REVIEWS

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Mechanisms underlying auditory processing deficits in Fragile X syndrome

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Abstract

Autism spectrum disorders (ASD) are strongly associated with auditory hypersensitivity or hyperacusis (difficulty tolerating sounds). Fragile X syndrome (FXS), the most common monogenetic cause of ASD, has emerged as a powerful gateway for exploring underlying mechanisms of hyperacusis and auditory dysfunction in ASD. This review discusses examples of disruption of the auditory pathways in FXS at molecular, synaptic, and circuit levels in animal models as well as in FXS individuals. These examples highlight the involvement of multiple mechanisms, from aberrant synaptic development and ion channel deregulation of auditory brainstem circuits, to impaired neuronal plasticity and network hyperexcitability in the auditory cortex. Though a relatively new area of research, recent discoveries have increased interest in auditory dysfunction and mechanisms underlying hyperacusis in this disorder. This rapidly growing body of data has yielded novel research directions addressing critical questions regarding the timing and possible outcomes of human therapies for auditory dysfunction in ASD.

KEYWORDS

auditory system, autism spectrum disorders, circuit development, Fragile X syndrome, hyperacusis, synaptic transmission

1 | INTRODUCTION

Communication disorders and sensory hypersensitivity are prominent features of autism spectrum disorder (ASD). ASD is frequently diagnosed in individuals with Fragile X syndrome (FXS), a leading cause of intellectual disability. FXS results from mutations in the *fragile X mental retardation 1 (FMR1)* gene, which is the most prevalent monogenic cause of ASD.[1] This gene encodes fragile X mental retardation protein (FMRP), an RNA binding protein that regulates expression of a select set of genes. The focus of this review is to understand the effects of *Fmr1* deletion in the auditory pathway, because these effects lie at the heart of the auditory hypersensitivity seen in FXS and likely contribute significantly to communication and other neurological deficits.

A large portion of the recent work discussed here has taken advantage of rodent models of FXS (*Fmr1* knockout mice and rats) and investigated structural, functional, and behavioral phenotypes at multiple stages of central auditory processing. Other studies have examined neuroanatomical and physiological abnormalities of human subjects with FXS. Additionally, the avian auditory brainstem has been used to investigate the FMRP regulation of auditory circuits that encode low frequency sounds, which are essential for acoustic communication in humans.

The significance of studying auditory processing in FXS is twofold. On the one hand, FMRP has the potential to influence a multitude of cellular processes at multiple stages of development. The diversity of FMRP function is of special significance in the auditory system, which carries out unique functions that require exceptionally fast and precise synapses. These requirements are fulfilled with specializations in axon terminations, dendritic branching, and synaptic and intrinsic properties of auditory neurons. Investigation of the roles for FMRP in each of these specializations will advance our understanding of auditory system development and function. Indeed, FMRP is found to be expressed throughout the auditory system and an array of distinct phenotypes has emerged in auditory circuits following FMRP deficiency that may account for auditory hypersensitivity and abnormal processing. At the same time, these findings highlight the auditory system as a powerful model system to study the FMRP function and FXS neuropathology. The

wealth of knowledge on connections and response properties in the healthy brain allows alterations to be relatively easy to characterize, thereby allowing for an in-depth analysis of the underlying etiologies that lead to problems with sensory processing.[2] Finally, the ability to stimulate neural responses in the auditory system on demand and the fact that many of these responses are independent of cognitive abilities or active participation opens the possibility to use these responses as biomarkers to characterize the effects of potential therapeutic approaches.

2 | FXS AND BIOLOGICAL ROLES OF FMRP

Fragile X syndrome is characterized by many similar symptoms to autism, namely, hyperactivity, anxiety, seizures, learning difficulties, and poor language development (reviewed in[3,4]). Most cases of FXS are caused by a trinucleotide repeat disorder, in which an increase in the numbers of a CGG motif in the 5′ UTR of the *Fmr1* gene (>200 repeats) leads to transcriptional silencing and the consequent loss of its product, FMRP. As a monogenetic disorder, FXS has come to be at the frontier of developing treatments for neurodevelopmental disorders. Loss of FMRP underlies the etiology of FXS, and most drug development has centered around rescuing specific biological effects caused by loss of FMRP. Evidence supporting the importance of FMRP in FXS includes (i) FMRP protein level is associated in a dose-dependent manner with brain anatomy, sensory function, autistic behaviors, and cognitive function of individuals with FXS,[5-8] (ii) genetically reintroducing FMRP rescues cellular and behavioral deficits in *Fmr1* knockout mice,[9] and (iii) point mutations in the *Fmr1* coding sequence lead to FXS symptoms similar to those of CGG expansion.[10,11] Thus, examining FMRP knockouts presents a suitable avenue for exploring FXS etiology.

FMRP regulates many aspects of RNA biology from RNA transport, stability, to mRNA translation.[12,13] The best-described biological role of FMRP in neurons is to bind a subset of mRNAs and to suppress their translation.[14,15] Appropriate stimulation of neurons can reverse this suppression, most likely by altering the phosphorylation state of FMRP, leading to increased synthesis of the protein products

of these mRNAs. There are on the order of ~1000 mRNAs that bind FMRP and these are termed targets of FMRP.[16,17] Thus, one central phenotype of *Fmr1* knockout animals is an increase in levels of some of these proteins, together with a lack of activity-dependent translation. This role for FMRP is commonly termed its canonical role. However, only a limited number of FMRP targets have been validated so far in specific circuits and FMRP binds to different sets of RNA targets in an age-dependent and cell type or brain region specific manner. As evidence, strikingly distinct sets of proteins exhibit altered expression in *Fmr1* knockout mice between the hippocampus and neocortex of adult brains,[18] and between the developing and adult neocortex.[19] In addition to RNA binding, FMRP directly binds proteins that control other aspects of neuronal activity. These include proteins required for RNA editing, RNA splicing, cytoskeletal rearrangement, regulation of protein kinases, as well as direct binding to ion channels that determine neuronal excitability.[20] In some cases, it is possible to link auditory phenotypes in *Fmr1* knockout animals to changes in mRNA targets or to other biological actions of FMRP. Indeed, key findings in FXS animal models include disturbances in circuit development, imbalanced inhibitory, and excitatory neuronal circuits, and altered synaptic plasticity.[21-23]

3 | FMRP REGULATES THE DEVELOPMENT OF SYNAPTIC TRANSMISSION IN THE AUDITORY BRAINSTEM

The auditory brainstem is where fundamental steps of auditory processing take place, including binaural computation for sound localization. Sounds arriving at our two ears from various locations along the azimuth create discrete interaural time and level differences (ITDs and ILDs).[24] These two binaural cues are first computed in the brainstem. In mammals, the major auditory nuclei involved in this process include the ventral cochlear nucleus (VCN), the lateral, ventral, and medial nucleus of the trapezoid body (LNTB, VNTB, and MNTB), and the medial and lateral superior olive (MSO and LSO; Figure 1). In reptiles and birds, nucleus magnocellularis (NM), and nucleus laminaris (NL) are structurally and functionally similar to the mammalian VCN and MSO, respectively.

Traditionally, the brainstem was thought to express low levels of FMRP[25,26] and received little attention in the field of FMRP research. Recent cellular analyses, however, reported strong expression of FMRP in auditory brainstem cell groups across a number of vertebrate species.[27] Quantitative analyses across the entire adult (5-6 weeks) mouse brain further confirmed that auditory neurons in the brainstem and thalamus have levels of FMRP immunoreactivity comparable to those in cortical neurons.[28] It is worth noting that FMRP is also expressed in spiral ganglion neurons, which convey acoustic signals from the inner ear to the brainstem, in both birds and mammals. In contrast, many cell groups in the midbrain and hypothalamus exhibit low FMRP levels.[28]

In this section, we first describe the expression and function of FMRP in the ITD circuit (3.1) and then discuss how *Fmr1* deletion affects the cell groups involved in ILD computation (3.2). Finally, we summarize effects of FMRP loss on auditory brainstem responses (ABRs), a noninvasive physiological output of the auditory brainstem (3.3).
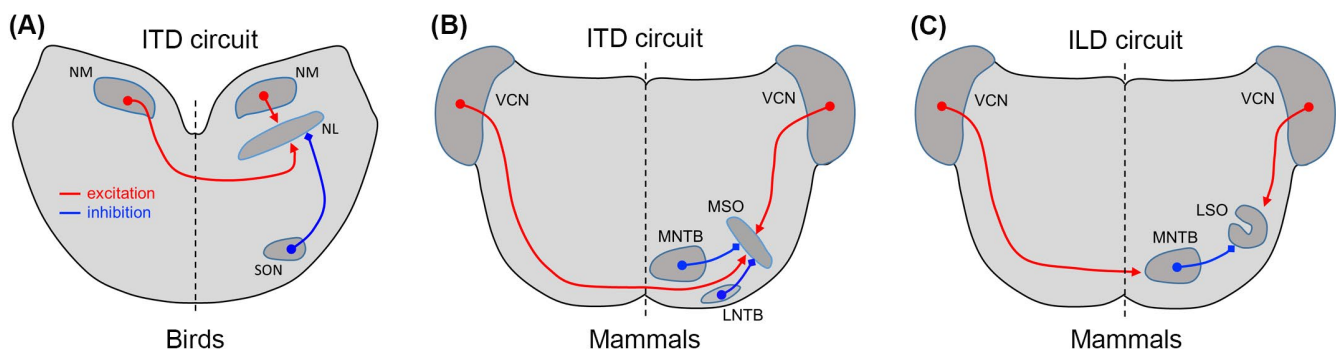

FIGURE 1 Sound localization circuits. Sound localization information is integrated in the auditory brainstem using interaural time and level differences (ITDs and ILDs). A-B, The ITD circuit is structurally and functionally comparable between birds (A) and mammals (B). The circuit is composed of spherical bushy cells in NM and VCN and fusiform bipolar cells in NL and MSO. The NL also receives an inhibitory input from the superior olive nucleus (SON). The MSO receives inhibitory inputs from MNTB and LNTB. C, In mammals, ILDs are computed in LSO through an excitatory projection from globular bushy cells in VCN and an inhibitory projection from MNTB

3.1 | FMRP regulates dendritic development and morphology in the ITD circuit

In the brainstem, ITD computation for low frequency sounds takes place in MSO and NL (Figure 1A,B). The MSO and NL are best developed in humans and other animal species most sensitive to low frequency sounds including reptiles, birds, some rodents such as gerbils, and primates. Dendrites of MSO and NL neurons segregate into two domains; each domain receives excitatory input from the ipsilateral or contralateral ear via VCN and NM (Figure 2A). This anatomic segregation, along with specialized synaptic and intrinsic physiology, enables MSO and NL neurons to detect ITDs in the microsecond range.[29]

Strong FMRP expression is detected in the VCN-MSO circuit across taxa, including gerbils,[27] rats,[30] and humans[31] Figure 2B). Similarly, almost all neurons of NM and NL in alligator and chicken brainstems contain exceptionally high levels of FMRP.[27] Perhaps surprisingly, postmortem human brains were found to have more variation of FMRP expression depending on the cell type.[31] In the human VCN (2 males, 5 females; average age 79 years), the vast majority (85%-95%) of bushy cells and octopus cells are FMRP immunoreactive, while only about 68% of stellate neurons express FMRP. It is

not clear whether this difference results from technical variations associated with postmortem tissue processing of human brains or reflects varied degrees of FMRP regulation in functionally distinct human VCN cell types.

Strong FMRP expression in normal developing MSO neurons is consistent with the remarkable changes seen in FMRP-deficient MSO. A morphological study of the human MSO was conducted in an age-matched (29-32 years of age, all males) series of subjects (one neurotypical subject, one subject with ASD, and one subject with FXS).[32] MSO neurons were significantly smaller in ASD and FXS. In fact, MSO neurons in the FXS subject were significantly smaller than both the control and ASD subjects, suggesting a direct link between FMRP and MSO development, although additional FXS patients are certainly needed for investigation. In control subjects, the MSO is composed of fusiform and stellate neurons, with only a small population of round/oval neurons. In the FXS patient, the MSO contained significantly more round/oval neurons, reflecting an immaturity of MSO neurons in this subject. Finally, in control subjects, individual MSO neurons are arranged with their long axis perpendicular to the dorsoventral axis of the brainstem. This orientation of neurons is lost in both ASD and FXS patients, implicating disrupted dendritic branching in MSO. Although only one human FXS subject has been examined so far, very similar cellular changes
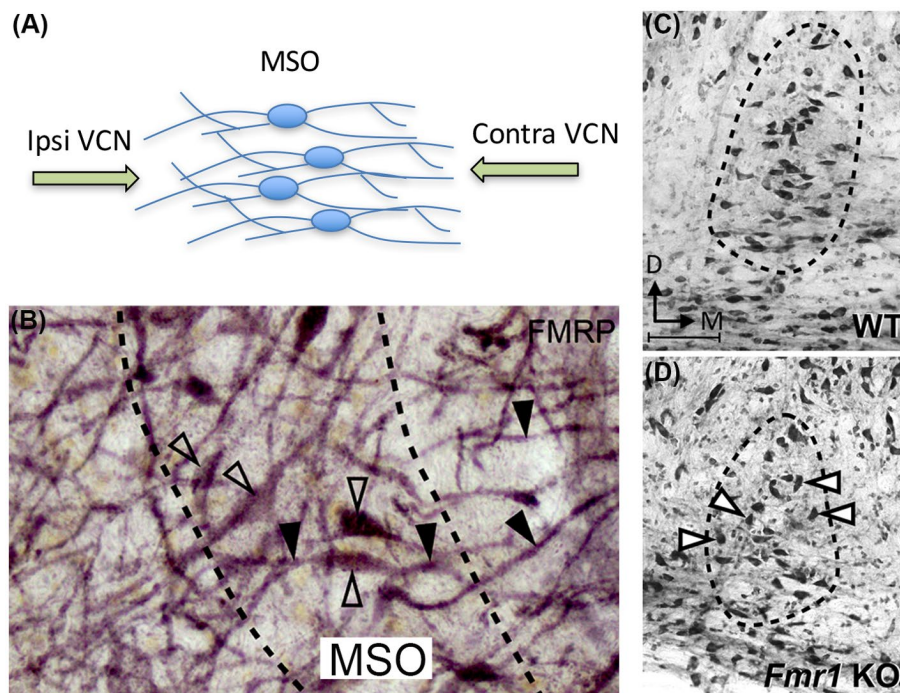

FIGURE 2 FMRP deficiency leads to altered cytoarchitecture in MSO. A, Schematic drawing of the normal organization of MSO. B, FMRP expression in the human MSO. Empty and solid arrowheads point to cell bodies and dendritic branches, respectively. C-D, Altered organization of MSO in *Fmr1* knockout rats (D) as compared to wild type (C). In *Fmr1* knockout animals, MSO somata were smaller, more round/oval, and more vertically oriented (arrows). Abbreviation: VCN, ventral cochlear nucleus; MSO, medial superior olive

induces a net change in the ratio of excitatory and inhibitory inputs to MNTB.

Direct in vitro slice recordings of synaptic transmission in MNTB of *Fmr1* knockout mice suggest that changes in synaptic excitation are subtle.[57] The maximal synaptic strength and kinetics of the glutamatergic input remain unchanged but the way the synapse responds to afferent stimulation with gradually increasing intensity becomes altered. In wild-type neurons, a typical all-or-none response pattern of evoked eEPSCs is observed in the majority of cells. Proportionally fewer *Fmr1* knockout than wild-type neurons showed this pattern. In *Fmr1* knockout animals, a smaller proportion of calyxes in the MNTB display simple morphology,[58] which is associated with synaptic depression.[59] Additionally, in vivo juxtacellular recordings detected only minor changes in excitatory spiking activity.[58] In vitro slice physiology on the inhibitory inputs has also revealed no significant changes in most of the parameters examined for both glycinergic and GABAergic transmission.[57] The discrepancy between anatomy and physiology suggests that the anatomical changes in the presynaptic terminals may not be sufficient to lead to significant functional changes. Alternatively, the electrophysiological changes may be attenuated by multiple anatomical alterations that balance out the overall effect on neurotransmission (for example, a decrease in GABA synthesis and an increase in GABA transport). A lack of change in synaptic strength at inhibitory synapses, measured by electrophysiological techniques, despite clear anatomical changes, has been also observed in other brain systems (eg,[60,61]).

One of the core mechanisms that have been proposed to account for the deficits in FXS in some parts of the nervous system is the "mGluR (metabotropic glutamate receptor) theory," specifically that group I mGluR activity is exaggerated.[62] While multiple transmitter systems may regulate auditory neurons, no significant differences have been reported in mGluR-mediated neuromodulation of the inhibitory inputs to MNTB.[63] Activation of group I mGluRs affected the inhibitory transmission in the same manner in both wild-type and *Fmr1* knockout neurons, enhancing spontaneous glycine release but suppressing the evoked inhibitory postsynaptic currents (eIPSCs), and suppressing the evoked GABAergic IPSCs without changing the spontaneous GABA release. These observations in MNTB contrast the more dramatic changes observed in other brain regions, supporting the view that the effects of FMRP on neural functions vary across brain regions, and potentially across development.

3.2.2 | Ion channels are regulated in a different way in the MNTB

Hyperexcitability in a circuit can result from changes in the intrinsic excitability of neurons, as well as changes in

synaptic E/I ratio. Direct recordings of the firing patterns of MNTB neurons in acute slice preparations have shown that loss of FMRP renders these neurons hyperexcitable. In wild-type mice, the normal response of MNTB neurons to a sustained depolarization is to fire only a single action potential at the onset of the depolarization. In MNTB neurons from *Fmr1* knockout animals, however, the same depolarization results in repetitive firing throughout the current pulse[64] (Figure 3A). Such changes in the intrinsic excitability of neurons produced by loss of FMRP must reflect changes in the numbers and types of ion channels expressed in neurons. This can be accomplished by at least two quite distinct mechanisms. The first is by changes in the rates of translation of mRNAs that encode channels.[65,66] The second is by altering the amplitude, gating or trafficking of the channel subunits that normally bind FMRP directly.

Ion channels are relatively minor cellular constituents compared to cytoplasmic and organellar proteins, and of the approximately 90 genes that encode Na^+, Ca^{2+}, or K^+ channel subunits, only very few of their mRNAs have been shown to bind FMRP.[16] A surprisingly high proportion of those that do bind FMRP are, however, expressed in neurons of the auditory system. Potassium channels that play major roles in regulating the intrinsic excitability of auditory brainstem neurons and whose mRNAs are targets of FMRP include the Kv3.1, Kv3.3, Kv1.2, Kv11.3, and $K_{Na}1.1$ channels.[67-75] The sodium channel Nav1.6[76] and the calcium channels Cav2.1, Cav2.2, and Cav 2.3[77] are also expressed in MNTB and their mRNAs are targets of FMRP.[16] These links between FMRP and its channel targets that directly determine intrinsic excitability have allowed analyses of how loss of FMRP contributes to changes in firing patterns such as those of Figure 3.

One clear example of how a change in the rate of synthesis of an ion channel caused by loss of FMRP disrupts auditory processing is provided by the Kv3.1 channel in MNTB neurons, as well as in the VCN. Messenger RNA for Kv3.1 was one of the very first mRNAs found to bind to FMRP.[17,78] This channel is absolutely required for neurons to fire at the very high rates required for phase-locking to physiological sound stimuli (up to 800 Hz).[72] Kv3.1 is normally expressed in a gradient along the tonotopic axis of MNTB, with highest levels in neurons that respond optimally to high-frequency sounds.[79,80] This gradient is absent and overall levels of this Kv3.1 protein and Kv3.1 currents are significantly higher in mice lacking FMRP (Figure 3B,C) .[64,78] Moreover, levels of Kv3.1 in VCN and MNTB are increased by physiological increases in auditory stimulation in vivo.[81] The ability of auditory stimulation to alter the already high level of Kv3.1 protein in these nuclei is abolished in *Fmr1* knockout mice.[78] These findings are consistent with a role for FMRP in the control of sound-induced translation of Kv3.1 mRNA, and with the enhanced firing rate of MTNB neurons in *Fmr1* knockout mice.
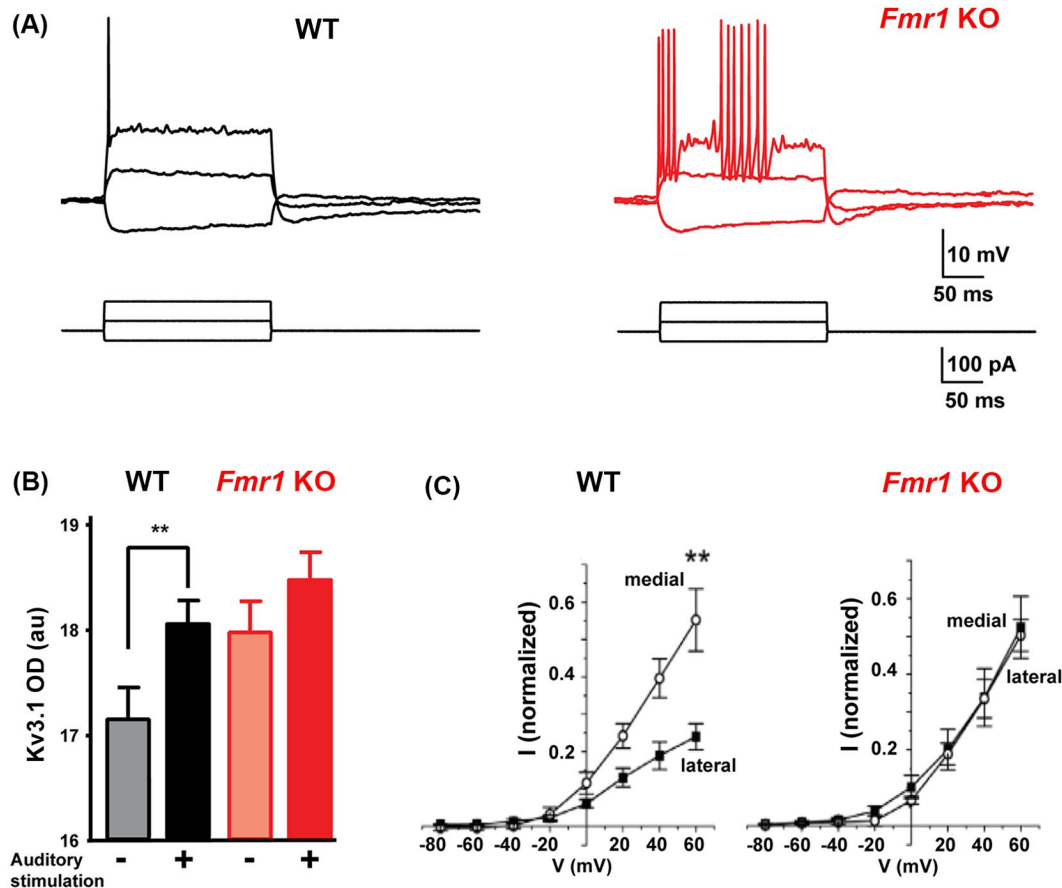
FIGURE 3 MNTB neurons from *Fmr1* knockout mice are hyperexcitable. A, Current-clamp recordings of action potentials in MNTB neurons from wild-type and *Fmr1* knockout mice in response to hyperpolarizing and depolarizing current pulses (modified from 64). B, Bar graphs showing quantification of Kv3.1 immunoreactivity in MNTB in silence or after 30 minutes of exposure to physiological sound stimulation. C, Mean current-voltage relations for high-threshold Kv3.1 currents in patch clamp recordings of neurons in lateral and medial aspects of MNTB. The tonotopic organization of currents present in wild-type neurons is absent in neurons from *Fmr1* knockout mice (*B* and *C* modified from 78). WT, wild type; KO, knockout

A second mechanism by which FMRP regulates neuronal excitability is through direct protein-protein interactions with ion channels subunits.[67,82-87] In each case, the interaction with FMRP alters the amplitude, kinetic behavior, or trafficking of the channel. Binding of FMRP to channels was first demonstrated for $K_{Na}1.1$ channels, also termed Slack channels,[67,82] and has also been documented for Kv1.2[87] and Cav2.2,[85] all of which are expressed in MNTB. The two potassium channels, Kv1.2 and $K_{Na}1.1$, that are regulated in this way play a very different function in the excitability of auditory brainstem neurons from that of the Kv3.1 channel. Both channels activate at negative membrane potentials close to the resting potential and contribute to the so-called low-threshold potassium currents. These are required for the high degree of temporal accuracy with which principal neurons of MNTB lock their action potentials to incoming stimuli.[68,75] In both cases, the direct binding of the channel subunit to FMRP promotes channel opening, increasing current amplitude.[67,82,87] Accordingly, the amplitude of

the low-threshold potassium currents is reduced in MNTB neurons from *Fmr1* knockout mice, promoting the abnormal firing patterns shown in Figure 3.[64] Novel drugs that alter the voltage-dependence of Kv3.1 channels have been shown to rectify these abnormal firing patterns by increasing low-threshold currents and decreasing high-threshold currents in *Fmr1* knockout animals.[64] These agents, therefore, represent a potential new therapy for auditory hypersensitivity in FXS patients

Loss of FMRP affects the levels and activity of several other types of ion channels both by translational mechanisms and by direct protein-protein interactions.[65] These include KCa1.1 (BK), Kv4.2, and HCN channels, which shape the excitability of many types of neurons in the auditory system, but their roles in auditory processing in FXS have not been investigated. Because FXS is a neurodevelopmental disorder, it is likely that there exist compensatory mechanisms that act over time to alter the excitability and synaptic transmission in the patients and in animal models of the disease.[58]

3.2.3 | Synaptic distribution is altered and excitation is enhanced in the LSO

In LSO, alterations in synaptic E/I balance directly translate into a measurable shift in ILD sensitivity. There is evidence of increases in both excitatory and inhibitory inputs as indicated by a larger area of LSO containing the vesicular glutamate transporter (VGLUT1 or 2) and VGAT.[55,88] Consistent with this, in vitro electrophysiological recordings from LSO neurons in acute brain slices of *Fmr1* knockout mice showed that maximal EPSCs evoked by stimulating the ipsilateral incoming fibers from VCN are more than twice as large in mature *Fmr1* knockout compared to wild-type animals (Figure 4A,B). This disproportionate increase in EPSCs occurs in the first week after hearing onset (Figure 4C). This increase is paralleled by a large increase in spontaneous EPSC frequency with no change in amplitude, and a similar increase in the number of synaptic endings labeled with VGLUT1 and VGLUT2.[88] Inhibitory synaptic transmission from MNTB to LSO is mainly unaltered.[56,88] These results suggest that LSO neurons of *Fmr1* knockout mice receive more synaptic inputs and input fibers from VCN, whereas the strength of each individual synapse is unaffected. One possible mechanism may be that the loss of FMRP disturbs the developmental pruning of excitatory inputs to LSO which in

wild-type animals results in a sharpening of frequency tuning in these neurons.[89,90]

Single unit recordings from LSO of anesthetized *Fmr1* knockout mice show specific changes in their spike responses to monaural and binaural acoustic stimulation. The number of spikes in response to stimulation at each neuron's characteristic frequency is increased and more neurons respond throughout the stimulus presentation[88] (Figure 4D). This is consistent with the larger number of excitatory input fibers and more excitatory synapses as observed in the in vitro experiments. Binaural stimulation at the characteristic frequency with different ILDs reveal that the E/I balance in LSO neurons in *Fmr1* knockout mice is shifted. On average, a louder stimulus at the contralateral, inhibitory ear is needed to cause complete suppression of spikes evoked by ipsilateral sound stimulation (Figure 4E). In addition, the slope of the ILD function, a measurement for how many decibels the sound at the contralateral ear has to change to go from no spiking to maximal spiking, was shallower. Shallower ILD slopes are considered to provide less accurate information on the azimuthal location of a sound.[24] Whether *Fmr1* knockout animals exhibit sound localization deficits needs to be tested in future experiments. Moreover, generating frequency response maps, where neurons are stimulated with a large range of different
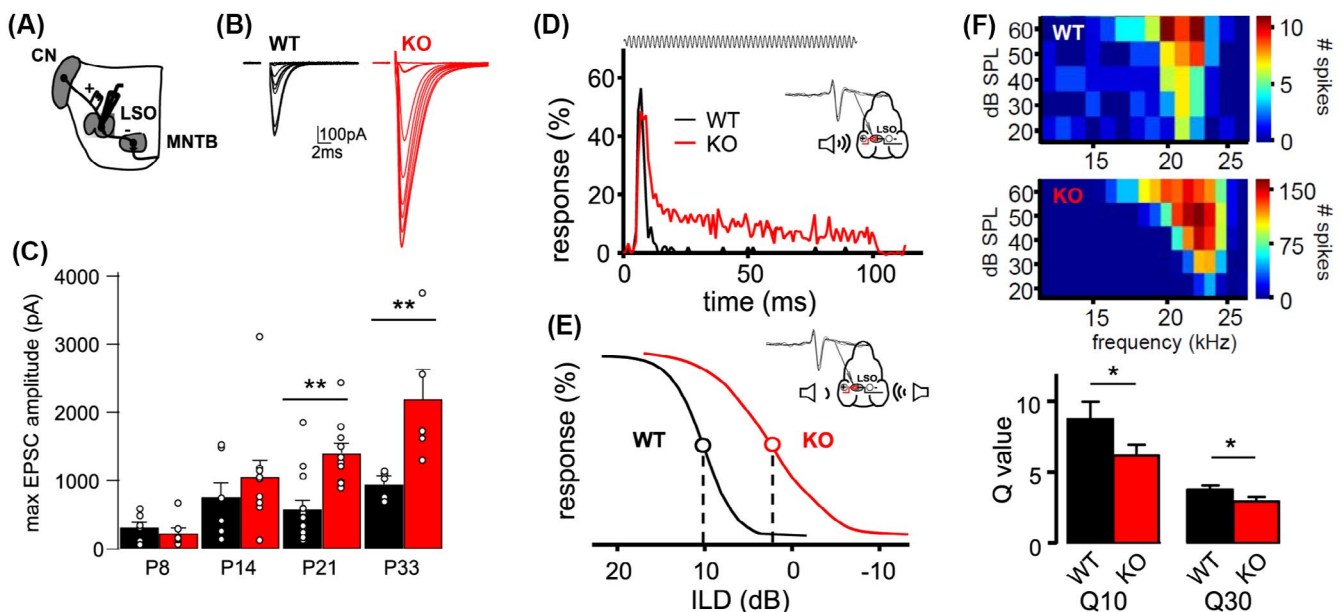
FIGURE 4 Enhanced excitation, altered E/I balance and broadened frequency tuning in LSO neurons of *Fmr1* knockout mice. A, Schematic of patch-clamp recordings from LSO neurons in acute brain slices. B, Examples of excitatory postsynaptic currents (EPSCs) evoked by stimulation of AVCN-LSO fibers with different intensities in wild-type (black) and *Fmr1* knockout mice (red). C, Averaged EPSC amplitudes evoked by maximal stimulation for different developmental stages (P8 to P33) in wild-type (black) and *Fmr1* knockout mice (red). D, Averaged spike poststimulus time histograms of wild-type and knockout LSO neurons in response to monaural, ipsilateral sound stimulation. E, Schematic of ILD function in wild-type and *Fmr1* knockout mice. Circles represent points of half-maximal inhibition. F, Frequency response areas of two representative LSO neurons, each patch showing the total number of spikes of four repetitions. Q–values as a measure for frequency tuning sharpness, expressing the bandwidth of the tuning curve 10 and 30 dB above threshold. Smaller Q10 and Q30 values indicate broader tuning in *Fmr1* knockout animals. Modified from 88 with permission

frequencies and intensities, show that frequency tuning of LSO neurons is broadened in *Fmr1* knockout compared to wild-type animals (Figure 4F).

In summary, current electrophysiological and immunostaining studies indicate that, despite clear changes in morphology and intrinsic excitability at the level of the VCN and MNTB, overall changes in the balance of excitation and inhibition in *Fmr1* knockout animals only become fully evident at the LSO where synaptic excitation is enhanced while the inhibitory input remains largely unchanged in strength. The disturbed E/I balance in LSO neurons may thus underlie broadened frequency tuning as well as shifted ILD responses in *Fmr1* knockout animals.

3.3 | FMRP loss affects ABR measurements in FXS and *Fmr1* knockout mice

Noninvasive measures of brainstem activity can be performed in both humans and animal models. ABRs are

electrophysiological representations of the population activity of auditory structures in the peripheral and central nervous system.[91] Each wave in an ABR corresponds to specific structures along the ascending auditory tract, with some differences between humans and rodents. Generally, the waves of the ABR correspond to the ascending auditory pathway through the areas of the brainstem responsible for sound localization (reviewed in 92,93).

Whether FMRP loss affects human ABRs is controversial. Early studies on patients with FXS showed significantly delayed wave peaks and prolonged interpeak latencies (IPLs; Table 1). Among the changes consistently detected, the absolute latency of peak III is delayed and the I-III IPL is prolonged.[96,97,99] Prolonged I-III IPL could be explained by a delay in the transmission of the auditory signal from the periphery to the brainstem nuclei, or a delay in the activation of the superior olivary complex itself. In these studies, the participants were not screened for sensorineural hearing loss and, because individuals with FXS are prone to otitis media, presumably had a wide range of

TABLE 1 ABR measurements in FXS

Study	Participants	Abnormal ABR/BAEP?	Results
Wisniewski et al[94]	n = 6 All male Mean age = 13.3 years	No	Normal ABRs; methodology was vague
Gillberg et al[95]	n = 10 All male Mean age unknown*	6 of 7 were abnormal	Longer latencies; ABR methodology was vague
Ferri et al[96]	n = 8 Males and females Mean age = 13.8 years	Yes	Delayed peak III; Prolonged I-III IPL; Shorter III-V IPL
Ferri et al[97]; Ferri[98]	n = 10 All male Mean age = 18.4 years	Yes	Delayed peak III; Prolonged I-III IPL; Shorter III-V IPL
Arinami et al[99]; Arinami[100]	n = 12 All male Mean age = 32.2 years	Yes	Longer peak V latencies; increased I-V and III-V IPL
Wisniewski et al[101]	n = 12 All male Mean age unknown**	5 of 12 were abnormal	Ambiguous late waves (peaks III-V) prolonged peak III-V IPL
Miezejeski et al[102]	n = 13 All male Mean age = 16.5 years	No	5 of 13 individuals with FXS sedated*** during ABR testing; Prolonged ABR latencies attributed to sedation
Roberts et al[103]	n = 23 All male Mean age = 11 years	No	No difference in FXS absolute or interpeak latencies

*7 participants were sampled from a pool of 10 FXS patients, mean age = 10.9 years.

**12 participants were sampled from a pool of 62 FXS patients, mean age = 23.1 +/− 14.3 years.

***Agents of sedation: chloral hydrate, thiopental, fentanyl citrate, diazepam, or droperidol. Sedation lasted less than 1 h.

peripheral hearing function.[104,105] It is thus possible that the detected ABR alterations may have resulted indirectly from impaired middle ear function. Indeed, more recent studies from FXS individuals without middle ear infection did not find any differences in the absolute peak latencies and IPLs of ABRs.[102,103,106] However, one should be careful in attempting to interpret this result as implying that ABRs and FMRP expression are independent. Because more than half of FXS children have otitis media,[105] individuals without this peripheral pathology present only a specific population of FXS who are probably less sensitive to FMRP loss in both the periphery and the central nervous system. Other work compared children with FXS, with or without receiving sedation, during ABR testing and argued that differences seen in ABR testing across studies may be caused by the administration of sedatives.[107] However, this study may have over-sampled from high-functional patients who may have been more tolerant of the ABR test.

Studies with mice are not confounded by some of the variables that affect human studies. Several ABR alterations have been identified by comparing *Fmr1* knockout mice and age-matched wild types under anesthesia. Similar to human studies, peak latencies appear to be a subject of FMRP loss in FVB mice,[108] but not in B6 mice.[55,64] Interestingly, although there is no report of altered ABR wave amplitudes in human FXS, the amplitudes of several ABR peaks are altered in *Fmr1* knockout mice, including decreased peak I and increased peak IV, suggesting impaired peripheral and central auditory systems. As the methodology for collecting ABRs can be quite variable, future work using similar experimental protocols on mice and humans may be useful for further characterization of the auditory brainstem phenotype in FXS.

4 | FMRP LOSS ALTERS AUDITORY CORTICAL ACTIVITY

Consequences of *Fmr1* loss at the cortical level have been primarily studied using physiological recordings at the single cell and population levels. These studies have made three main contributions to our understanding of sensory phenotypes in FXS. First, auditory cortex exhibits increased sound evoked responses, and one potential mechanism for this involves changes in levels of matrix metalloprotease-9 (MMP-9), a protein whose mRNA is a target of FMRP. This finding points to a potential therapeutic avenue that targets MMP-9 for treating FXS. Second, deficits in cortical processing are generated early in development. Third, humans with FXS and *Fmr1* knockout mice exhibit remarkably similar changes in their electroencephalogram (EEG), which may prove a useful biomarker in FXS translational studies.

Studies in humans with FXS reveal a number of electrophysiological differences in auditory cortex compared to control participants.[2] Increased activation of left hemispheric circuitry, including superior temporal gyrus, was observed in FXS subjects during an auditory temporal discrimination task.[46] FXS subjects also show reduced synchronization of neural responses to time-varying signals, suggesting impairments in temporal processing.[109] Rojas et al[110] showed that tone-evoked responses measured using magnetic fields are higher in the auditory cortex of individuals with FXS.[110] EEG recordings revealed increased gamma frequency band power in resting state EEG of FXS patients.[111] Auditory event related potential (ERP) studies report abnormally high amplitudes of the N1 wave in response to tones and reduced habituation to repeated sound in FXS.[110,112-115] These data indicate a noisy resting state of auditory cortex in FXS that may lead to abnormal amplitude and synchronization of evoked responses.

Electroencephalogram recordings from the auditory cortex of the *Fmr1* knockout mouse show remarkably similar phenotypes to those seen in humans.[116,117] The gamma frequency band power is increased in resting state EEG in auditory cortex of *Fmr1* knockout mice. The N1 amplitude of ERP is elevated and shows reduced habituation to repeated sounds. The auditory cortex shows a reduced ability to synchronize responses to time-varying signals. In fact, almost every EEG phenotype that have been measured in both *Fmr1* knockout mice and humans with FXS has shown a similar change in both species, indicating a potential use of these deficits as biomarkers to facilitate the translation pipeline. Moreover, EEG deficits in *Fmr1* knockout mice are present from early in development.[118] At the single neuron level, Rotschafer and Razak [119] showed that adult mouse cortical neurons produce a stronger response to brief tones in *Fmr1* knockout mice compared to wild-type mice.[119] Developmental studies further demonstrated that the auditory cortex develops hyper-responsiveness between P14 and P21, and that this is maintained in adulthood.[118] In addition, the adult knockout neurons showed broader frequency tuning (similar to LSO – see above) and reduced selectivity for frequency modulated (FM) sweeps suggesting abnormal spectrotemporal processing.[119]

In addition to potential changes in synaptic transmission and ion channel regulation similar to those described for the auditory brainstem, there is evidence that an alteration in MMP-9 dependent regulation of perineuronal nets (PNNs) may contribute to hyperactivity of the auditory cortex, as well as perhaps lower brainstem structures. PNNs are extracellular matrix components that are often associated with parvalbumin (PV)-expressing cortical cells and regulate their development and functions.[121-124] MMP-9 is a secreted endopeptidase that regulates PNN formation and organization.[125] FMRP negatively regulates MMP-9 translation and MMP-9 levels in neurons, including those in the auditory cortex, are

elevated in FXS.[120,126-128] Consistently, the auditory cortex exhibits delayed development of PNNs and PV cells in *Fmr1* knockout mice.[120] Genetic reduction of MMP-9 in *Fmr1* knockout mice restored the formation of PNNs around PV cells and rescued spontaneous and sound-driven responses to normal level in the auditory cortex.[120] Together, these studies demonstrate that elevated MMP-9 levels contribute to the development of auditory processing deficits by influencing the development of PNNs and PV cells in the auditory cortex of *Fmr1* knockout mice (Figure 5). Whether altered synaptic transmission and ion channel dysregulation as described in the auditory brainstem is also present in the auditory cortex requires further investigation.

Given that FMRP is expressed across multiple auditory system nuclei, the question arises as to whether abnormal cortical responses reflect local circuit dysfunction. This is a complex issue because of the nature of ascending and descending connections in the auditory system. To begin to evaluate the relative contribution of subcortical inputs and local cortical circuits to observed changes in cortical recordings, a conditional knockout in which FMRP was deleted specifically from forebrain excitatory neurons using the *Nex1* promoter was generated.[129] Similar to global knockout animals, this conditional knockout strain exhibits elevated gamma frequency band power in EEGs as well as delayed development of PNN and PV cells, indicating that these deficits arise locally in the cortex. However, the deficit in temporal synchronization of responses to time

varying stimuli was absent when FMRP expression is preserved at subcortical levels, demonstrating that the change following global *Fmr1* knockout is a secondary consequence of altered subcortical inputs. Together, these observations suggest that FMRP deficiency at both subcortical and cortical levels contribute to the physiological deficits recorded from the auditory cortex.

5 | AUDITION-ASSOCIATED BEHAVIORAL CHANGES IN FXS

Three main groups of auditory-associated behavioral phenotypes have been described in *Fmr1* knockout rodents: (i) alterations to the acoustic startle response (ASR) and/or prepulse inhibition (PPI) of the ASR, (ii) increased audiogenic seizure susceptibility, and (iii) impaired auditory-cued fear conditioning.

5.1 | Altered ASR and PPI

Deletion of *Fmr1* impacts acoustic processing in both rodents and humans, as evaluated by altered ASR and PPI.[130] ASR is a full body reflexive response elicited by a loud sound stimulus. PPI is thought to occur when a weak nonstartling sound presented immediately before a startling sound that suppresses the ASR. ASR provides a robust and high throughput
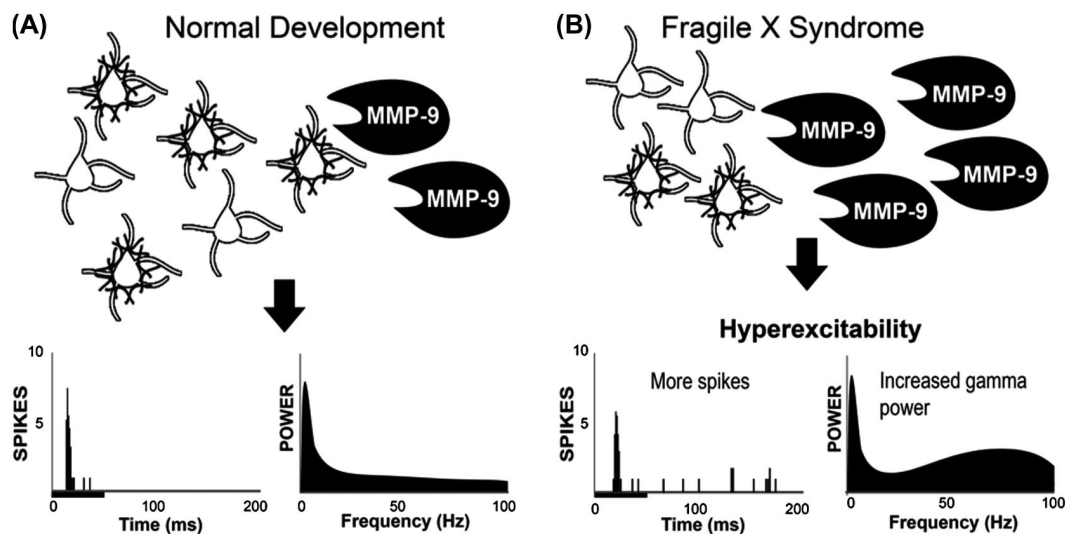
FIGURE 5 Mechanisms underlying cortical hyper-responsiveness in FXS involve local circuit deficits. A, In normal development, MMP-9 provides a certain level of control over PNN integrity around PV cells leading to normal balance between inhibition and excitation, auditory cortical responses as shown below with the poststimulus time histogram and power spectral density plots in the lower left panels. B, In FXS, the MMP-9 level is increased leading to greater breakdown of PNN around PV cells leading to increased spiking and increased resting EEG gamma band power. MMP-9/PV/PNN defects are present in global *Fmr1* knockout mice, as well as mice in which FMRP is removed only from forebrain excitatory neurons. This suggests that local PV neuron functional deficits can lead to cortical hyper-responsiveness. Moreover, even though FMRP was affected only in excitatory neurons, deficits were observed at the network level involving PV cells and PNN that surround them. Image modified from 120

assay for measuring sound sensitivity, but it is difficult to differentiate changes to sound perception from generalized hyperactivity or motor abnormalities.[131,132] Additionally, ASR only occurs for high-intensity sounds, which limits its sensitivity for identifying perceptual abnormalities at moderate sound levels. On the contrary, PPI can be used to assess behavioral responses to softer stimuli that do not elicit a startle response themselves. Enhanced or reduced PPI indicates increased or decreased sensitivity to a sound stimulus and implicates sensorimotor gating disruptions that are specific to the auditory system.

In contrast to the variability in human measurements of the ABR, studies of ASR and PP1 in humans have given clear-cut results. Two clinical studies have compared ASR and PPI responses between individuals with FXS and control subjects.[130,133] Both studies agree on unchanged threshold and magnitude of the baseline ASR but a reduced PPI in FXS individuals. Interestingly, studies of *Fmr1* knockout mice show mixed results, perhaps because of the very different hearing range of humans and mice. Many studies found reduced ASR to startle sounds at the level of 90-120 dB, along with *enhanced* PPI, as compared to age-matched wild-type mice.[130,134-145] Other studies reported unaltered, enhanced ASR, or reduced PPI.[134,138,139,142] Potential contributors to these discrepancies include, but are not limited to, the genetic background,[140,146] sex (147; but also see137), age,[145] and the sound level for triggering ASR.[140] Particularly, strain-specific differences have been reported in a variety of behavioral phenotypes following *Fmr1* deletion[142] and it is known that different mouse strains exhibit varying degrees of ASR in wild-type mouse strains.[148] Despite the variability in ASR and PPI phenotypes, ASR changes in *Fmr1* knockout mice are developmentally regulated[145] and can be rescued with re-introduction of the *Fmr1* gene,[141] supporting a direct involvement of *Fmr1* gene in these auditory-mediated behaviors. Because their hearing range overlaps with that of humans, it is likely that *Fmr1* rats will prove a better model for studying these auditory phonotypes.

5.2 | Increased audiogenic seizure susceptibility

A high proportion of FXS patients have childhood seizures, which typically abate with development.[149] In *Fmr1* knockout mice, a continuous loud sound lasting longer than 30 seconds leads to audiogenic seizures (AGS).[136,150] AGS are generalized convulsive seizures characterized by wild running, loss of righting reflex, generalized tonic-clonic seizing and, in some cases, death of the animal. As the seizure phenotype is robust, it is frequently used as a marker for testing drug efficacy in mouse models of FXS. Indeed, many therapeutics for FXS have been shown to reduce or abolish AGS in *Fmr1* knockout mice

(metformin: 151; CTEP: 152; Lovastatin: 153,154; Baclofen: 155; PAK inhibitor: 156). Similar to ASR and PPI, there are strain-specific differences in both susceptibility and developmental timing of AGS, with the most robust seizure phenotype seen in *Fmr1* knockout mice on the FVB background.[136,150] Animals from the C57BL/6 background are most susceptible to seizures during a restricted developmental window around postnatal day 21-25.[137] Determining the contribution of genetic background to audiogenic seizure susceptibility in *Fmr1* knockout mice could potentially help explain epilepsy variability seen across individuals with FXS.[149]

Cell type specific *Fmr1* deletion demonstrates that lack of FMRP in subcortical regions is sufficient to cause AGS. Moreover, restoration of FMRP expression solely in the inferior colliculus is sufficient to prevent AGS in the global knockout mouse strain.[157] At the molecular level, generation of AGS likely involves the common signal pathways underlying other FXS phenotypes, because the frequency of AGS in *Fmr1* knockout mice is reduced by correcting mGluR5 signaling[143,158] or ERK1/2 activity.[159,160] Although the AGS in *Fmr1* knockout mice are frequently compared to epilepsy in FXS individuals, it is not clear that the underlying seizures are similar in humans and mice.[149] Moreover, the relationship between AGS and sound tolerance issues in FXS individuals has not been established, presenting questions for future research.

5.3 | Perceptual learning and decision making

Recent studies have provided evidence for perceptual learning or discrimination deficits in *Fmr1* knockout mice in both the tactile[161,162] and visual domains.[163] Although fewer studies have examined auditory perceptual learning in FXS models, there is evidence for impaired auditory-cued fear conditioning in *Fmr1* knockout mice.[164,165] It is not clear, however, whether this impairment is more cognitive than perceptual in nature, because *Fmr1* knockout mice exhibit impairment on a variety of associative learning paradigms that are not dependent on auditory cues.[166] Examination of auditory learning and/or discrimination in *Fmr1* knockout mice has the potential to shed light onto the mechanistic basis of sound sensitivity and processing deficits in FXS, as well as fuel development of behavioral paradigms that can be directly translated to human studies.

6 | CONCLUSIONS AND FUTURE DIRECTIONS

The studies of the auditory system in FXS in humans and animal models represent one of the most detailed examinations

across a sensory system in an ASD. The results discussed here demonstrate that FMRP deficiency affects the auditory system at multiple stages along the ascending pathway, starting with the spiral ganglion and very first nuclei in the brainstem and extending all the way to auditory cortex. This wide-spread involvement strongly supports the notion that FXS is not purely a cognitive condition but alters fundamental sensory information processing that is required for a subject to perceive, separate, and localize sounds. Interestingly, striking changes in morphology or intrinsic neuronal excitability caused directly by loss of FMRP may be compensated by other mechanisms, such that changes in overall synaptic strength may be little changed at lower levels but become progressively clearer at higher levels. In some cases, studies of auditory neurons have also revealed new potential therapeutic strategies for hypersensitivity to sounds in FXS, including treatments that alter the activity of potassium channels or metalloproteases.

Fmr1 knockout rodents have provided numerous insights into specific cellular and molecular changes that contribute to the auditory problems of FXS patients. Similar genetic approaches are likely to provide even more insights in the future. It is important to note that although *Fmr1* knockout mice recapture some phenotypes of the auditory system of human FXS, large variations and even completely opposite changes are frequently encountered across mouse studies as well as between mouse and human studies. In part, these problems may arise because some of the observed changes are the result of compensatory neuronal plasticity rather than true FMRP-dependent biological pathways, and may therefore, differ with age, sex, and genetic background. More systematic future studies of the role of FMRP at different developmental states in multiple auditory nuclei may, therefore, resolve some of these issues.[167]

Finally, it may be possible to "borrow" useful approaches in other fields for studying FMRP regulation of auditory processing. For example, hearing loss often results in auditory perceptual disruptions, including hyperacusis, where moderate-intensity "everyday" sounds are perceived as intolerably loud or aversive.[168] While sound hypersensitivity due to hearing loss or FMRP deficiency are likely to be mechanistically distinct, there are well-developed assays for assessing sound sensitivity following hearing loss that can be applied to FXS and ASD models. Potential approaches include operant conditioning paradigms that assess perceptual decision making,[169-171] which likely engage a more extensive range of auditory structures than startle-based measures, or innate place-preference paradigms that reflect the aversive nature of sounds without extensive training or conditioning.[172,173]

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
All authors contributed to the writing and editing of the manuscript and have given final approval for all edits. E.A. McCullagh and Y. Wang organized and edited the final versions of the manuscript in addition to writing and editing the main text.

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