trains of single frequency tones as stimuli. A study using MEG also revealed enlargement and reduced latency of the N100m (the MEG equivalent of the N1 in EEG)(Rojas et al., 2001). FXS patients also failed to demonstrate the N100m anterior right to left asymmetry seen in typically developing adults (Rojas et al., 2001). As FXS patients are unusually sensitive to auditory stimulus (Frankland et al., 2004; Hessl et al., 2009; Yuhas et al., 2011), N1 enhancement is conceivably an artifact of the auditory hypersensitivity associated with FXS. Moreover, individuals with FXS demonstrate neuroanatomical abnormalities which may modulate N1 magnitude. In FXS patients, hippocampal size is increased, however an age related decrease in superior temporal gyrus (STG) size is also seen (Reiss et al., 1994). Despite decreased STG size, MRI studies have found white matter enlargement localized specifically to the temporal lobe (Hazlett et al., 2012). Additionally, fMRI research shows that the STG, along with the medial frontal gyrus, middle temporal gyrus, cerebellum, and pons display higher levels of activation in FXS patients, possibly contributing to a larger N1 component (Hall et al., 2009).

Generation of the P2 component has been broadly localized to the temporal lobe, but the specific structures which generate the P2 are somewhat more diffuse (Hari et al.,

1980). MEG, EEG, and implanted depth electrode evidence suggest that planum temporale and the auditory association cortex (Area 22) are heavily involved in P2 generation (Godey et al., 2001; Crowley and Colrain, 2004). There is also evidence that auditory input to the mesencephalic reticular activating system contribute to the P2 component (Rif et al., 1991; Crowley and Colrain, 2004). P2 amplitude has been shown to decrease in amplitude as attention devoted to a stimulus increases (Crowley and Colrain, 2004). Accordingly, the P2 has been shown to act as an index of task-devoted attention.

Studies that showed FXS-related N1 enhancement typically showed P2 enhancement as well (St Clair et al., 1987; Castren et al., 2003; Van der Molen et al., 2012a, b). Because both components seem to stem from temporal lobe activity, it is possible that the structural anomalies and increased temporal lobe activity that likely drives N1 augmentation also contribute to P2 enlargement. Additionally, P2 component enhancement may suggest abnormal activation of the mesencephalic reticular activating system, which may contribute to the hyperactivity seen in FXS. Interestingly, alterations in P2 may drive MMN, N2b, and P3a abnormalities (Van der Molen et al., 2012b). Structures linked to P2 generation are also responsible for early auditory processing. As such, malfunction of P2-associated structures may create an incorrect memory trace of the target stimulus which may impair performance on stimulus detection tasks (Naatanen et al., 2007; Naatanen et al., 2011b).

While the N1 and P2 components are readily modulated by altering the spectral components of auditory stimulation, the N2 and P3 component families are generally more heavily involved in task-related selective attention or novelty detection (Breton et al., 1988; Patel and Azzam, 2005). The N2 family is composed of three main components, the N2a/mismatch negativity, the N2b, and the N2c (Patel and Azzam, 2005). N2 components can be elicited with auditory or visual stimulus, and are often probed with an 'oddball' paradigm (Patel and Azzam, 2005). Oddball tasks typically involve presenting repetitive trains of a primary stimulus with deviant stimuli interspersed at unpredictable intervals (Breton et al., 1988). N2a, also called the mismatch negativity (MMN), is a feature unique to auditory attention tasks (Cone-Wesson and Wunderlich, 2003). It is associated with bilateral supratemporal processing and right hemisphere frontal lobe activity and is typically seen during tasks which require participants to attend or ignore deviant stimulus (Naatanen et al., 1978; Luck, 2005; Naatanen et al., 2007). Notably, the MMN is present when subjects passively listen to deviant stimuli and when subjects are asked to provide a response to deviant stimuli (Cone-Wesson and Wunderlich, 2003). As MMN is responsive to changes in frequency and intensity of sound, it likely represents the change in attention associated with comparing a deviant tone to the sensory-memory of the control tone (Cone-Wesson and Wunderlich, 2003; Patel and Azzam, 2005). The MMN is also responsive to language-specific speech sounds (Cone-Wesson and Wunderlich, 2003). The N2b wave can also be generated through oddball tasks, but it is most prominent during voluntary processing of deviant stimuli or when a stimulus is otherwise selectively attended (Patel and Azzam,

2005). The N2b has also been shown to be modulated by phonological and semantic changes in language (Sanquist et al., 1980). The N2c wave is most strongly associated with visual attention and stimulus context (Folstein 2008).

Enlargement of the N2b wave (Van der Molen et al., 2012a, b) and increased N2 latency (St Clair et al., 1987; Van der Molen et al., 2012a) was consistently reported in FXS patients. Despite a general increase in N2 amplitude, the MMN was reduced in individuals with FXS (Van der Molen et al., 2012b). The most likely cause for MMN component attenuation is poor memory trace formation of control stimulus (Naatanen et al., 2007). In addition to the decline in MMN amplitude, Van der Molen et al. (2012b) also reported exaggerated N1 and P2 components. Because N1 and P2 are generated by structures involved in early auditory processing, their aberrant profile may reflect altered perception of auditory stimulus, and therefore an inaccurate representation of the control stimulus (Naatanen et al., 2007). Without an accurate memory trace to compare against deviant tones, individuals with FXS may be less able to identify unexpected stimuli (Naatanen et al., 2007). Interestingly, in studies where participants were asked to respond to, rather than to passively attend deviant stimuli, FXS patients provided more false positives and were slower to respond, suggesting confusion as to the veracity of a stimulus (Scerif et al., 2012; Van der Molen et al., 2012a).

Though the N2b component is typically seen in response to oddball tasks which require participants to attend deviant stimuli, enhancement of N2b may result from a general hypersensitivity to stimuli. In control subjects, the N2b generated in response to

the deviant tone was typically larger than the N2b generated by the standard stimulus (Van der Molen et al., 2012b). In FXS subjects however, there was little difference in the N2b peak amplitudes generated by the deviant and standard stimuli (Van der Molen et al., 2012b). As the N2b peaks generated in response to both the standard and deviant stimuli in FXS participants had greater amplitudes than those of control participants, N2b enhancement in FXS participants may stem from a general increase in sensitivity to any auditory stimuli. Taken together with the reduction in MMN amplitude, oversensitivity to auditory stimulus may impair the ability of FXS participants to discriminate between standard and deviant tones. Increased N2b latency (St Clair et al., 1987; Van der Molen et al., 2012a) may be inherited from auditory processing deficiencies in auditory brainstem nuclei, result from white matter irregularities, or possibly be related to confusion regarding the nature of the stimulus. Functionally, auditory brainstem response (ABR) research has demonstrated that many FXS patients show notable differences in auditory brainstem activity. ABRs are based upon the sequential activation of neuron populations in peripheral and brainstem auditory system in response to sound. Individuals with FXS show increased latency to the peak associated with inferior colliculus activity, as well as an increase in the amount of time between peaks of activity (Arinami et al., 1988). ABR anomalies may partially explain changes in N2b activity, but given that the auditory brainstem structures are heavily involved in sound acquisition, any effects of altered ABRs should also create delays in the N1 and P2 component. Diffuser tensor imaging (DTI) revealed abnormal white matter tracts in the frontal-

caudate pathway and in parietal sensory-motor pathway, which may affect the speed of auditory processing in FXS (Barnea-Goraly et al., 2003).

The P3 family is comprised to the P3a and P3b components, which are both elicited by infrequent or unpredictable elements introduced into otherwise predictable trains of stimuli. The P3 component typically occurs 300 – 500ms after stimulus presentation and is readily evoked with oddball tasks (Hruby and Marsalek, 2003). The structures which generate the P3 component are not well understood, though there is evidence of hippocampal and temperoparietal structure involvement (Hruby and Marsalek, 2003). The P3a component occurs at 250-280ms, and is present when infrequent or unpredictable shifts occur during a train of otherwise predictable stimuli regardless of where the participant is asked to direct his or her attention (Squires et al., 1975; Hruby and Marsalek, 2003). As such, the P3a is often described as a 'novelty detector' (Comerchero and Polich, 1999; Hruby and Marsalek, 2003). The P3b is also evoked by oddball tasks, and is observed at 250-500ms (Polich, 2007). Like the P3a, the P3b component is elicited by improbable events. However, the amplitude of the P3b is dependent upon how improbable a stimulus is, with more improbable stimulus resulting in larger amplitude responses (Sutton et al., 1965; Polich, 2007). The P3b response is thought to be distributed across the prefrontal cortex, anterior insula, cingulate gyrus, medial temporal cortex, and hippocampus (Van der Molen et al., 2012a). To gauge the predictability of a given stimulus, the ability to recall variations of that stimulus is necessary. As such, short term memory is required for tasks with unpredictable stimuli (Hruby and Marsalek, 2003). Dolchin and Coles (1988), Verleger (1988) and Desmedt

(1980) offer three prominent theories of P3 generation (Desmedt, 1980; Dolchin and Coles, 1988; Verleger, 1988). In the Dolchin and Coles theory, P3 acts as an index of how frequently working memory is updated, with P3 amplitude and latency reflecting expectations regarding new information, and speed of processing, respectively (Dolchin and Coles, 1988; Hruby and Marsalek, 2003). The Verleger theory suggests that when an infrequent stimulus is presented, the expectation of a pending infrequent stimulus ends, and neuronal activity associated with maintaining a template of the expected infrequent stimulus is deactivated (Verleger, 1988; Hruby and Marsalek, 2003). Lastly, Desmedt suggests that P3 results from the activity of transitory inhibitory connections from the mesencephalic reticular formation to the prefrontal cortex which are putatively responsible for modulating the activation of appropriate brain structures in response to sensory stimuli (Desmedt, 1980; Hruby and Marsalek, 2003).

In individuals with FXS, the amplitude of the P3 component was consistently reduced and the latencies to the components were longer (St Clair et al., 1987; Van der Molen et al., 2012a, b). St. Clair and colleagues found a general reduction in P3 amplitude in individuals with FXS, but did not discriminate between P3a and P3b (1987). Van der Molen et al. revealed reduced P3a (2012b) and P3b (2012b) components in FXS patients. Though the precise source of P3 generation is uncertain, modulation of P3 amplitude or latencies suggest difficulty identifying or responding to infrequent stimuli in FXS patients (Hruby and Marsalek, 2003). Decreased P3b amplitude specifically, may reflect a failure to identify a stimulus as improbable (Sutton et al., 1965), possibly resulting from improper stimulus representation at lower levels of processing, or from

short term memory impairments (Polich, 2007). Altered short term memory function may obstruct a FXS patients' ability to recall previous stimulus (Polich, 2007).

1.3 *Fmr1* KO Mouse Phenotype

The *Fmr1* knockout mouse model (*Fmr1* KO) was developed as a tool for studying the mechanisms underlying FXS (Bakker et al., 1994). *Fmr1* KO mice not only lack FMRP, but also manifest several FXS-associated symptoms (Moy and Nadler, 2008; Bernardet and Crusio, 2006). Of particular significance, *Fmr1* KO mice replicate several symptoms consistent with the diagnostic criteria for autism. *Fmr1* KO mice show evidence of social impairments, as demonstrated by social dominance and social interaction tasks (Spencer et al., 2005). In the social dominance task, two mice are introduced to opposite ends of a hollow tube, such that one of the mice must retreat out of the tube to allow the other mouse through. *Fmr1* KO mice retreat more frequently than their control counterparts, and display a longer latency before approaching other mice (Spencer et al., 2005). Furthermore, when presented with female mice, male *Fmr1* KO mice show fewer incidences of social interactions than control male mice (Mineur et al., 2002). Repetitive behaviors have been demonstrated in *Fmr1* KO mice using marble burying tasks. In marble burying tasks, *Fmr1* KO mice are presented with an array of marbles in a cage with bedding. *Fmr1* KO mice show a higher propensity to bury marbles than do their control counterparts (Crawley, 2004, 2007). Through studying ultrasonic vocalization production, evidence has been found that *Fmr1* KO mice

experience communication deficits. As pups, *Fmr1* KO mice produce wriggling calls at a higher frequency than their littermate controls, and as adults male *Fmr1* KO mice produce mating calls at a slower rate than male wild type mice (Rotschafer et al., 2012; Roy et al., 2012).

There is also evidence that *Fmr1* KO mice may replicate the heightened anxiety associated with FXS. Elevated plus mazes are a common test for anxiety in mice, but show variable results in *Fmr1* KO mice. Elevated plus mazes usually have four arms, two of which are enclosed, while two remain open. Reluctance to enter the open arms is suggestive of heightened anxiety, and so fewer entries into open arms can serve as a measure of anxiety (Bilousova et al., 2009). While some studies report *Fmr1* KO mice spend less time in the open arms (Bilousova et al., 2009), others do not (Mineur et al., 2002; Zhao et al., 2005). Open field mazes are used to probe exploratory behavior in *Fmr1* KO mice. Results of open field maze tasks are also mixed, but do consistently show increased locomotor activity in *Fmr1* KO mice (Bakker et al., 1994; Mineur et al., 2002). Increased locomotor activity may be an artifact of FXS-related hyperactivity (Bakker et al., 1994; Mineur et al., 2002). Variability in elevates plus maze tests results may be an artifact of increased locomotor activity or hyperactivity in FXS. Entering the open arms of the plus maze at control levels may reflect *Fmr1* KO mice moving more vigorously within the maze rather than any anomaly in the anxiety the mouse experiences.

1.4 *Fmr1* KO Mouse Auditory Behavior

Fmr1 KO mouse show unusual behavior in response to auditory stimulus, as seen in audiogenic seizure, prepulse inhibition (PPI), and auditory startle response (ASR) paradigms. In *Fmr1* KO mice, intense auditory stimuli typically induce a period of wild running, clonic – tonic seizing, and can result in the death of the animal (Musumeci et al., 2000; Chen and Toth, 2001; Musumeci et al., 2007). Though the mechanism results in audiogenic seizure is not well understood, evidence of enhanced immediate-early gene induction has been found in the dorsal nucleus of the lateral lemniscus and the posterior intralaminar nucleus of the thalamus of *Fmr1* KO mice following seizure induction. Those nuclei play roles in inhibiting other auditory nuclei and receiving input form the inferior colliculus, respectively (Chen and Toth, 2001). Additionally, reintroduction of FMRP to *Fmr1* KO mice significantly reduced the incidents of audiogenic seizure (Musumeci et al., 2007). Taken together, this research suggests FMRP-associated abnormalities in auditory inhibitory nuclei.

Contrary to the human response on PPI test, *Fmr1* KO mice typically show enhanced startle response on PPI tests (Chen and Toth, 2001; Nielsen et al., 2002; Frankland et al., 2004). The stimulus protocol on mouse PPI tests is similar to what is used in human testing, with an intense startle stimulus proceeded by a less intense prepulse stimulus (Frankland et al., 2004; Bray et al., 2011). As *Fmr1* KO mice reliably show enhanced PPI response, it is routinely used as a behavioral measure for the efficacy of possible FXS treatments. When treated with the $GABA_A$ receptor agonist THIP, *Fmr1*

KO mice showed a significant reduction in PPI magnitude (Olmos-Serrano et al., 2011) suggesting that disregulation of inhibitory input may drive the PPI response in *Fmr1* KO mice. The role of mGluR activity in gating PPI is somewhat uncertain, as gauged by tests with various mGluR antagonists. Treatment of *Fmr1* KO mice with the mGluR1 antagonist (JNJ16259685) failed to affect PPI in *Fmr1* KO mice (Thomas et al., 2012). The mGluR5 antagonist MPEP, however has had variable effects on PPI severity, with some studies showing reduced PPI magnitude (de Vrij et al., 2008), and others showing no difference (Thomas et al., 2012). Inconsistency in MPEP results may be an artifact of its lack of mGluR5 specificity or tendency to bind to NMDA receptors at high concentrations (O'Leary et al., 2000; Lea et al., 2005; Levenga et al., 2011). Testing with a highly specific mGluR5 antagonist, AFQ056, resulted in PPI magnitude returning to control levels, implying that heightened mGluR5 activity plays a role in the *Fmr1* KO PPI response (Levenga et al., 2011). Though *Fmr1* KO mice and humans show incongruous PPI responses, respective enhancements and deficits in startle response are both attributed to an underlying aberration in sensory gating (Frankland et al., 2004).

When presented with startle stimulus alone, *Fmr1* KO mice generally show enhanced ASR (Nielsen et al., 2002; Yun et al., 2006). ASR enhancement was readily found in *Fmr1* KO mice after 3 weeks of age (Yun et al. 2006). Interestingly, the degree of ASR magnitude was uneffected by stimulus intensity in *Fmr1* KO mice. The *Fmr1* KO mouse ASR was relatively large when presented with low intensity stimuli, and relatively small in response to high intensity stimuli (Nielsen et al., 2002). Failure to

modulate behavior in response to the magnitude of sensory input is also indicative sensorimotor gating deficits (Nielsen et al., 2002).

1.5 Altered Cortical Circuitry in *Fmr1* KO Mice

Fmr1 KO mice are an essential tool for understanding the alterations in neuronal function in FXS. Consistent with research demonstrating mGluR antagonists and GABA receptor agonists improve *Fmr1* KO mouse responses to auditory stimuli, evidence of both GABA and glutamate imbalances have been found in *Fmr1* KO mice. Notably, GABA receptor subunit expression appears to be altered in *Fmr1* KO mice. Specifically, mRNA of the α_1, α_3, α_4, β_1, β_2, γ_1, and γ_2 GABA receptor subunits were down regulated by 35 to 50%, and the actual α_1, β_2, and δ GABA receptor subunits were down-regulated in *Fmr1* KO mice (D'Hulst et al., 2006; D'Hulst et al., 2009; Adusei et al., 2010). The β subunit is required for the assembly of functional $GABA_A$ receptors and was associated with a decrease in the number of GABA receptors on pyramidal cell in cortex of *Fmr1* KO mice. Regions that showed a decrease in the number of $GABA_A$ receptors also demonstrated increased glutamic acid decarboxylase (GAD) levels (El Idrissi et al., 2005). In the hippocampus, elevated GAD levels are associated with increased excitatory input and may represent an increase in there excitability of cortical cells here (Ramirez and Gutierrez, 2001). Additionally, a 20% reduction in the number of parvelbumin positive (PV+) cells was found in the somatosensory cortex of *Fmr1* KO mice. Specifically, a notable reduction in PV+ cells was found in layers II/III and IV of the

cortex, while deeper layers demonstrated an increase in PV+ cells (Selby et al., 2007). Underlying alterations in GABA receptor function and excessive excitatory input may result in some changes in auditory behavior seen in *Fmr1* KO mice.

Like FXS patients, *Fmr1* KO mice exhibit abnormal cortical and hippocampal dendritic spine morphology. Dendritic spines on pyramidal cells in the visual cortex of *Fmr1* KO mice are longer, thinner, and generally display a more immature morphology than control mice, with increased spine density along dendrites (Comery et al., 1997; Irwin et al., 2002; McKinney et al., 2005). Pyramidal cells in the temporal cortex are less readily studied, but also showed morphological changes similar to those seen in the visual cortex (Irwin et al., 2000). The *Fmr1* KO mouse barrel cortex has been studied more extensively and shows a host of dendritic spine abnormalities. Examination of dendritic spine development over the course of cortical development revealed that relatively young mice (P25) did not show dendritic spine abnormalities, but older mice (P73 – P76) show fewer short/ mature spines and more long/immature spines (Galvez and Greenough, 2005; Till et al., 2012). Transcranial two-photon imaging revealed that dendritic spines in the barrel cortex of *Fmr1* KO mice also display a higher turnover rate, with more pools of new, transient spines (Pan et al., 2010). Dendritic spines in the hippocampus of *Fmr1* KO mice were altered, with pyramidal cells showing longer, more immature spines, and fewer mature, mushroom shaped spines (Grossman et al., 2006).

The *Fmr1* KO mouse barrel cortex also demonstrates delayed formation, and abnormal dendrite pruning. In mice, each vibrissa (whisker) is represented by a cortical

barrel that has a cell body dense septa and cell-sparse hollow. During development, the number of dendrites at the septa decreases, while the number of dendrites growing to the hollow increases. Pruning of dendrites growing toward within the septa results in asymmetrical dendrite distribution in wild type adult animals (Greenough and Chang, 1988). In *Fmr1* KO mice, spiny stellate cells in the barrel cortex have an excessive number of dendrites oriented toward the septa, resulting in less asymmetrical cells (Galvez et al., 2003; Till et al., 2012). Mouse somatosensory cortex development has been well characterized, and the morphological anomalies seen in *Fmr1* KO mouse dendritic development is consistent with a developmental delay in barrel cortex map formation (Till et al., 2012). Consistent with other cortical areas, the cells in the somatosensory cortex of *Fmr1* KO mice also show unusual dendritic spine morphology and a higher spine turnover rate (Pan et al., 2010). Functionally, dendritic spines in the *Fmr1* KO mouse barrel cortex are less sensitive to sensory experience modulation. Sensory deprivation (all whiskers on one side of the facial pad were trimmed) resulted in a reduced the rate of dendritic spine elimination in wild type mice, but was unaltered in *Fmr1* KO mice (Zuo et al., 2005; Pan et al., 2010). Alternatively, dendritic spine formation was enhanced in wild type mice when the whiskers were trimmed in a chessboard pattern, while *Fmr1* KO mice failed to show any difference in spine formation. Failure to form or eliminate dendritic spines in response to changing sensory input suggests that barrel cortex neurons in *Fmr1* KO mice may be improperly tuned to sensory stimuli (Pan et al., 2010).

Neurons in the *Fmr1* KO mouse somatosensory cortex also show an altered balance between inhibitory and excitatory input. Specifically, neurons within the *Fmr1* KO mouse somatosensory cortex show disorganized or weakened inhibitory interneuron activity and more excitable pyramidal neurons (Gibson et al., 2008; Hays et al., 2011; Paluszkiewicz et al., 2011). Following group 1 mGluR stimulation in *Fmr1* KO mice, somatostatin-expressing low-threshold spiking (LTS) interneuron activation was reduced (Paluszkiewicz et al., 2011). Paired pyramidal cell recordings then displayed a decrease in synchrony of synaptic inhibition and spiking (Paluszkiewicz et al., 2011). Similarly, monosynaptic GABAergic transmission in the barrel cortex of *Fmr1* KO mice is unaffected, but fast spiking (inhibitory) interneurons experience an approximate 50% decrease in excitatory drive (Gibson et al., 2008). *Fmr1* KO mouse somatosensory neurons also display hyperexcitability, as characterized by longer, less synchronous UP states (Gibson et al., 2008; Hays et al., 2011). UP states were found to be 38-67% longer in *Fmr1* KO mice in the somatosensory cortex, and are likely driven by excessive mGluR5 signaling, as application of mGluR5 antagonist rescues UP state duration. Interestingly, prolonged UP states seem to be caused by *Fmr1* loss alone, rather than impaired inhibitory neuronal activity (Hays et al., 2011). To elucidate the role of *Fmr1* on inhibitory and excitatory neuron activity, mouse models were created which expressed *Fmr1* in GABAergic, but not glutamatergic neurons, and a second mouse model which expressed *Fmr1* in glutamatergic but not GABAergic neurons. UP states were prolonged in cortical slices that did not express *Fmr1* in glutamatergic neuron, while deletion of *Fmr1* in GABAergic neurons had no effect on UP state duration (Hays et al., 2011).

Prolonged UP states are generated by the over activity of mGluR5 receptors on excitatory neurons which lack *Fmr1*, rather than any *Fmr1* deficiency in inhibitory neurons (Hays et al., 2011).

Aberrant plasticity is a prominent feature in the hippocampus of *Fmr1* KO mice. Long term depression (LTD) is a protein synthesis dependent form of plasticity which is triggered by mGluR5 activation (Huber et al., 2002b; Bear et al., 2004; Huber, 2007; Bassell and Warren, 2008). Typically, FMRP is synthesized in response to mGluR activation and represses the translation of several mRNAs which encode proteins associated with AMPA receptor endocytosis at the synapse (Bear et al., 2004; Bassell and Warren, 2008). When FMRP dissociates from mRNA targets, AMPA receptor internalization is facilitated. Removal of AMPA receptors from the post synaptic membrane then reduces the magnitude of the postsynaptic response (Bear et al., 2004; Bassell and Warren, 2008). Excessive mGluR activity and loss of FMRP in *Fmr1* KO mice is thought to drive excessive synthesis of proteins associated with the internalization of AMPA receptors (Huber et al., 2002a; Bear et al., 2004; Huber, 2007). As a result, *Fmr1* KO mouse hippocampal neurons experience greater LTD (Huber et al., 2002a). In addition to LTD enhancement, reduced long term plasticity (LTP) is a recurring finding in *Fmr1* KO mice. LTP deficits are found in the anterior pyriform cortex and deep layers of the visual cortex (Wilson and Cox, 2007; Larson et al., 2008), though LTP did not seem to be altered in the hippocampus of *Fmr1* KO mice (Larson et al., 2008).

1.6 Conclusion

Individuals with FXS show evidence of auditory processing deficits. Notably, ERP research revealed enhanced N1 and P2 peaks in FXS patients presented will an oddball task (St Clair et al., 1987; Rojas et al., 2001; Castren et al., 2003; Van der Molen et al., 2012a, b). The N1 and P2 components are typically attributed to temporal lobe activity, making greater N1 and P2 magnitude in FXS patients suggestive of excessive temporal lobe activity. The N2 and P3 components are also altered in FXS patients, reflecting altered responses to unexpected stimuli. The underlying cellular sources of auditory processing deficits in FXS patients however remain elusive. *Fmr1* KO mice provide a means of studying alterations in sensory processing in FXS, however little work has been done to investigate whether *Fmr1* KO mice show auditory processing deficits or the nature of possible auditory processing deficits.

In probing spectrotemporal processing in the *Fmr1* KO mouse auditory cortex, we hope to establish whether *Fmr1* KO mice process display processing abnormalities and whether the *Fmr1* KO mouse is a fit model for developing treatments for FXS-specific communication deficits. To that end, we will test *Fmr1* KO mouse auditory processing using single frequency and frequency modulated (FM) sweep stimuli. Processing of both single frequency tones and FM sweeps require measured interaction between inhibitory and excitatory inputs, and are therefore appropriate test stimuli for possible imbalances of excitation and inhibition in the mouse auditory cortex (Fuzessery and Hall, 1996; Gordon and O'Neill, 1998; Ma and Suga, 2004; Razak and Fuzessery, 2006, 2007; Wu et al., 2008). Identifying how auditory processing may be altered *in vivo* may assist researchers

in developing targeted treatments for the symptoms of FXS and in devising more focused assessments of auditory processing in FXS.

Patients with FXS also demonstrate aberrant communication (Roberts et al., 2001; Fidler et al., 2007; Price et al., 2007; Roberts et al., 2007a; Roberts et al., 2007b; Price et al., 2008; Barnes et al., 2009). Currently, there are few assays for assessing communication is *Fmr1* KO mice (Rotschafer et al., 2012; Roy et al., 2012). Here, we establish a means of assessing vocalization production deficiencies in *Fmr1* KO mice, and provide a possible treatment of communication deficiencies in FXS (Rotschafer et al., 2012).

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Chapter 2: Altered Auditory Processing in a Mouse Model of Fragile X Syndrome

Abstract

Fragile X syndrome (FXS) is the most common cause of inherited intellectual impairment and autism. Evoked potentials in response to sound stimuli indicate that FXS is associated with abnormal auditory processing, but the underlying mechanisms are not clear The *Fmr1* KO mouse is a useful model for studying FXS as many neural abnormalities and symptoms associated with FXS are also seen in these mice. These mice also exhibit acoustic hypersensitivity and propensity for audiogenic seizures suggesting altered auditory responses, but the nature of such changes are not known. In order to elucidate possible abnormalities in auditory processing in *Fmr1* KO mice, we performed single unit extracellular electrophysiological recordings in the auditory cortex of urethane/xylazine-anesthetized *Fmr1* KO mice in response to tones and frequency modulated (FM) sweeps. Using tones as stimuli, we report expanded frequency tuning, enhanced response magnitude, and more variable first spike latencies in *Fmr1* KO mice compared to wild-type control. FM sweep stimuli revealed altered sensitivity to the rate of frequency change indicating abnormal spectrotemporal processing. There were no differences in FM sweep direction selectivity. The differences in *Fmr1* KO mouse auditory processing provide insight into the possible neuronal mechanisms which underlie auditory processing abnormalities in FXS and could serve as outcome measures for the efficacy of treatments for FXS.

2.1 Introduction

Fragile X Syndrome (FXS) is a genetic disorder that affects 1 in every 4000 males and 1 in every 8000 females (Hagerman, 2008). FXS results from elongated CGG trinucleotide repeats in the promoter region of the *FMR1* gene which become hypermethylated, leading to inactivation of the *FMR1* gene and a failure to produce fragile X mental retardation protein (FMRP) (O'Donnell and Warren, 2002). FMRP acts to inhibit the translation of several synaptic mRNAs, and loss of FMRP typically results in an over-production of associated synaptic proteins (Bassell and Warren, 2008). The symptoms of FXS include hyperactivity, altered social interactions, macro-orchidism, repetitive behavior, abnormal dendritic spine formation, intellectual disability, language deficits and seizures (Largo and Schinzel, 1985; Hanson et al., 1986; Roberts et al., 2001; Fidler et al., 2007; Roberts et al., 2007; Barnes et al., 2009). FXS is a leading known genetic cause of autism spectrum disorders (Hagerman, 2008).

FXS patients also display an array of auditory cortex structural and functional abnormalities. There is a reduction in size of the superior temporal gyrus in FXS (Reiss et al., 1994) and a temporal lobe-specific white matter enlargement (Hazlett et al., 2012). Brain activity of FXS patients is diffuse due to activation of more areas in the brain than typically seen in controls when presented with a series of tones (Hall et al., 2009). Electroencephalogram (EEG) studies have demonstrated that FXS patients have enlarged N1 and N2 components when presented with deviant tone stimuli (Castren et al., 2003; Van der Molen et al., 2012b, a), and show unusually slow background rhythm (Wisniewski et al., 1991). Using magnetoencephalography (MEG), Rojas and colleagues

showed that the N100 (equivalent of N1 in EEG) was also enhanced during an auditory oddball task (Rojas et al., 2001). N1 is typically associated with activity within the superior temporal gyrus (Scherg and von Cramon, 1986; O'Connor, 2012). The nature of deficits in the auditory cortex that may lead to aberrant EEG and MEG signals remains unclear.

The goal of the present study was to determine auditory response selectivity at the level of individual neurons in the primary auditory cortex (A1) and the anterior auditory field (AAF) of the *Fmr1* knock-out (KO) mouse and compare responses with wild-type (WT) controls. *Fmr1* KO mice display several symptoms associated with FXS, and is the commonly used disease model for FXS. Like their human counterparts, *Fmr1* KO mice display abnormal dendritic spine formation in cortex and hippocampus (Galvez et al., 2003; McKinney et al., 2005; Grossman et al., 2006; Cruz-Martin et al., 2010; Grossman et al., 2010; Pan et al., 2010). Social communication abnormalities have been demonstrated in *Fmr1* KO mice as a reduced tendency to vocalize during mating (Rotschafer et al., 2012). There is also evidence that *Fmr1* KO mice show social deficits when interacting with other mice, and engage in repetitive behaviors (Mineur et al., 2002; Spencer et al., 2005; Crawley, 2007). In the auditory brainstem, FMRP contributes to maintenance of tonotopic gradients in potassium ion channels and KO mice show deficits in experience-dependent plasticity (Strumbos et al., 2010). *Fmr1* KO mice also show audiogenic seizures and hypersensitivity to auditory stimuli (Musumeci et al., 2000; Chen and Toth, 2001; Errijgers et al., 2008) as seen in human FXS patients (Hagerman et al., 1986; Hagerman et al., 1991; Miller et al., 1999; Frankland et al., 2004; Hessl et al.,

2009; Yuhas et al., 2011). This suggests abnormal responses in the auditory system of the *Fmr1* KO mice, but the nature of such deficits are not known.

In vitro studies of the *Fmr1* KO mice somatosensory cortex have shown abnormalities in the balance between inhibition and excitation (Gibson et al., 2008, Hays et al., 2011, Paluszkiewicz et al., 2011). Such an imbalance has been postulated as an important mechanism underlying symptoms in several neurodevelopmental disorders (Rubenstein and Merzenich, 2003). It is not known if the auditory cortex in *Fmr1* KO mice shows similar changes, but the auditory processing deficits seen in both FXS patients and the *Fmr1* KO mice formed the motivation to examine *in vivo* response selectivity in the auditory cortex. Specific emphasis was on properties known to depend on interactions between excitatory and inhibitory components of the receptive field.

The frequency receptive fields of auditory cortical neurons contain both excitatory and inhibitory components (Calford et al., 1983; Sutter and Loftus, 2003; Kaur et al., 2004; Tan et al., 2004; Oswald et al., 2006; Tan et al., 2007). Inhibitory input is typically broader than excitatory input and may serve to sharpen excitatory frequency tuning because antagonists of $GABA_A$ receptors broaden frequency tuning of cortical neurons (Muller and Scheich, 1988; Foeller et al., 2001; Wang et al., 2002; Kaur et al., 2004; Wu et al., 2008). The first property we compared between KO and WT mouse cortex, therefore, was excitatory frequency tuning. The response of cortical neurons to excitatory tones is characterized by an early excitation that produces an onset response, followed by inhibition that shortens the time course of excitation (Wehr and Zador,

2003). The hypersensitivity and seizures in response to sounds seen in KO mice may arise in part due to increased excitation of auditory neurons. Therefore, we compared the magnitude of response to excitatory tones between KO and WT neurons.

A third class of response property known to depend on interactions between inhibitory and excitatory components of the receptive field is selectivity for frequency modulated (FM) sweeps (Zhang et al., 2003; Razak and Fuzessery, 2006, 2009). Hall et al. (2009) showed differences in brain regions activated in response to a tone sequence in FXS patients, suggesting possible changes in temporal processing. FM sweeps are relatively simple and useful stimuli to test spectrotemporal processing in the auditory system. Moreover, the auditory cortex of every species examined, including mice (Trujillo et al., in review), contains neurons selective for the rate and direction of FM sweeps (Mendelson et al., 1993; Godey et al., 2005; Razak and Fuzessery, 2006; Brown and Harrison, 2009; Shechter et al., 2009). The third goal was to compare response selectivity for FM sweep rate and direction across KO and WT mice cortex. The spectral and temporal properties of sideband inhibition shape selectivity for FM sweeps across species including mice (Gordon and O'Neill, 1998; Razak and Fuzessery, 2006, 2007a). Therefore, we also compared the bandwidth and timing of sideband inhibition across the two groups of mice. We found *Fmr1* KO mouse neurons had significantly broader frequency tuning curves, a larger jitter in first spike latency, stronger excitatory response to tones and altered selectivity for FM sweep. However, there was no difference in FM sweep direction selectivity and sideband inhibition properties. The changes in auditory

response selectivity may explain observed changes in auditory processing in FXS patients.

2.2 Methods

2.2.1 Animals

Fmr1 KO mice are available on both FVB and C57bl/6 background strains (Bernardet and Crusio, 2006). The latter strain is subject to accelerated hearing loss. FVB mice do not show early onset hearing loss and hearing thresholds are low up to at least 7 months of age (Zheng et al., 1999). Therefore, we chose the FVB strain in this study. All mice used here were between 1 and 4 months old. 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 hour light/dark cycle. All studies were performed in accordance with the National Institutes of Health and with Institutional Animal Care and Use Committee guidelines. Mice were housed with 1 – 3 littermates and fed *ad libitum*. Fifty control mice and 37 *Fmr1* KO mice (both males and females) were used in this study.

2.2.2 Surgery

Mice were anesthetized with a combination of 1 g/kg urethane and 20 mg/kg xylazine. A toe pinch was administered every half hour to assess anesthetic state, and supplemental doses of urethane and xylazine were given as needed. When an areflexic anesthetic state was reached, a midline incision was made to expose the skull. The skull

was then cleaned and the temporalis muscle was reflected. A dental drill was used to perform a craniotomy to expose the auditory cortex, identified using vascular landmarks and the Paxinos mouse brain atlas. At the conclusion of the experiment, mice were euthanized with a lethal dose of 125 mg/kg sodium pentobarbital.

2.2.3 Electrophysiology

Mice were secured on a bite bar and placed in a stereotaxic apparatus (model 930; Kopf, Tujunga, CA). Experiments were performed in a sound-attenuated chamber lined with anechoic foam (Gretch-Ken Industries, Lakeview, OR). Electrophysiological recordings were obtained using glass electrodes filled with 1 M NaCl (impedance 2-10 MΩ). Electrodes were maintained orthogonal to the auditory cortex and driven into the cortex using a Kopf direct drive 2660 micropositioner. Recordings were gathered at depths of 200 – 700 μm (Table 1) with most neurons recorded between 300-700 μm. The majority of our recordings were therefore gathered in the granular (layers III and IV) and infragranular (layers V and VI) layers of the auditory cortex (Anderson et al., 2009; Christianson et al., 2011). Single unit recordings were isolated using a window discriminator and identified by the waveform displayed and consistency of the spike amplitude. Each stimulus was repeated 20 times and the number of action potentials elicited within 200 msec of stimulus onset was counted.

2.2.4 Acoustic Stimulation

Sounds were presented through a free-field speaker (LCY-K100 ribbon tweeter; Madisound, Middleton, WI) maintained 6 inches and 45 degrees from the left ear. All recordings were obtained from the right cortex. A1/AAF location was confirmed using tonotopy, vascular landmarks and robust, short latency responses to pure tones. The frequency response of the sound delivery system, assessed with a ¼ inch Bruel and Kjaer microphone and measuring amplifier, was flat within ±3 dB between 7 and 40 kHz. The roll-off at higher frequencies was gradual at ~20dB/octave. Acoustic stimulation and data acquisition were done with custom-written software (Batlab, Dr. Dan Gans, Kent State University, OH) and a Microstar digital signal processing board. Sound intensity was controlled by programmable attenuators (PA5; Tucker-Davis Technologies, Gainesville, FL).

2.2.5 Frequency Tuning

Isolated single units were probed with 50 ms pure tones (1 msec rise/fall times). At a given intensity, the frequency of the tone was increased in 1 or 5 kHz increments. A neuron was counted as responding to a given frequency if it produced action potentials to at least 4 of 5 consecutive stimulus presentations. Frequencies between 5 and 50 kHz were tested because the vast majority of neurons in the lemniscal auditory pathway of mice are tuned <50 kHz (Portfors and Felix, 2005; Willott, 2006). Both A1 and AAF are considered core auditory cortical fields in the lemniscal pathway. Tone intensity was increased or decreased by 10 dB to determine frequency-intensity tuning curves. The

range of intensity tested was between 30 and 90 dB SPL. The characteristic frequency (CF) was defined as the frequency to which the neuron responded at the lowest intensity tested. The intensity level at which CF was revealed was termed 'minimum threshold'. Q-values were used to compare the tuning curve bandwidths by dividing the CF by the bandwidth of tuning at 10, 20, or 30 dB above minimum threshold. Larger Q-values indicate narrower tuning curves.

2.2.6 Response Magnitude and variability of first spike latency

In order to compare the magnitude of neuronal responses in WT and KO mouse neurons, a 10 msec single frequency tone at the CF was repeated 20 times. The total response was then averaged over the 20 trials. To assess variability in first spike latency in response to repetitions of the same tone, the Fano factor was calculated for neurons from both WT and KO mice using the following equation:

$$\text{Fano Factor} = \text{Variance} / \text{Mean}$$

In this equation, 'variance' refers to the variance of first spike latency over 20 trials, and 'mean' referred to the average first spike latency.

2.2.7 Frequency-Modulated (FM) sweep rate selectivity

FM sweeps are relatively simple sounds used to study spectrotemporal processing. Neurons sensitive to the direction and/or rate of FM sweeps are present in the mouse cortex (Trujillo et al., 2011). To test FM sweep rate selectivity, linear FM sweeps were presented at a fixed bandwidth (centered near the CF) and various durations.

FM sweep rates (kHz/msec) were calculated by dividing the bandwidth of a sweep (kHz) by the duration of the sweep (msec). The bandwidth of an FM sweep was chosen to include all excitatory frequencies of a given neuron at the intensity tested and to exceed the bandwidth of the tuning curve by at least 5 kHz. This method of choosing a bandwidth captures putative inhibitory sidebands that are known to shape selectivity for FM sweep rate/direction (Razak and Fuzessery, 2006; Zhang et al., 2003). FM sweeps were presented at 10-20 dB above minimum threshold. Sweep rates between 0.2 and 45 kHz/msec were tested.

Neuronal responses to FM sweeps were classified according to four different response types: all pass (AP), band pass (BP), fast pass (FP), or slow pass (SP) (Trujillo et al., 2011). All pass neurons responded within 50% of maximum response to all FM sweep rates presented. Band pass neurons responded maximally to an intermediate range of rates, with the responses falling below 50% of the maximum at rates both greater than and less than the preferred rate. Fast pass neuron responded maximally to relatively fast rates, with response falling below 50% of the maximum as the sweep rate was decreased. Slow pass neurons responded best to relatively slow rates, with response falling below 50% as the sweep rate was increased.

The '50% cutoff rate' was calculated for FP and BP neurons. In FP neurons, the 50% cutoff rate was the rate at which the response fell to 50% of the maximum response as the sweep rate was slowed. In BP neurons there were two possible 50% cutoff rates: the 50% cutoff for increasing rates (termed 'fast 50% cut-off rate'), and the 50% cutoff for decreasing rates (termed 'slow 50% cut-off rate'). The 'best rate' of BP neurons was

calculated as the geometric mean of the neuronal response at 80% of the maximum response.

A rate tuning index (RTI) was quantified to determine the degree of rate selectivity (Godey et al., 2005; Trujillo et al., 2011):

$$RTI = [(n /(n-1)] \times [1 – (mean/maximum)]$$

where, *n* was the number of sweep rates tested, *mean* was the average response across all rates presented, and *maximum* was the maximum response. Values closer to 1 describe neurons with a high degree of rate selectivity. A value near 0 indicates a non-selective neuron.

2.2.8 FM sweep direction selectivity

FM sweep direction selectivity was determined by comparing responses to linear upward and downward sweeps of the same bandwidth. The direction selectivity index (DSI) was calculated using the following equation:

$$DSI = (D – U)/(D + U)$$

D and U were the trapezoidal area under the curve in response to downward and upward FM sweeps, respectively. As direction selectivity can change with sweep rate (Zhang et al., 2003; Razak et al., 2008a), DSI was evaluated at three different ranges of rate. DSI was found for ‘slow’ rates (0.18 – 1 kHz/msec), for ‘medium’ rates (1.1 – 3.0 kHz/msec), and for ‘fast’ rates (3.1 – 10.0 kHz/msec). DSI values near 1 suggest a preference for

downward FM sweeps, while DSI values near -1 suggest a preference for upward FM sweeps.

2.2.9 Sideband Inhibition

High frequency and low frequency sideband inhibition play a significant role in shaping the neuronal response to FM sweeps (Zhang et al., 2003; Ma and Suga, 2004; Sadagopan and Wang, 2010). Notably, the bandwidth and arrival time of sideband inhibition can be used to predict FM sweep selectivity in bats (Razak and Fuzessery, 2006) and rodents (Trujillo et al., In review). Here, the two-tone inhibition paradigm was used to determine the bandwidth and the arrival time of inhibitory sidebands in a manner similar to Razak and Fuzessery (2006). A 5-10 msec tone was played at a neuron's CF and acted as a control response. A second 5-10 msec tone was played at putative inhibitory frequencies and was delayed or advanced relative the control tone. The intensity of both tones was the same at 10-20 dB above minimum threshold. The frequency of the putative inhibitory tone began at either end of the tuning curve and was increased or decreased with a 1 kHz resolution. A tone was counted as inhibitory if it caused a reduction of at least 50% of response to the control tone for at least one of the following delays: 0, 1, 3, 5, and 7 msec (control tone delayed). The range of frequencies producing inhibition was noted as the bandwidth of inhibition. The shortest delay that produced an inhibition of 50% relative to control tone was taken as arrival time of inhibition.

2.3 Results

The main goal of this study was to compare cortical responses to tones and FM sweeps between the *Fmr1* knockout (KO) mice and wild-type controls (WT).

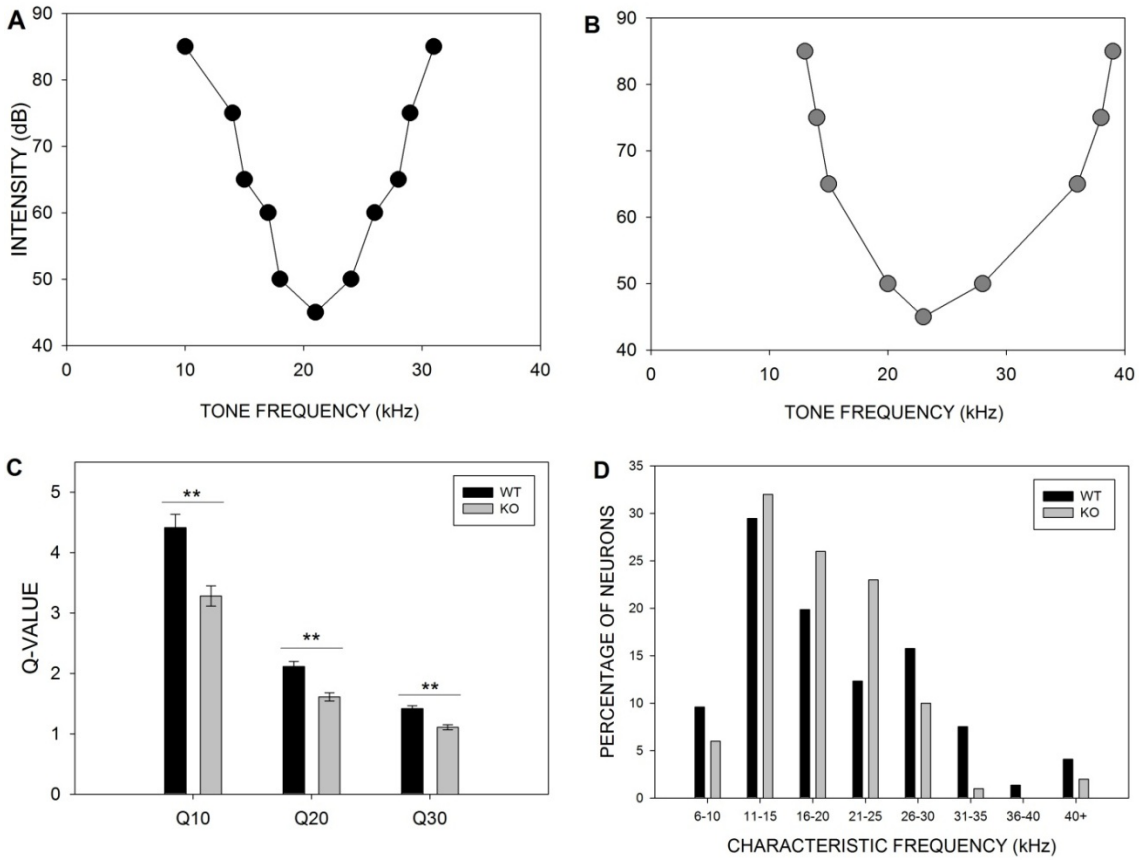
Tone responses

2.3.1 Fmr1 KO neurons are more broadly tuned than WT neurons

Q-values for 205 neurons from WT mice and 134 neurons from KO mice at 10, 20, and 30 dB above threshold were compared. Example tuning curves typical of WT and KO mouse cortex are shown in Figure 1A and 1B, respectively. Across the population, frequency tuning in KO mice was broader (e.g., Figure 1B) than in WT mice (Figure 1C, one-way ANOVA $F(5,782) = 86.063$, $p < 0.001$). Neither the distribution of CF ($\chi^2 = 28$, $p = 0.260$, Figure 1D) nor the cortical depths of recording ($\chi^2 = 6$, $p = 0.199$, Table 1) were significantly different between WT and KO mice, indicating that the broader tuning in the KO mice was not an artifact of differences in recording depth or tonotopic location sampling bias. The minimum threshold for tone responses were not significantly different between the two groups (t-test, $p = 0.231$).

Figure 1: Neurons from Fmr1 KO mice show significantly broader frequency tuning and lower Q-values than WT mice.

Examples of frequency tuning curves from a WT mouse neuron (A) and a KO mouse neuron (B). The example neurons are of comparable CF and threshold, however, the KO neuron is more broadly tuned. C. Q-values at 10, 20, and 30 dB above threshold were significantly smaller in KO mice neurons compared to WT mice neurons (WT: n = 127 neurons, KO: n = 97 neurons). This indicates broader frequency tuning in KO mouse cortex. (D) WT and KO neurons sampled did not significant differences in best frequency.

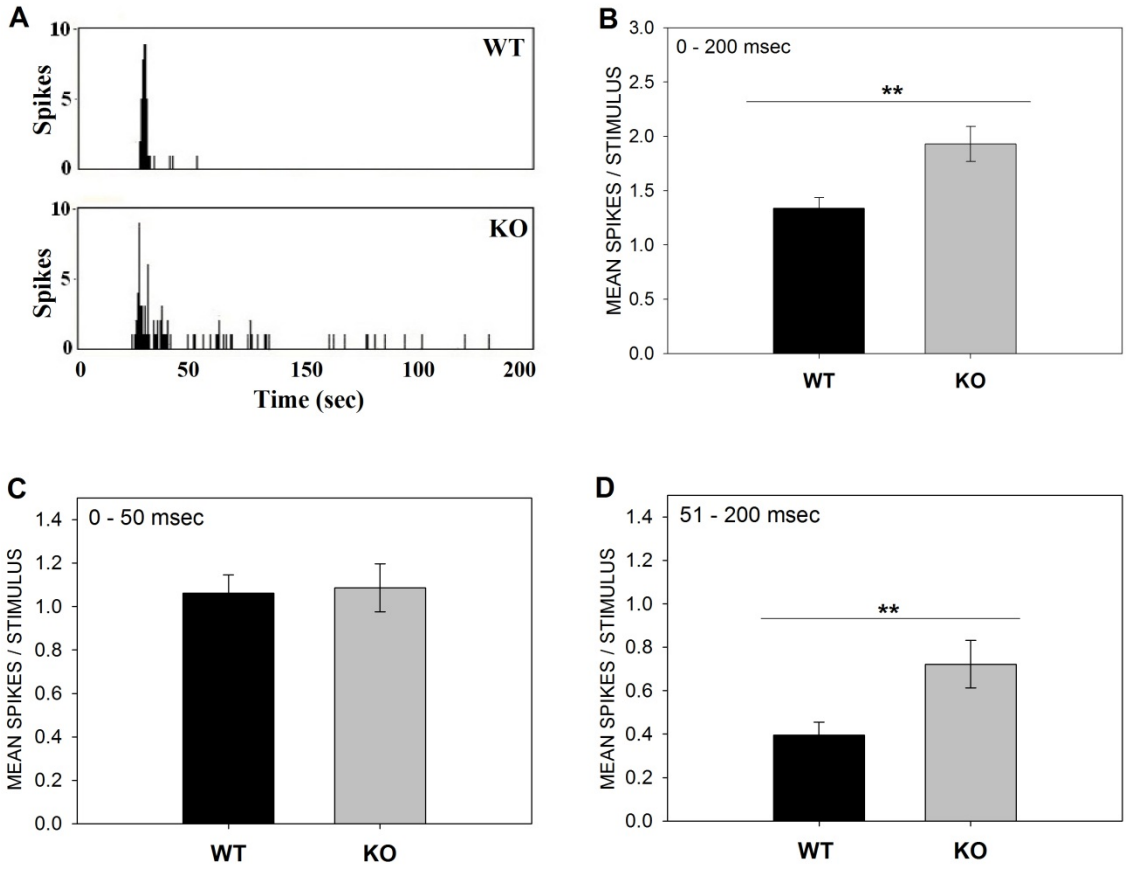


2.3.2 Fmr1 KO neurons produce more spikes and show a larger variability in first spike latency

Fmr1 KO mice are acoustically hypersensitive and are prone to audiogenic seizures (Chen and Toth, 2001; Nielsen et al., 2002; Frankland et al., 2004), suggesting enhanced excitability in the auditory system. To test this, the magnitude of response to the CF tone was compared. Figures 2A and 2B provide examples of neuronal responses to 10 msec single frequency tone stimulus in WT and KO mice respectively. When response magnitude was compared across the entire 200 msec recording window, neurons from KO mice showed a significant enhancement in response magnitude (Figure 2C, Mann-Whitney Rank Sum Test, $p = 0.002$). This enhancement was carried by changes in response magnitude during the latter portion (51-200 msec, Figure 2E, Mann-Whitney Rank Sum Test, $p = 0.002$) of response and not due to changes in the first 50 msec of responses (Figure 2D, Mann-Whitney Rank Sum Test, $p = 0.916$).

Figure 2: Fmr1 KO neurons show a significantly larger response magnitude than WT neurons in response to the CF tone presented at an intensity of 20 dB above threshold.

Presenting pure tone stimuli elicits a response in both wild type and *Fmr1* KO neurons, with KO neurons displaying a more prolonged response (A). KO neurons (n=51) show a greater response magnitude than WT neurons (n=67) when the entire 200 msec recording windows were compared (B). There was no significant difference between groups in the first 50 msec following stimulus onset (C). The enhanced response seen in KO neurons was the result of increased activity in the 51 – 200 msec window following stimulus onset (D).

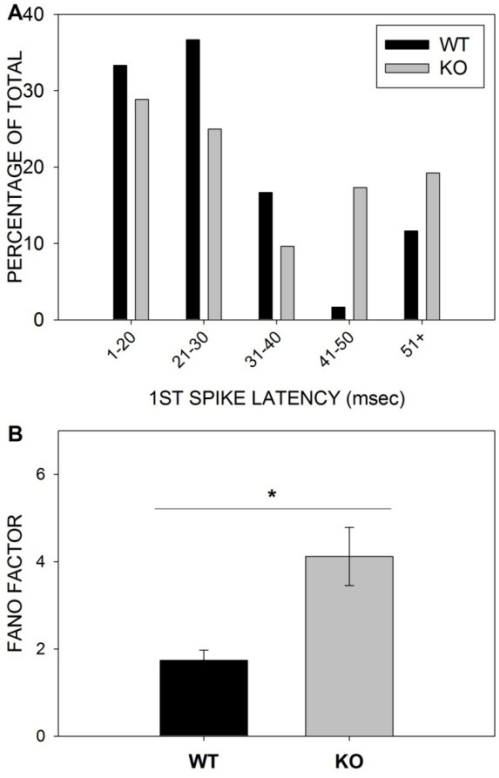


The mean first spike latency was not significantly different in KO mice (Figure 3A, Mann-Whitney Rank Sum Test, p = 0.088), and was less than 50 msec in the majority of

neurons. These data suggest an inability of KO neurons to shut down activity following an initial onset-related burst. First spike latency was also more variable in KO mouse neurons. Fano factor was larger in KO neurons (Mann-Whitney Rank Sum Test, p = 0.027, Figure 3B) suggesting reduced temporal precision of excitatory tone representation in KO neurons.

Figure 3: Distribution of first spike latency and first spike variability in WT and KO mouse neurons.

A. In both groups, the first spike occurred within the first 50msec in the majority of neurons. B. *Fmr1* KO neurons show greater variability in first spike latency. KO neurons (n=51) demonstrate a larger Fano factor than WT neurons (n = 67).



Spectrotemporal processing

2.3.3 FM sweep rate selectivity

FM sweep rate selectivity was tested in 155 WT neurons and 101 KO neurons, using upward and downward FM sweeps with sweep rates between 0.2 to 45 kHz/msec. Neuronal response was classified as being fast pass (FP, Figure 4A), band pass (BP, Figure 4B), all pass (AP, Figure 4C), or slow pass (SP Figure 4D). No significant difference was found for the distribution of FM rate selectivity types to upward (χ^2 = 13.543, p = 0.140) or downward (χ^2 = 5.890, p = 0.751) FM sweeps across WT and KO mice (Figures 4E and 4F).

Cortical neurons in KO mice were, however, less selective for FM sweep rates compared to WT neurons. The rate tuning index (RTI) was used as a measure of FM rate selectivity (Godey et al., 2005; Trujillo et al., 2011). Both BP (one-way ANOVA F(3,80) = 6.988, p < 0.001) and FP (one-way ANOVA F(3,133) = 21.584, p < 0.001) neurons in KO mice demonstrated smaller mean RTI values in response to upward and downward FM sweeps than WT neurons (Figure 5A and 5B, respectively). No significant differences were seen in the RTI values of SP (Figure 4C, one-way ANOVA F(3,72) = 0.795, p = 0.501) or AP neurons (Figure 5D, one-way ANOVA F(3,138) = 0.416, p = 0.742).

Figure 4: Neuron classification according to FM sweep rate selectivity (A – D) and the distribution of FM rate selectivity classes in WT and KO cortex (E – F).

Rates between 0.18 and 45 kHz/msec were tested for each neuron. Neurons that favored fast rates were classified as 'fast pass' (A); neurons that responded best to an intermediate range of sweep rates were classified as 'band pass' (B); neurons that did not respond preferentially to any rate were 'all pass' (C); and, neurons that preferred slow rates were 'slow pass' (D). In A-D, 'Number of spikes' is in response to 20 repetitions of each sweep. FM sweep rate selectivity was measured in response to upward and downward FM sweeps. There was no difference in the distribution of FM rate selectivity classes between KO and WT mice for either upward (E) or downward (F) sweeps.

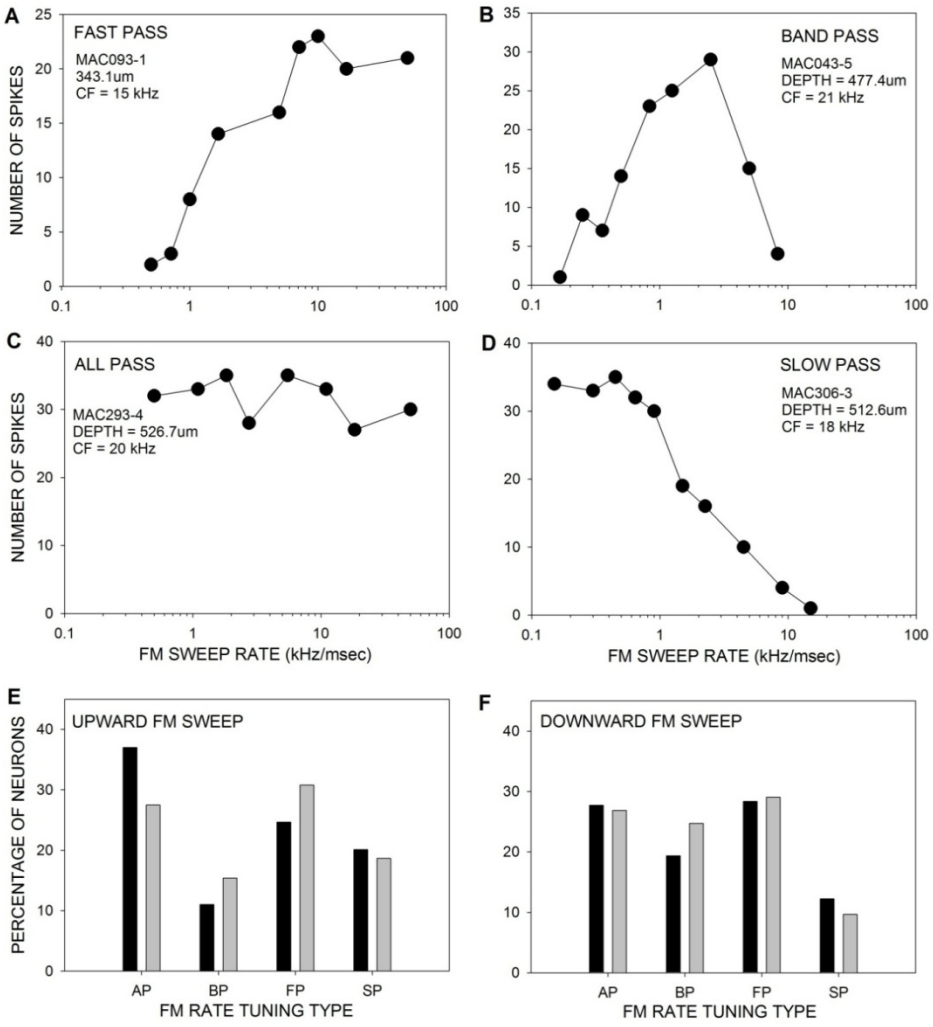
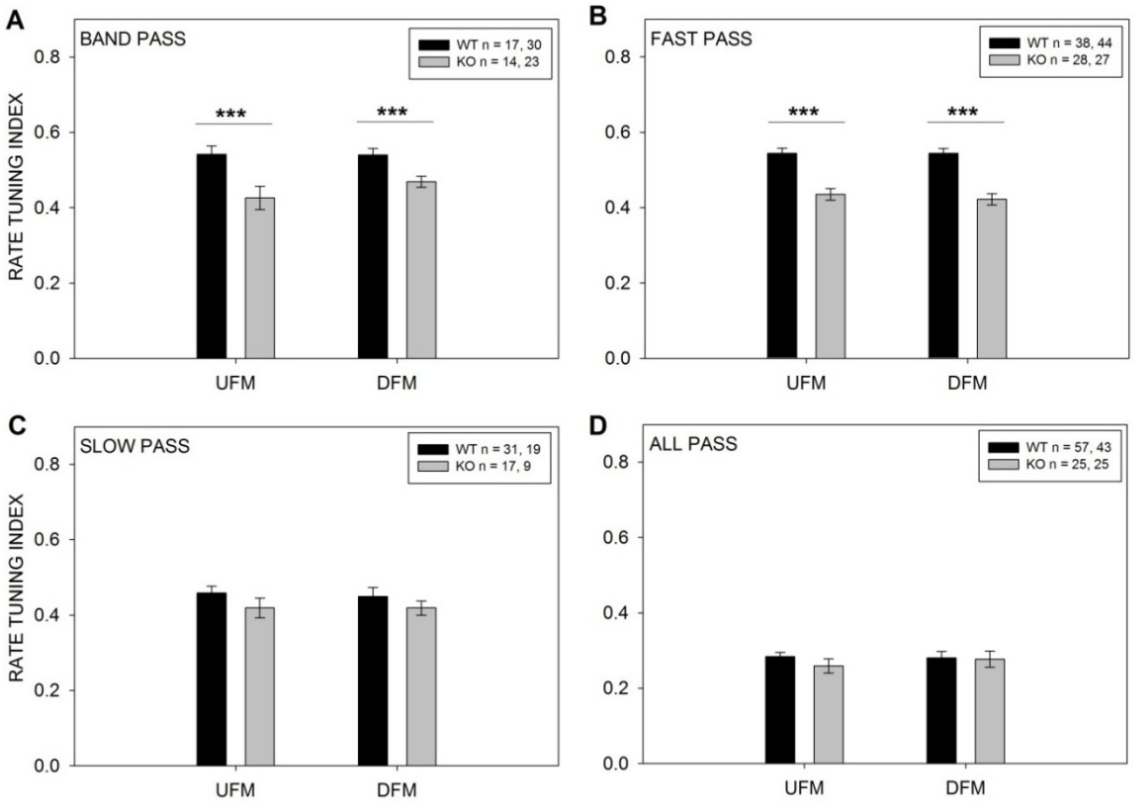


Figure 5: Fmr1 KO mouse band-pass and fast-pass neurons show reduced rate tuning index in response to FM sweeps.

Band pass (A) and fast pass (B) neurons from KO mice show decreased rate tuning in response to both upward and downward FM sweeps. Slow pass (C) and all pass (D) neurons however, did not show significant difference in rate tuning between groups.



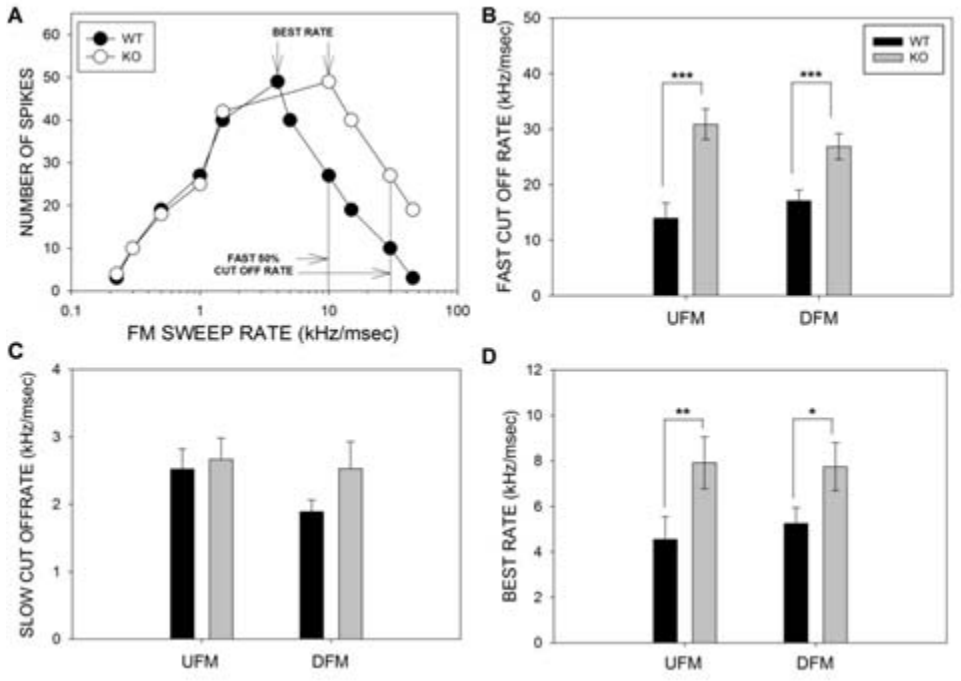
Cortical neurons in KO mice were, however, less selective for FM sweep rates compared to WT neurons. The rate tuning index (RTI) was used as a measure of FM rate selectivity (Godey et al., 2005; Trujillo et al., 2011). Both BP (one-way ANOVA $F(3,80) = 6.988$, $p < 0.001$) and FP (one-way ANOVA $F(3,133) = 21.584$, $p < 0.001$) neurons in KO mice demonstrated smaller mean RTI values in response to upward and downward FM sweeps than WT neurons (Figure 5A and 5B, respectively). No significant

differences were seen in the RTI values of SP (Figure 4C, one-way ANOVA $F(3,72) = 0.795$, $p = 0.501$) or AP neurons (Figure 5D, one-way ANOVA $F(3,138) = 0.416$, $p = 0.742$).

The reduced rate selectivity may arise due to the fact that neurons respond better to slower and/or faster sweeps in KO mice than WT mice. To distinguish between these possibilities, the 50% cut-off rate was measured (e.g., Figure 6A). This measure provides information on the fastest and the slowest sweep rates that produce more than 50% of maximum response. For fast-pass neurons only the 'slow' 50% cut-off rate is present. For band-pass neurons, both 'fast' and 'slow' 50% cut-off rates are present. The 'slow' 50% rate was combined for fast-pass and band-pass neurons in the following analysis. As in the example shown (Figure 6A), the population average 'fast' 50% cut-off rate in band-pass neurons was significantly higher in KO neurons than WT neurons (Figure 6B, one-way ANOVA $F(3,99) = 9.532$, $p < 0.001$). There was no difference in the 'slow' 50% cut-off rate in band-pass and fast-pass neurons (Figure 6C, one-way ANOVA $F(3,99) = 1.768$, $p = 0.158$). This asymmetric expansion of rate selectivity graphs towards the faster rates also resulted in band-pass neurons exhibiting faster best rates (Figure 6D, one-way ANOVA $F(3,99) = 2.974$, $p = 0.035$). The population level differences in 50% cut-offs and best rates were similar for upward and downward sweeps.

Figure 6: Band pass and fast pass neurons in Fmr1 KO mice show altered rate selectivity.

A. Example of a band pass neurons WT and KO neuron. The KO neuronal response has a higher best rate and 'fast' 50% cut off rate. The 'slow' 50% cut-off rate was not different. B. Band pass neurons from KO mice show a greater 'fast' 50% cut off rate than WT neurons (WT n = 31, KO n = 24). C. The 50% cut off to slow sweep rates in band pass neurons and 50% cut off rate of fast pass neurons were grouped. No significant difference was found between groups (WT n = 70, KO n = 53). D. The best rate of band pass neurons was significantly faster in KO neurons.



2.3.4 FM sweep direction selectivity

Direction selectivity index (DSI) was calculated at three different ranges of rates to compare direction selectivity between WT and KO mouse neurons. DSI values closer

to -1 indicate upward sweep selectivity and values closer to +1 indicate downward sweep selectivity. Up or down sweep direction selective and non-direction selective neurons were found in both WT and KO mice. Figure 7A is an example of a WT neuron that responds preferentially to upward FM sweeps, with figure 7B showing a WT neuron that responds similarly to upward and downward FM sweeps. Figure 7C shows a KO neuron that responds more strongly to downward FM sweeps, while figure 7D represents a KO neuron that is not selective for sweep direction. DSI was found for slow rates (0.1 – 1.0 kHz/msec, Figure 8A), medium rates (1.1 – 3.0 kHz/msec, Figure 8B), and fast rates (3.1 – 10.0 kHz/msec, Figure 8C). A one-way ANOVA revealed significant differences in distribution of DSI across different rates (one-way ANOVA, $p< 0.001$), but a pairwise comparison did not reveal any significant differences between the WT and KO groups within each sweep rate range (Figure 8D). A trend for more upward selectivity in WT compared to KO mice was seen, but this did not reach significance. Comparing DSI according to FM sweep rate selectivity type also did not reveal a difference in direction selectivity (data not shown). Linear regression analysis did not reveal a significant relationship between the CF and DSI value at any FM sweep rate range tested in either WT (slow $R^2 = 0.0143$, $p = 0.220$; medium $R^2 = 0.0063$, $p = 0.419$; fast $R^2 = 0.0049$, $p = 0.482$) or KO (slow $R^2 = 0.0182$, $p = 0.331$; medium $R^2 = 0.0075$, $p = 0.509$; fast $R^2 = 0.0022$, $p = 0.722$) mice.

2.3.5 Sideband inhibition is not significantly different in KO neurons

Both DSI and the 'slow' 50% cut-off rate are predicted by the spectrotemporal interactions between the excitatory and inhibitory components of a neuron's frequency receptive field (Razak and Fuzessery, 2006; Razak et al., 2008b; Razak and Fuzessery, 2009). Because neither DSI nor the slow 50% cut-off rate was altered in KO mice, we

Figure 7: Direction selective and non-direction selective neurons in control and Fmr1 KO mice.

DSI was calculated by finding the response magnitude at three different ranges of FM sweep rates (slow, medium, and fast) in response to both upward and downward FM sweeps. Both CT (A and B) and KO (C and D) mice displayed direction selective (A and C) and non-direction selective neurons (B and D).

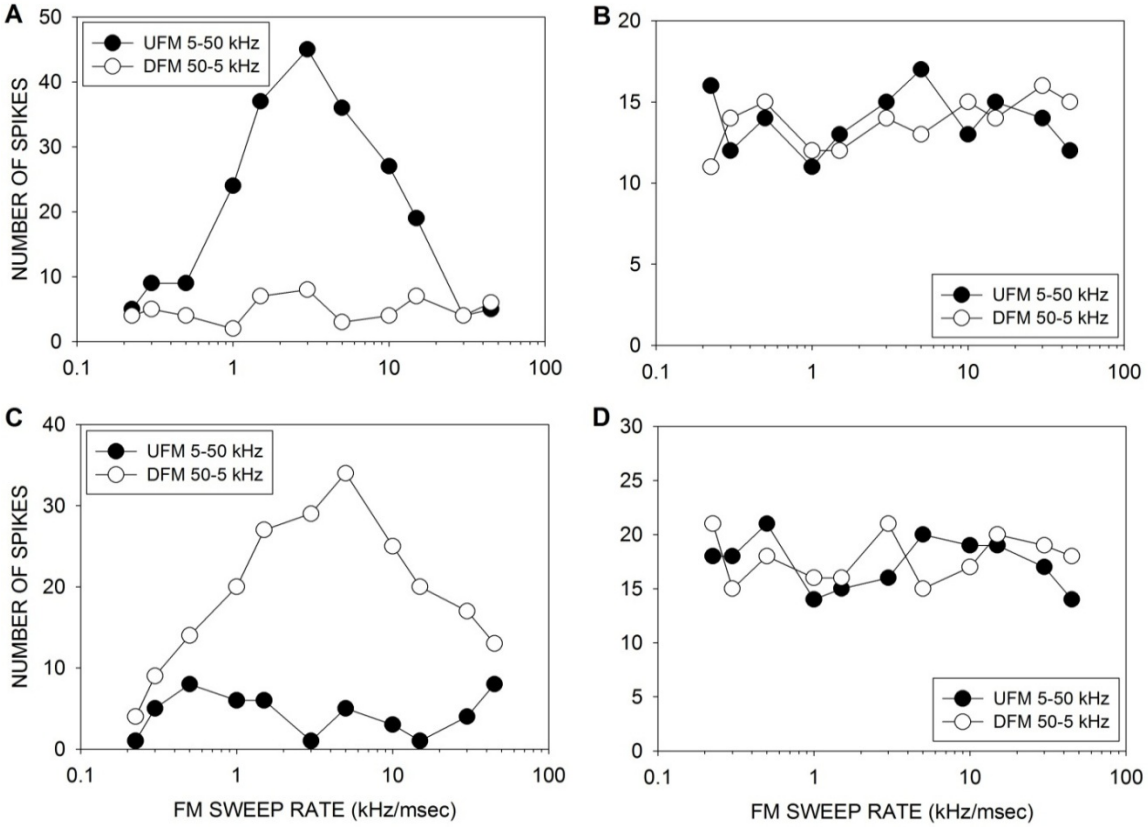
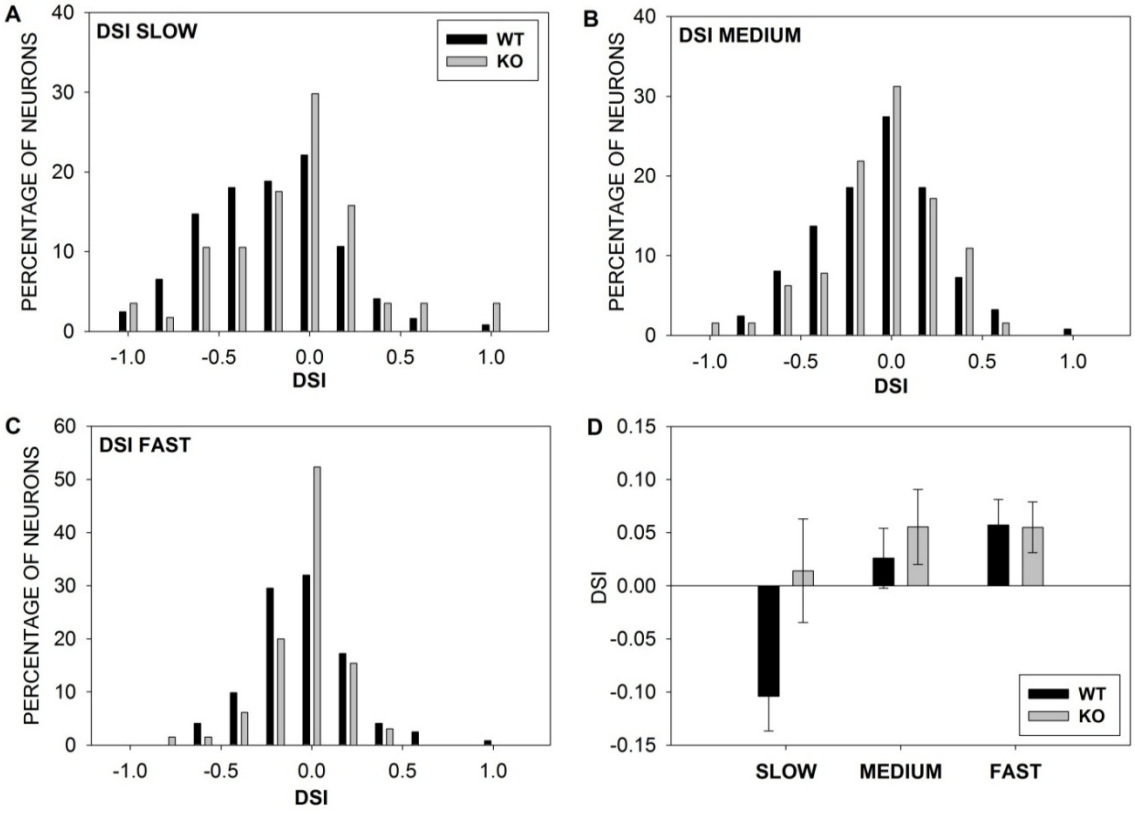


Figure 8: No differences in FM direction selectivity between Fmr1 KO and wild type mice.

Direction selectivity index (DSI) was used to assess direction selectivity in neuron of WT and KO mice. DSI was calculated at slow (A), medium (B), and fast (C) ranges of FM sweep rates. (D) DSI in WT and KO mouse neurons were not significantly different at any rate (WT, n = 131, KO, n = 68).



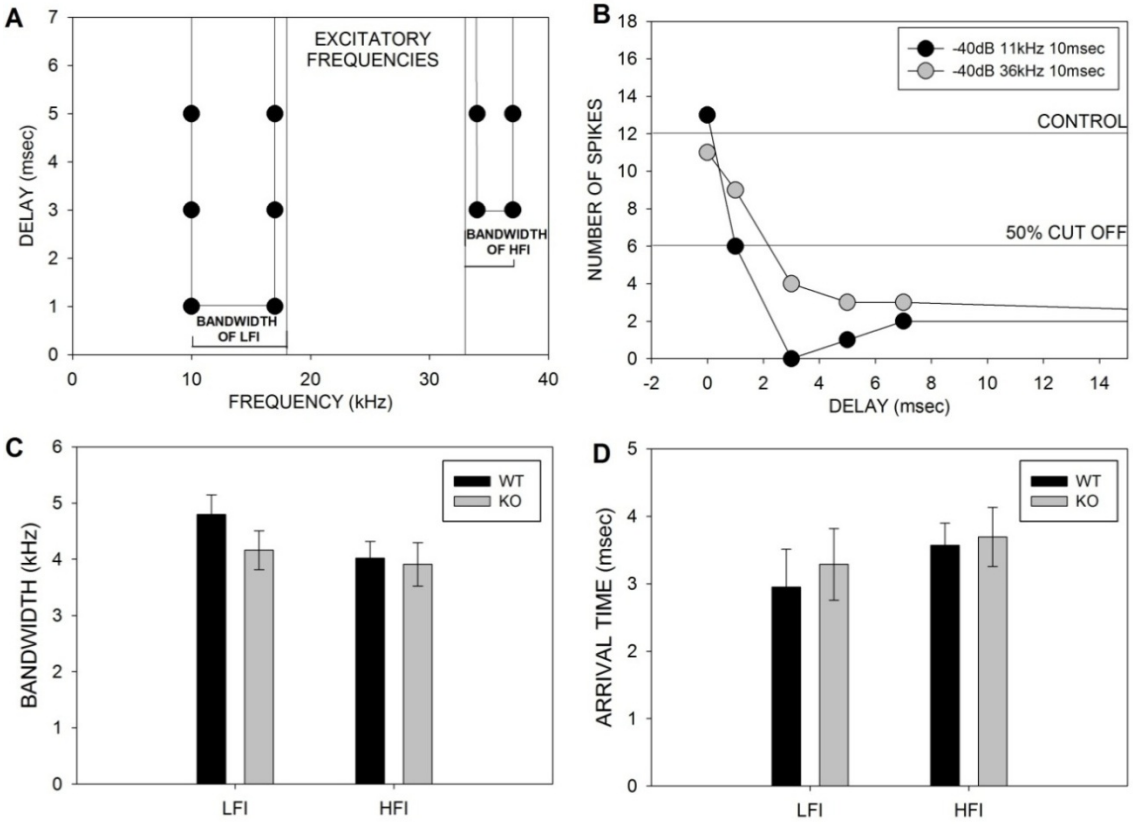
predicted sideband inhibition in both groups will be similar. Sideband inhibition was determined using the two-tone inhibition paradigm. Two tones, one at CF, and another whose frequency was varied, were presented with different delays between them. A tone frequency was noted as inhibitory if it reduced responses to the CF tone by at least 50%

of response to the CF tone alone. The delay-frequency combinations of tones that produced this 50% criterion inhibition were noted as inhibitory sidebands (e.g., Fig. 9A). The bandwidth of sideband is the range of inhibitory frequencies. Inhibitory frequencies lower than the excitatory frequencies were considered low-frequency inhibition (LFI). Frequencies above the excitatory frequencies were considered high-frequency inhibition (HFI).

Once bandwidth of inhibitory frequencies was established, arrival time of inhibition was found by playing a tone at the center of the inhibitory bandwidth against the CF (excitatory) control tone with different delays between the two (Fig. 9B). The delay at which response declined to 50% of control response was noted as the arrival time of inhibition. Arrival times were found for both LFI and HFI (Figure 9B). On average, there were no differences in the bandwidth (Figure 9C) and arrival time (Figure 9D) between KO and WT neurons as well as between low and high frequency inhibition within each group (bandwidth: two-way ANOVA $F(3,130) = 0.180$, $p = 0.910$; arrival time: two-way ANOVA $F(3,130) = 0.965$, $p = 0.412$).

Figure 9: Sideband inhibition is unaltered in Fmr1 KO mice.

(A) This neuron has both low and high frequency inhibitory sidebands on either side of excitatory frequencies. The low and high frequency inhibitory sidebands have bandwidths of 7 kHz and 3 kHz, respectively. (B) Two-tone inhibition revealed that low frequency inhibition has an arrival time of 0.98 msec, and high frequency inhibition an arrival time of 2.22 msec. The bandwidth (kHz) of the inhibitory sidebands and the arrival time (msec) of inhibition predict that the neuronal response will fall to 50% of maximum at 7.14 kHz/msec to upward FM sweeps and 1.35 kHz/msec to downward FM sweeps. No significant differences were found between WT and KO mouse neurons in the bandwidth of inhibitory sidebands (C), or the arrival time of inhibition (D). WT: Low frequency inhibition (LFI) n = 64, High frequency inhibition (HFI) = 93; KO LFI n = 48, HFI n = 59).



2.4 Discussion

The goal of this study was to compare cortical responses to tones and FM sweeps between *Fmr1* KO mice and WT controls. Compared to neurons from WT mice, neurons in KO mice showed broader frequency tuning, larger response magnitude and more variability of first spike latency when tested with tones. There was no difference in minimum thresholds and in the first spike latency between the groups. In response to FM sweeps, KO neurons were less selective for sweep rates but did not show differences in

direction selectivity. The reduction in rate selectivity was due to enhanced responses to fast sweep rates in KO mice. There was no difference in the slow 50% cut-off rate, a measure of neural selectivity to slower sweeps. There was also no difference in the spectral and temporal properties of sideband inhibition. Sideband inhibition shapes direction selectivity and the slow 50% cut-off rate and direction selectivity in bats and rodents (Zhang et al., 2003; Razak and Fuzessery, 2006; Trujillo et al., *in review*). The lack of difference in sideband inhibition is therefore consistent with lack of difference in slow 50% cut-off rate and direction selectivity. Taken together, the data suggest changes in cortical processing of both spectral and spectrotemporal properties in the *Fmr1* KO mice. It must be noted that these data do not indicate that the auditory cortex is the origin of observed deficits as abnormal cortical responses can be inherited from sub-cortical sites. As with other phenotypes seen in the *Fmr1* KO mice (reviewed in Bernardet and Crusio, 2006), the data do not point to gross pathology of the auditory system.

2.4.1 Frequency tuning and response to pure tones

Frequency tuning is shaped by overlapping inhibitory and excitatory inputs (Wehr and Zador, 2003; Wu et al., 2008; Tan and Wehr, 2009). Iontophoresis of GABA receptor antagonists on neurons cause expansion of tuning curves in many cases indicating contribution of inhibitory input in shaping frequency tuning (Muller and Scheich, 1988; Fuzessery and Hall, 1996; Wang et al., 2002; Sutter and Loftus, 2003; Kaur et al., 2004; Wu et al., 2008). As excitatory tuning broadens in the KO mice without a change in the bandwidth of sideband inhibition, we hypothesize that neurons in

the auditory cortex of KO mice receive an expanded range of both inhibitory and excitatory inputs. This will cause expanded frequency tuning without a change in the bandwidth of inhibitory sidebands.

Neurons in the KO mice also responded more than WT neurons to CF tone stimulation at similar sound levels. Over-active neurons may be associated with auditory behavior changes seen in *Fmr1* KO mice. *Fmr1* KO mice are susceptible to audiogenic seizures and show aberrant prepulse inhibition and auditory startle responses (Chen and Toth, 2001; Nielsen et al., 2002). When FMRP is reintroduced to *Fmr1* KO mice, the number of audiogenic seizures is reduced (Musumeci et al., 2007). Whether cortical responses to sounds are also reduced under such conditions will be important to test in the future. The larger response magnitude, particularly during the latter portion of the response window, may arise due to increased intrinsic excitability and/or reduced synaptic inhibition in response to a CF tone (de Vrij et al., 2008; Errijgers et al., 2008; Levenga et al., 2011; Olmos-Serrano et al., 2011; Thomas et al., 2012). Gibson et al. (2008) found the principle cells of the *Fmr1* KO somatosensory cortex were intrinsically more excitable then cells found in control mice. Gross et al., (2011) suggested that increased excitability may be related to impaired regulation of potassium ion channels in the KO mice. In *Fmr1* KO mice, excitatory neurons were stimulated by lower intensity stimulus, produced more spikes upon stimulation, and produced more frequent and prolonged UP states (Gibson et al., 2008). Of particular note, loss of *Fmr1* alone, rather than impaired inhibition, was shown to produce prolonged UP states (Hays et al., 2011).

Wehr and Zador (2001) showed that the onset response to a tone is an excitatory post-synaptic potential followed by a delayed inhibitory post-synaptic potential. The function of the latter was to reduce responses following the initial burst. Increased response magnitude may therefore result from a reduction in this CF-generated inhibition. Deficits are present in cortical $GABA_A$ receptor structure and function in *Fmr1* KO mice. Altered $GABA_A$ receptor subunit expression and unusual inhibitory interneuron activity has been found in *Fmr1* KO mice. Specifically, the α_1, β_2, and δ subunits of $GABA_A$ receptors are underrepresented, implying that GABA receptor function may be impaired in the cortex of *Fmr1* KO mice (D'Hulst et al., 2006; D'Hulst et al., 2009). The β subunit is required for the assembly of functional $GABA_A$ receptors and is associated with a decrease in the number of GABA receptors on pyramidal cell in cortex of *Fmr1* KO mice.

Functionally abnormal inhibition has also been found in *Fmr1* KO mice. Whole cell recordings in response to stimulation of thalamocortical axons projecting to the somatosentory cortex of *Fmr1* KO mice show decreased excitatory drive to fast spiking inhibitory interneurons, resulting in decreased inhibitory output (Gibson et al., 2008). Impaired inhibitory interneuron network function was also found in *Fmr1* KO mice (Paluszkiewicz et al., 2011). Whole cell recordings performed on pyramidal cells in the somatosensory cortex of *Fmr1* KO mice showed decreased activation of somatostatin expressing low-threshold-spiking interneurons upon mGluR1/5 stimulation, which resulted in less synchronized synaptic inhibition, and less coordinated pyramidal cell spiking (Paluszkiewicz et al., 2011). Such changes in the auditory cortex may explain the

observed variability in first-spike latency in the KO mice. It is also conceivable that excessive dendritic spine formation, a consistent phenotype in *Fmr1* KO mice and in FXS patients, could produce altered cortical circuitry in *Fmr1* KO mice (Comery et al., 1997; Irwin et al., 2002; Galvez et al., 2003; McKinney et al., 2005; Till et al., 2012).

2.4.2 FM sweep rate and direction selectivity

Using FM sweeps as stimuli, we did not find any difference in the distribution of FM rate tuning type, but we did find altered rate tuning in *Fmr1* KO mice. Band pass and fast pass neurons in *Fmr1* KO mice were less sharply rate tuned, and were tuned to faster rates than wild type mouse neurons. All pass and slow pass neurons were unaffected in *Fmr1* KO mice. In BP and FP neurons, the shift to faster best rates and fast 50% cutoff rates may be attributed to broader frequency tuning. In a study of the AI response properties of squirrel monkeys, a significant correlation was found between the bandwidth of frequency tuning and best sweep rate. Neurons that were more broadly tuned tended to respond maximally to faster FM sweep rates (Godey et al., 2005). Though no correlation between tuning bandwidth and rate tuning index (RTI) was found in the squirrel monkey (Godey et al., 2005), research done in the auditory cortex of chinchillas showed a negative correlation between frequency tuning and RTI (Brown and Harrison, 2009) implying that neurons with narrower frequency tuning are likely to be more selective for sweep rates than more broadly tuned neurons (Brown and Harrison, 2009). Consistent with these findings, *Fmr1* KO mouse neurons demonstrate broader frequency tuning, faster best FM sweep rates, and reduced rate tuning overall.

There was no difference in FM sweep direction selectivity between the two groups. A comparison of Figures 8A, B and C shows that more direction selective responses (DSI > 0.3 or DSI < -0.3) are present when tested with slow sweep rates. The histogram compresses towards DSI = 0 at faster sweep rates. There is a tendency for selectivity to upward sweeps at the slow rates. However, both these trends were seen in KO and WT mice. The lack of difference in DSI is consistent with the lack of difference in the properties of sideband inhibition. Direction selectivity is associated with asymmetries in sideband inhibition (Suga, 1965, Zhang et al., 2003, Razak and Fuzessery, 2006). In the mouse cortex, both arrival time and bandwidth of low and high frequency inhibition were similar (symmetrical sidebands). This was true in both KO and WT mice. Unlike the rat A1 (Zhang et al., 2003), we did not find a relationship between the CF and DSI at any sweep rate. This may be at least in part due to the restricted range of CFs that was sampled in both groups.

2.4.3 Methodological considerations

The combination of urethane and xylazine used to anesthetize mice will alter auditory responses. Urethane acts by enhancing $GABA_A$, glycine, and acetylcholine receptor function and reducing NMDA and AMPA receptor function (Hara and Harris, 2002). Xylazine works by stimulating α_2-adrenergic receptors and inhibiting noradrendergic transmission (Hsu, 1981). Urethane and xylazine may reduce neuronal response magnitude, but as the anesthetic regimen was applied to both wild type and *Fmr1* KO mice, the differences in selectivity observed here are unlikely to be an artifact

of anesthesia. A second caveat to consider while interpreting data is the fact that littermate controls were not used in this study. Differences in maternal pup care may result from *Fmr1* KO mice being raised by *Fmr1* KO mothers and wild type mice being raised by wild type mothers. Though research is limited, differences in maternal care of pups have not been reported in *Fmr1* KO mice to our knowledge. We did not observe differences in body weight between the groups. The KO and WT pups were raised in the same vivarium room (and shelves) and were therefore in similar auditory environments until the day of electrophysiology.

2.4.4 Conclusions

Impaired spectral resolution, greater response magnitude, decreased temporal fidelity of responses and altered FM sweep rate selectivity may underlie auditory processing deficits seen in FXS patients. Individuals with FXS show excessive ERP responses on oddball tasks using pure tones as stimuli (Rojas et al., 2001; Castren et al., 2003; Van der Molen et al., 2012b, a). Notably, FXS patients consistently show enhancement of components that are associated with early processing of auditory stimuli (Rojas et al., 2001; Castren et al., 2003; Van der Molen et al., 2012b, a). Subsequent aberrations in auditory processing may stem from improper sound-memory trace formation, and may underlie some aspects of FXS-related language abnormalities (Cone-Wesson and Wunderlich, 2003; Naatanen et al., 2007; Naatanen et al., 2011). Presently, only tasks with pure tones as stimulus are used in assessments of auditory processing in individuals with FXS. On the other side of the stimulus spectrum, speech and language

tasks are used as outcome measures for clinical trials of potential therapeutics. Our data suggest that a battery of auditory tests that encompass a broad range of spectral, temporal and spectrotemporal complexities may provide a potentially rich source of relevant biomarkers in FXS. Our data also show that the *Fmr1* KO mouse is a useful model to study auditory processing-based biomarkers relevant to FXS. Future studies will investigate the development of auditory processing in the KO mice, the role of experience-dependent plasticity and the effects of potential therapeutics on auditory responses.

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Chapter 3: Minocycline treatment reverses ultrasonic vocalization production deficit in a mouse model of Fragile X Syndrome

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 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.

3.1 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, macroorchidism, abnormal dendritic spine formation, and visuomotor impairments (Hagerman et al., 2009; McNaughton et al., 2008; Rudelli et al., 1985). Additionally, 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; Roberts et al., 2002).

The *Fmr1* knock-out (*Fmr1* KO) mouse displays a wide range of symptoms typical of FXS, making it a useful model system (Bernardet and Crusio, 2006; 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/sec) 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 parents of the patients.

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 *Fmr1* KO mouse social vocalizations may serve as a useful biomarker in FXS to study potential therapies for communication problems in FXS.

3.2 Experimental Procedure

3.2.1 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 hour light/dark cycle. All studies were performed in accordance with the National Institutes of Health and with Institutional Animal Care and Use Committee guidelines.

3.2.2 Minocycline Administration

Minocycline (30mg/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 treatment prior to mating.

3.2.3 Recording mouse vocalizations

USV produced during mating behavior were recorded using a full spectrum Petterssen D1000x bat detector (250 kHz sampling rate) maintained 5cm 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 x 21.6 x 28.8 cm enclosure. Recording continued until mating occurred or until 20 minutes 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 was obtained by mating 17 pairs of WT mice and 14 pairs of KO mice. All mice used were virgins between 2 and 3 months old. 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 0.04mg/mL estradiol benzoate solution 36 to 48 hours prior to mating and 0.018 mL of 0.60mg/mL progesterone solution 4 hours 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.

3.2.4 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 msec 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 msec). Vocalizations in which less than 3 msec 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 background noise and used to determine the number of calls per second.

3.2.5 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 msec 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 figure 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 minutes of each mating trial. A two-way ANOVA for treatment group x time 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.

3.2.6 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. Mouse mating behavior has been well characterized (Bialy et al., 2000; McGill 1962; Nyby, 1983; White et al., 1998) allowing

us to isolate and identify specific mating behaviors. JWatcher software was used to mark to time points at which each behavior occurred and the amount of time spent performing each behavior. Two raters scored 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 twenty minutes 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 (Vale et al., 1971, 1972). Ano-genital sniffing was identified as the male mouse sniffing the ano-genital region of the female (Cassaing and Isaac, 2007). 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 (McGill, 1962). 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 $p = 0.158$).

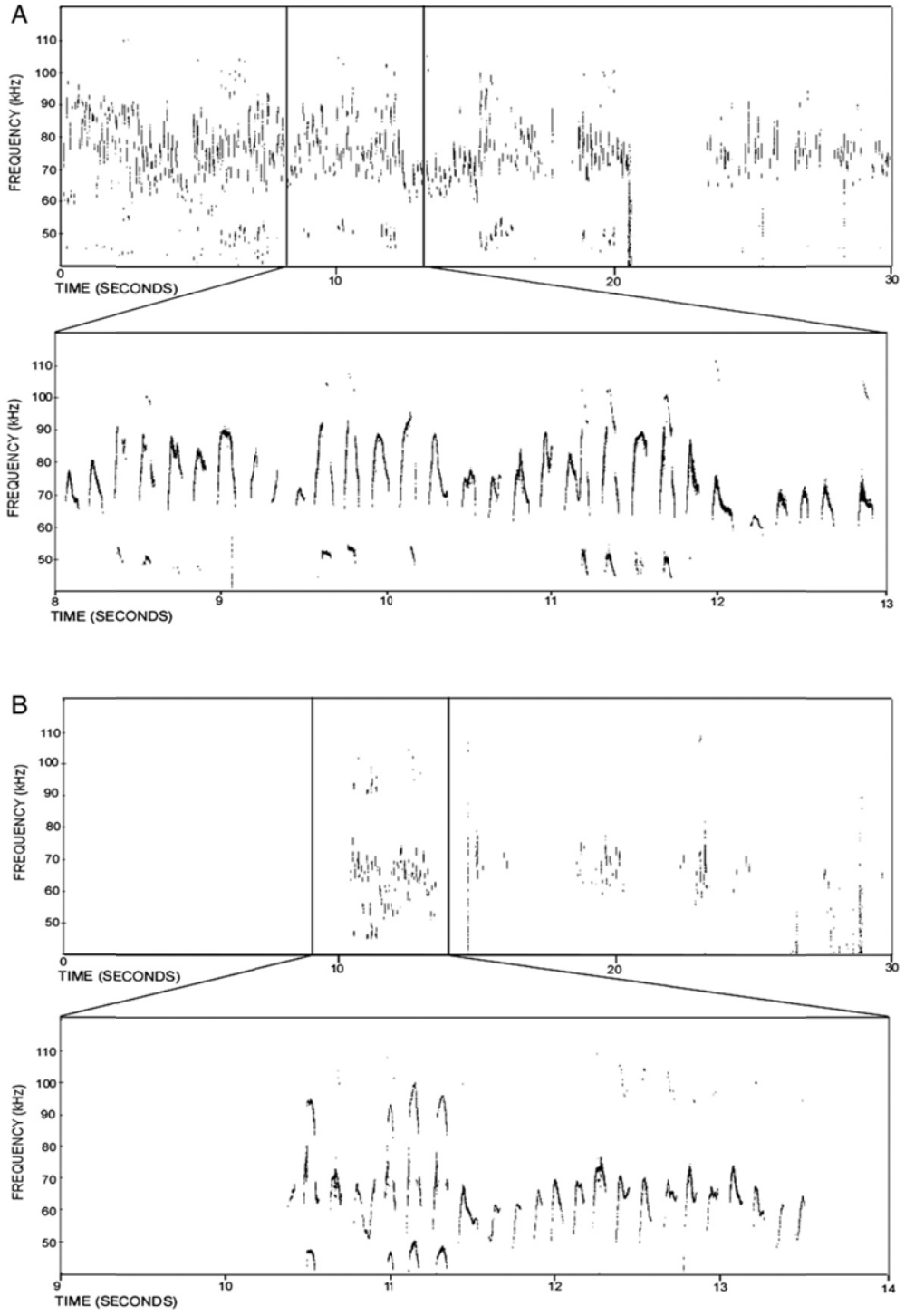
3.3 Results

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.

3.3.1 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 Figure 1). We compared USVs production across WT, KO, MTWT, and MTKO groups during the first ten minutes 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 (Figure 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

Figure 1: Example ultrasonic vocalizations recorded from WT (A) and Fmr1 KO mouse (B) during mating.



significant difference was observed between WT, MTWT, and MTKO groups. The calling rate collapsed across the first 10 minutes of a mating session shows the difference between the KO and the other three groups (Figure 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. While it has been established that USV production is required to attract female mice (Pomerantz et al., 1983), the importance of USV production rate in successful mouse mating has not yet been described.

3.3.2 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 (Figure 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 $F(3,36) = 3.207$, $p = 0.361$), average frequency (one-way ANOVA $F(3,36) = 2.549$, $p = 0.070$), or dynamic range (one-way ANOVA $F(3,36) = 0.278$, $p = 0.841$). These findings

Figure 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.

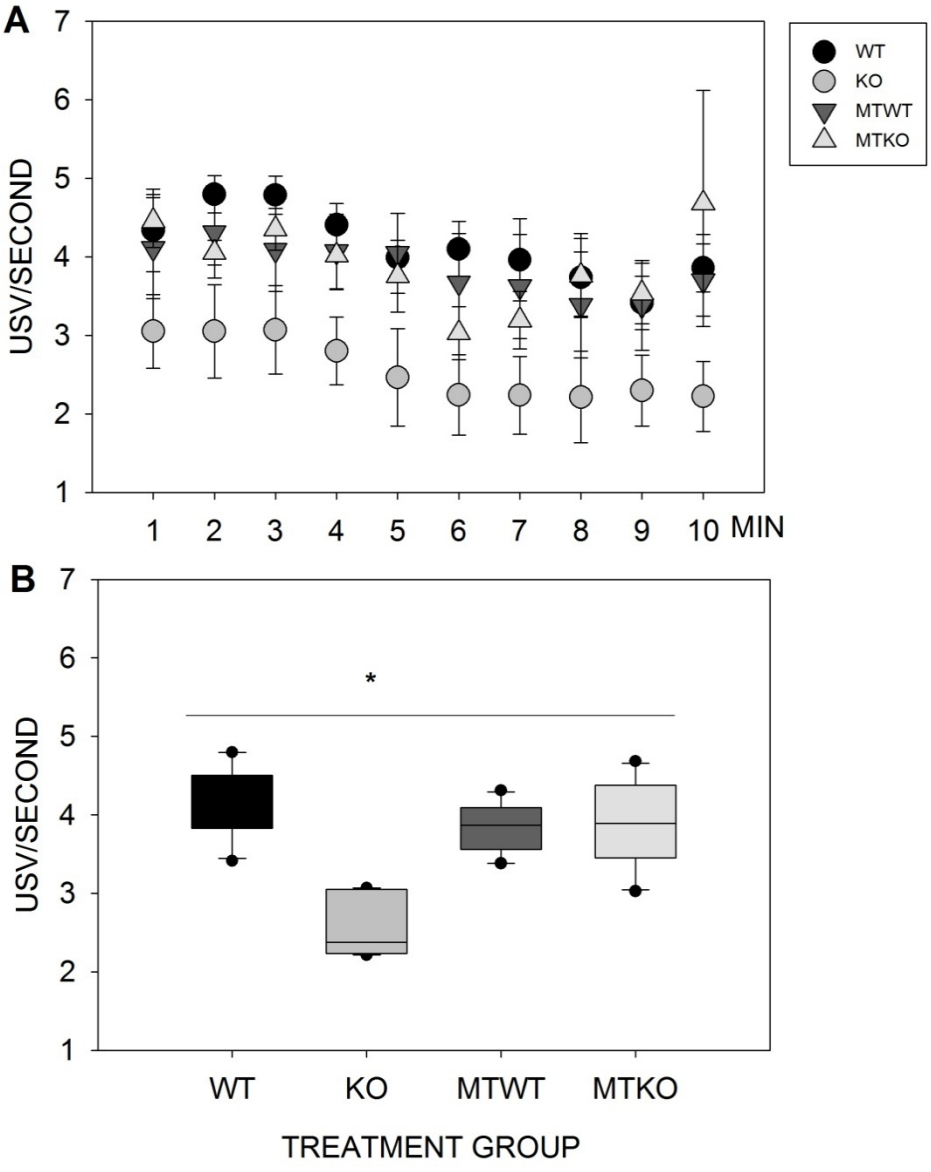
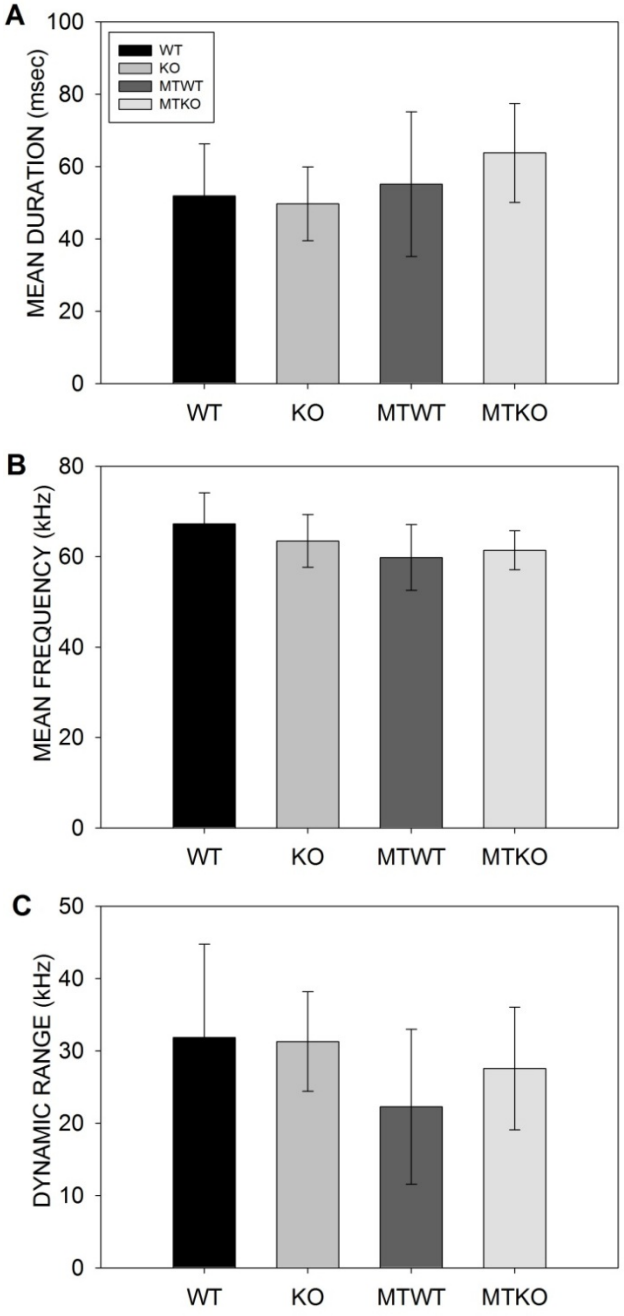


Figure 3: Acoustic properties of USVs are not different across groups.

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.



demonstrate that the main difference in control and *Fmr1* KO mouse calling lies in vocalization production rate rather than the acoustic properties of calls.

3.3.3 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 (Figure 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.

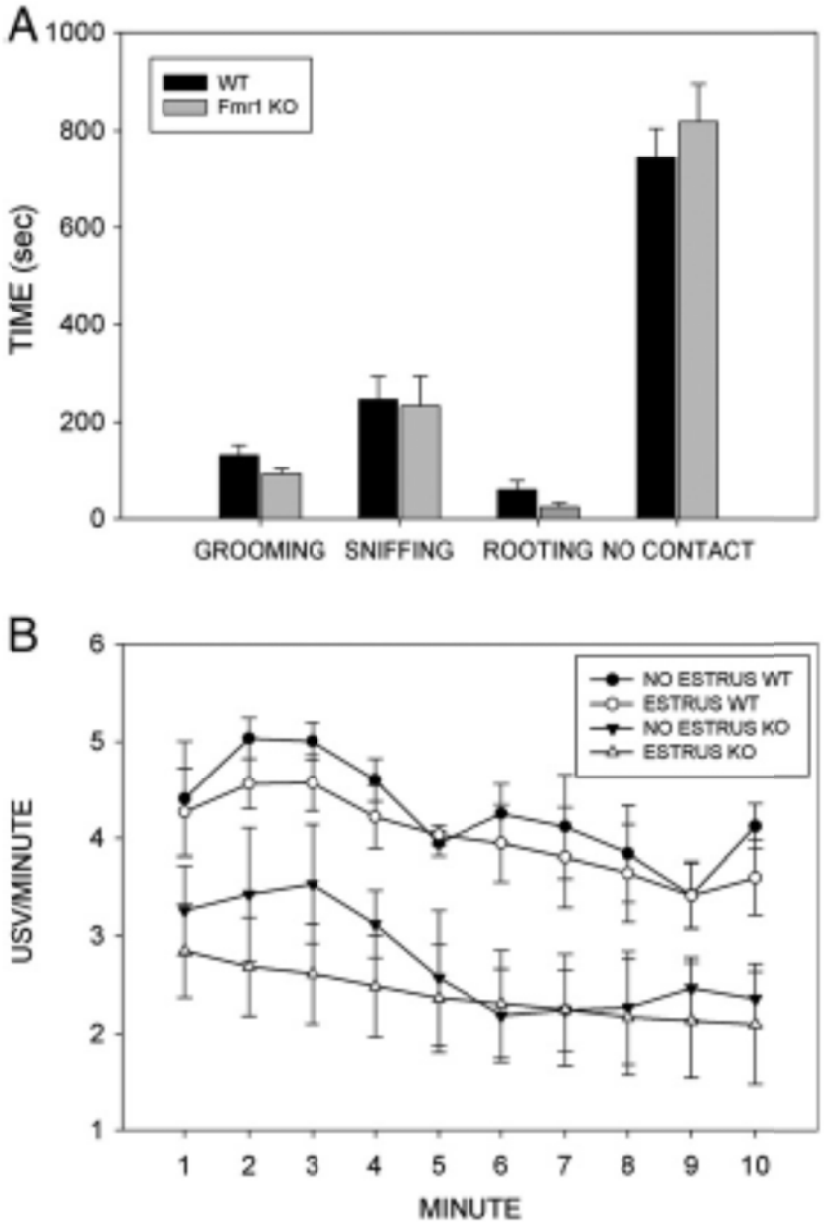
3.3.4 Estrus state of females

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 (Figure 4B). There was no significant differences in the number of USVs produced per second between the estrus induced 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.

Figure 4: Mating behavior was unaltered in Fmr1 KO mice and did not vary with reproductive status.

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.



3.4 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 (Figure 2B) indicting 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 possible biomarker relevant to communication disorders associated with FXS or other autistic spectrum disorders (ASDs). 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 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 indicting 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). In addition, treated KO mice demonstrated a reduction in anxious behavior as assessed through elevated plus maze performance (Bilousova et al., 2009). 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 are 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 (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). We previously demonstrated that MMPs can influence dendritic spine development and hence synaptic stability (Bilousova et al., 2006). Our previous studies also indicate that MMP-9 activity is up-regulated in the hippocampus of *Fmr1* KO mice and may be partially responsible for abnormal dendritic spine development, whereas minocycline treatment reduces MMP-9 levels and activity. MMPs play an important role in both normal development of dendritic spines in the brain and their ability to remodel (Ethell and Ethell, 2007). 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 et al., 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 et al., 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., 2007). 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., 2007). Therefore, social vocalizations may serve as a useful biomarker to study potential therapies for these communication disorders as well. In this study, minocycline treatment began at birth and continued to P28. Although mating sessions occurred after minocycline treatment had ceased (ages 2-3 month), the calling rate showed improvement. While some studies use mouse pups to test mouse communication, we used the mouse mating paradigm for two

reasons. First, there is some evidence suggesting that rodent pup vocalizations are the result of a physiological response to cold temperatures (Blumberg and Stolba, 1996; Blumberg and Alberts, 1990), and therefore not a meaningful attempt at communication. Second, communication is an inherently social behavior. It is well established that *Fmr1* KO display a host social abnormalities (Hagerman et al., 2009; McNaughton et al., 2008) so eliciting USVs with male-female pairs enabled us to determine if communication deficiencies were the result of aberrant social interactions or could be attributed to other factors. An understanding of how effective minocycline treatment is and how long the benefits will last when treatment is begun at a later age are currently unclear. Future studies will address the optimal time course (age and length) of minocycline treatment to improve calling rate in the KO mice. Studies will be especially concerned with the efficacy of minocycline treatment on adult animals as opposed to pups. These studies will be informative for clinical studies since most candidates for minocycline treatment are adolescents and adults.

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Conclusion

Fmr1 KO mice demonstrate reduced ultrasonic vocalization (USV) production rate and alterations in auditory processing making them a useful model for the communication and sensory processing abnormalities common to FXS. Decreased USV production is found in *Fmr1* KO mice despite unaltered male-to-female mouse social behavior, suggesting that decreased USV production is not a result of unusual social interactions. Reduced USV production then, is a useful biomarker for the communication deficits associated with FXS. Accordingly, USV production in *Fmr1* KO mice may serve as a model for the communication disorders seen in FXS, and may acts as an outcome measure for possible FXS therapies.

Additionally, electrophysiological techniques reveal that *Fmr1* KO mice show broader frequency tuning and enhanced neuronal response magnitude when presented with single frequency tone stimulus. Single frequency tones are often used as stimulus in oddball paradigms to probe auditory processing in FXS patients using event-related potential (ERP) electrophysiology. Typically, oddball tests generate enhancement of and delays in ERP components associated with early cortical processing of auditory stimuli. As such, the loss of spectral resolution and increase in response magnitude seen in *Fmr1* KO mice may reflect the altered latencies and response magnitude of auditory components typical of FXS. Interestingly, the degree to which neuronal response is attenuated by changes in the frequency modulated (FM) sweep rate is smaller in *Fmr1* KO mice. This suggests that impaired FM sweep processing may be a symptom of FXS,

and that FM sweep stimulus may serve as a metric for assessing auditory processing in individuals with FXS.