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Cochlear Nucleus Transcriptome of a Fragile X Mouse Model Reveals Candidate Genes for Hyperacusis

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Abstract

Objective: Fragile X Syndrome (FXS) is a hereditary form of autism spectrum disorder. It is caused by a trinucleotide repeat expansion in the *Fmr1* gene, leading to a loss of Fragile X Protein (FMRP) expression. The loss of FMRP causes auditory hypersensitivity: FXS patients display hyperacusis and the *Fmr1*- knock-out (KO) mouse model for FXS exhibits auditory seizures. FMRP is strongly expressed in the cochlear nucleus and other auditory brainstem nuclei. We hypothesize that the *Fmr1*-KO mouse has altered gene expression in the cochlear nucleus that may contribute to auditory hypersensitivity.

Methods: RNA was isolated from cochlear nuclei of *Fmr1*-KO and WT mice. Using next-generation sequencing (RNA-seq), the transcriptomes of *Fmr1*-KO mice and WT mice ($n = 3$ each) were compared and analyzed using gene ontology programs.

Results: We identified 270 unique, differentially expressed genes between *Fmr1*-KO and WT cochlear nuclei. Upregulated genes (67%) are enriched in those encoding secreted molecules. Downregulated genes (33%) are enriched in neuronal function, including synaptic pathways, some of which are ideal candidate genes that may contribute to hyperacusis.

Conclusion: The loss of FMRP can affect the expression of genes in the cochlear nucleus that are important for neuronal signaling. One of these, *Kcnab2*, which encodes a subunit of the Shaker voltage-gated potassium channel, is expressed at an abnormally low level in the *Fmr1*-KO

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Level N/A

cochlear nucleus. *Kcnab2* and other differentially expressed genes may represent pathways for the development of hyperacusis. Future studies will be aimed at investigating the effects of these altered genes on hyperacusis.

Keywords

cochlear nucleus; hyperacusis; FMRP; Fragile X; KCNAB2

INTRODUCTION

The *Fmr1* gene encodes Fragile X Protein (FMRP), which is an RNA-binding protein that is best characterized as a translational repressor[1] and is important for proper synaptic development.[2] However, a trinucleotide repeat expansion in the 5′-untranslated region of the *Fmr1* gene results in the failure to produce FMRP[3] and is the most common single-gene cause of autism spectrum disorder called Fragile X Syndrome (FXS) with a prevalence of at least 1 in 4000.[4] FXS patients as well as rodent models for FXS can exhibit hypersensitivity to sensory stimulation, including hyperacusis.[5] The well-studied *Fmr1* knock-out (*Fmr1*-KO) mouse[6] exhibits audiogenic seizures in response to loud sounds of 120dB[7,8] and abnormal auditory startle reflexes.[9] Thus, this mouse may serve as a useful model for studying auditory hypersensitivity, a condition whose mechanism of pathogenesis is not well understood.

A recent study suggests that subcortical excitatory neurons of the auditory pathway are necessary for the audiogenic seizure phenotype of *Fmr1*-KO mice.[10] Although FMRP is widely expressed throughout the brain, it has previously been shown that FMRP is highly concentrated in auditory brainstem nuclei of mice, including the cochlear nucleus.[11] We hypothesized that the loss of FMRP may result in gene expression changes in the auditory brainstem that may contribute to auditory hypersensitivity.

Here, we present a transcriptomic comparison of the *Fmr1*-KO and wild-type (WT) cochlear nucleus to provide an initial survey of potential genes whose dysregulation upon *Fmr1*-KO may contribute to hyperacusis. Candidate genes of interest encode proteins that are important for neuronal activity or synaptic modulation. Among these, we further characterize *Kcnab2* which encodes a ß-subunit that modulates the activity of the α subunits of the Shaker potassium channel family that are important for the faithful transmission of auditory signals along the auditory pathway.[12–14] Thus, the reduction of *Kcnab2* may contribute to auditory hypersensitivity seen in the *Fmr1*-KO mouse.

MATERIALS AND METHODS

Animals

Animals were used in compliance with a protocol approved by the University Committee on Animal Resources. Two-month-old male mice were used for all experiments unless otherwise specified. *Fmr1*-KO mice from Jackson Laboratory (B6.129P2-Fmr1^{tm1Cgr}/J, #003025) in the C57Bl/6J strain (#000664) were maintained with intermittent backcrossing to C57Bl/6J.

RNA Sequencing/Analysis

Cochlear nuclei (dorsal and ventral) were dissected from fresh brainstem sections. For each *Fmr1*-KO and WT, three biological replicates were prepared (two male mice pooled for each replicate). Tissues were homogenized in lysis buffer (10 mM TrisHCl, 150 mM NaCl, 10 mM EDTA, 0.5% Triton™ X-100) and RNA purified with RNeasy Mini kits with DNase I digestion (QIAGEN). RNA-seq libraries were constructed using TruSeq Stranded mRNA Library Prep Kit (Illumina) with 200 ng of RNA. The quantity and quality of libraries were measured using a Qubit 4 Fluorometer (Thermo Fisher Scientific) and a 5300 Fragment Analyzer System (Agilent), respectively. Sequencing performed on Illumina NextSeq550. Raw reads generated from the Illumina base calls were demultiplexed using bcl2fastq version 2.19.1. Quality filtering and adapter removal were performed using FastP version 0.20.0 with parameters "--length_required 35 --cut_front_window_size 1 --cut_front_mean_quality 13 --cut_front --cut_tail_window_size 1 --cut_tail_mean_quality 13 --cut_tail -w 8 -y -r -j". Cleaned reads were aligned to the Mus musculus reference genome (GRCm38.p6 + Gencode-M22 Annotation) using STAR_2.7.0f and parameters "--twopassMode Basic --runMode alignReads --genomeDir ${GENOME} --readFilesIn ${SAMPLE} --outSAMtype BAM Unsorted –outSAMstrandField intronMotif voutFilterIntronMotifs RemoveNoncanonical". For RNA-seq analyses, gene-level read quantification was derived using the subread-1.6.4 package (featureCounts) with a GTF annotation file (Gencode M22) and parameters "-s 2 -t exon –g gene_name". For comparative transcript expression analyses, transcript-level reads were quantified with Salmon-0.13.1. Data normalization and differential expression analysis between wildtype and *Fmr1*-KO groups were performed using DESeq2–1.22.1 using median of ratios normalization, an adjusted P-value threshold of 0.05 within R version 3.5.1 (https://www.R-project.org/). Pheatmap version 1.0.12 (https://CRAN.R-project.org/package=pheatmap) was used to produce the heatmap of differentially expressed genes. The RNA-seq raw data is available through GEO, accession number GSE236056 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE236056). Gene ontology analysis utilized DAVID program (https://david.ncifcrf.gov/),[15] The Human Protein Atlas (https://www.proteinatlas.org/) and EMBL-EBI expression atlas (https://www.ebi.ac.uk/gxa/home).

Rreverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA (250 ng), isolated as described above, was reverse transcribed using qScript cDNA SuperMix (Quantabio), and 1/100th of it was used in 10 µl quantitative (q)PCR reactions that employed SYBR™ Select Master Mix (Thermo Fisher Scientific). PCR was undertaken using a QuantStudio™ Real-Time PCR System (Thermo Fischer Scientific) using serial dilution control using WT samples. Quantitation was performed using the standard curve method and Pfaffl calculation using Microsoft Excel. The following primers were utilized: *Kcnab2* forward (CTGCAGCTGGAGTACGTGG), *Kcnab2* reverse (CATCCCCTGGTTGATGACA), *Actb* forward (CGCCACCAGTTCGCCATGGA), and *Actb* reverse (TACAGCCCGGGGAGCATCGT). Cycling conditions: 94°C for 2 min, followed by 40 cycles at 94°C for 15 s and 60°C for 1 min.

Western Blotting

Dissected cochlear nuclei were homogenized with a pestle in 6 M urea, sonicated for 10 s, and centrifuged at 14,000 × g for 10 min to remove cellular debris. Protein was quantitated using Bradford Assay (Bio-Rad). Western blotting was performed as described[16] using 10 μg of protein per lane. See Table I for primary antibody concentrations. Horseradish peroxidase-conjugated goat secondary antibody was used at 1:30,000 dilution (Abcam ab6721). Blots were developed using Pierce™ SuperSignal™ West Pico Western Blot Kit (Thermo Scientific) and imaged with ChemiDoc™ Touch Imaging System (Bio-Rad).

Immunostaining

CO_2 euthanized mice were perfused with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO, and 1.8 mM KH_2PO4) followed by fixative (4% paraformaldehyde in PBS). Brains were postfixed for 2 h, dehydrated in 30% sucrose/PBS for 72 h, and frozen in O.C.T. Medium (Sakura). Cryosections (14 μm) of cochlear nucleus were collected (Leica CM1860 UV) and mounted on SuperFrost glass slides (Fisher Scientific). Slides were incubated sequentially in Tris-buffered saline (TBS) (50 mM Tris HCl, 150 mM NaCl, pH 7.5), TBS with 0.1% Triton™ X-100 and TNB Blocking Buffer (Perkin Elmer) with 0.1% Triton™ X-100. See Table I for the primary antibodies used. Antibody incubations were performed in TNB Blocking Buffer. Rabbit antibodies were detected with Alexa-488-conjugated goat antibody (1:500, Invitrogen A11034). Mouse antibodies were detected with an M.O.M. kit (Vector) and streptavidin-Cy3 (1:1000). Following washing in TBS, slides were mounted using Fluoromount-G™ (Invitrogen) and imaged using a Zeiss Palm MicroBeam microscope. Images were analyzed with Image J software. For each WT and *Fmr1*-KO, 20× images of four ventral cochlear nuclei from equivalent cross-sections were analyzed. Within each nucleus, a 600 × 600 pixel square region was selected. For cell intensity measurement, the same fixed circle shape was used to measure average pixel intensity within the cell body of a stained neuron. Statistics were performed using Microsoft Excel.

RESULTS

Consistent with previous literature,[8] *Fmr1*-KO mice exhibit seizures to >120 dB sound. Wild running and/or grand mal seizures were observed in 10/14 (71%) *Fmr1*-KO mice and in 0/11 (0%) WT mice (Supplemental Table 1). FMRP is readily detected by immunostaining in the cochlear nucleus of adult WT mice (Fig. 1A) but not of *Fmr1*-KO (Fig. 1B). We dissected cochlear nuclei (both ventral and dorsal) from two-month-old WT and *Fmr1*-KO male mice (Fig. 1C, dotted outline, identified in comparison to an intact nucleus Fig. 1D).

Transcriptome analysis of cochlear nuclei between WT and *Fmr1*-KO (n = 3 each) reveal 286 transcripts that were differentially expressed between the two genotypes (Fig. 2A), of which, 270 were unique (Supplemental Table 2). FMRP is known to bind mRNA transcripts which have been previously defined by cross-linking immunoprecipitation using FMRP specific antibody and mouse brain.[17,18] We found that only 21 of 270 transcripts are known FMRP bound targets, therefore, a majority of altered gene expression is likely indirectly of FMRP binding. Interestingly, FMRP target genes tended to be downregulated more than

non-FMRP target genes (leftward shift in the distribution of genes with given log2 fold difference in Fig. 2B), suggesting that loss of FMRP binding may reduce the stability of FMRP target mRNAs.

To select the most differentially expressed genes, cut-off differentials were set to |log2 fold change| ≥0.5 and p-value of <0.001 (Fig. 2C, red and blue box on the volcano plot). This yielded 142 genes, 67% of which were upregulated, and 33% of which were downregulated in *Fmr1*-KO relative to WT mice (Fig. 2D). Gene ontology analysis reveals that upregulated genes were enriched in those encoding secreted proteins. Interestingly, among the 38 genes that fell into the category of secreted and extracellular pathways, 30 are expressed in immune or glial cells (Supplemental Table 3), which are involved in the supportive and maintenance function of the nervous system. Several other upregulated genes encode proteins that function in transcriptional regulation.

Downregulated genes in *Fmr1*-KO cochlear nucleus were enriched in those involved in synaptic pathways. Genes encoding proteins in the synapse were of particular interest because of their ability to affect neuronal activity. Among them include: *Piezo1, Kcnip4, Gria3, Kcnab2, Clcn7* and *Pacsin1* (Table II). Gene search through the Allen Brain Atlas[21] (online in-situ hybridization database) reveals that all genes are expressed in mouse cochlear nucleus (data not shown).

We chose to further characterize *Kcnab2* which had the highest expression level among the candidate genes. RT-qPCR confirmed the decreased gene expression from 1.06 (95% CI [0.88,1.23], $n = 6$) in WT to 0.75 (95% CI [0.66,0.84], $n = 7$) in *Fmr1*-KO cochlear nuclei, ($p < 0.01$, Student's t-test) (Fig. 3). Transcript abundance was normalized to the abundance of a control gene *Actb* (a.k.a., ß-actin) transcript that was stable between samples.

We examined the tissue expression of KCNAB2 protein using immunostaining. The specificity of the KCNAB2 antibody used was confirmed by western blotting, which recognized the expected 44-kDa band that was decreased in the *Fmr1*-KO cochlear nucleus lysate (Fig. 4A). Immunostaining showed that only a subset of neurons in the cochlear nucleus express KCNAB2 (Fig. 4B, arrows) in both ventral and dorsal cochlear nuclei. There is also punctate staining along neurites (Fig. 4B, arrowhead). Furthermore, a comparison of *Fmr1*-KO to WT animals showed a similar pattern of expression but a general reduction in the intensity of KCNAB2 immunoreactivity within cells (Fig. 4C). Average pixel intensities were measured from the cell bodies of WT (152.95, 95% CI [146.24,159.66], $n = 41$) and *Fmr1*-KO cells (131.51, 95% CI [127.33,135.69], $n = 35$), revealing a 21 point reduction in the pixel intensity within immunopositive cells that is statistically significant ($p < 0.01$, Student's t-test) (Fig. 4D).

Previous studies have shown that KCNAB2 associates *in vitro* with multiple Shaker channel α subunits, (KCNA1–3, 5 and 6).[24] We sought to determine which α subunits may be co-expressed to partner with KCNAB2. We mined the RNA-seq data and found that *Kcna1* and *Kcna2* were highly expressed while *Kcna6* is expressed at much lower levels (Fig. 5A). There was no statistically significant difference between *Fmr1*-KO and WT mice by Student's t-test ($n = 3$ each). Co-immunohistochemistry in WT ventral cochlear nuclei

was performed to detect KCNAB2 and KCNA1, 2 or 6 (Fig. 5B–J). KCNAB2+ cells (Fig. 5B,D) frequently co-expressed KCNA1 (Fig. 5B,C). KCNAB2+ cells (Fig. 5E,G) also frequently co-expressed KCNA2 (Fig. 5E,F). The distribution of KCNA2 was more localized to the plasma membrane and less so in the cytoplasm when compared with KCNA1 staining pattern. KCNA6 was not detected to be co-expressed with KCNAB2 (Fig. 5H–J). KCNAB2+ cell counting reveals that approximately one-third of co-stains for KCNA1 and one-third for KCNA2 (Fig. 5K).

DISCUSSION

The *Fmr1*-KO mouse is a well-studied and widely used animal model for FXS. There are numerous reported abnormalities in the *Fmr1*-KO mouse that suggest an auditory processing defect. For example, the *Fmr1*-KO mouse exhibits elevated auditory brainstem response thresholds,[26] abnormalities in acoustic startle reflex,[9] enhanced pre-pulse inhibition,[27] and seizures in response to loud sounds.[7] Loss of FMRP specifically in glutamatergic neurons of the inferior colliculus and brainstem has been reported to be necessary for the audiogenic seizure phenotype.[10] Although the mechanism leading to tinnitus and hyperacusis are not well understood, there is growing evidence to support the central gain enhancement model, where hearing loss or ototoxicity lead to abnormally increased responses in the auditory and central pathways.[20] Consistent with this, the *Fmr1*-KO mouse cortical electrophysiological recordings show hyper-responsiveness to sounds.[26]

To begin to identify candidate genes that contribute to auditory hypersensitivity in the *Fmr1*-KO mouse, we performed transcriptome analyses to define genes that are differentially expressed in the cochlear nucleus of this mouse relative to its WT counterpart. Differentially expressed genes that are upregulated in the *Fmr1*-KO are enriched in those encoding secreted proteins. Interestingly, gene ontology reveals that most of these secreted molecules are expressed in glial cells. There is increasing evidence that glia plays an important role in modulating neuronal activity by pruning and modifying synapses.[28] One-third of genes are found to be downregulated in the *Fmr1*-KO mouse and were enriched in genes encoding proteins that function in synaptic pathways.

Interestingly, transcripts known to be bound by FMRP tended to be more downregulated (50%) compared to those not bound by FMRP (33%), which suggests that the loss of FMRP binding may destabilize or reduce the abundance of FMRP-bound transcripts. Although the sample size is small, this observation is consistent with other transcriptome studies in brain.[19] Downregulated genes also were enriched in those involved in synaptic pathways. Among these, we chose *Kcnab2* as a promising candidate because of its relatively abundant expression and its potential to affect neuronal activity in the cochlear nucleus as a voltage-gated potassium channel subunit. In this study, *Kcnab2* mRNA and KCNAB2 protein were indeed moderately decreased in the *Fmr1*-KO mouse with statistical significance. *Kcnab2* mRNA has not been shown to be bound by FMRP in the brain, thus its reduction in abundance could be due to the indirect effects of FMRP on gene expression. More studies will be needed to determine the exact mechanism.

What is KCNAB2?

KCNAB2 is an auxiliary ß subunit that interacts with multiple members of the pore-forming α subunits of the Shaker family voltage-gated potassium channels.[24] Shaker channels are important for maintaining the fidelity of high spiking signals along the auditory pathway that can affect auditory behavior.[12–14] Dendrotoxin inhibition of Shaker channel currents results in hyperexcitability of auditory neurons.[12] The α subunits have six transmembrane domains: the first four are the voltage sensing, and the last two form the pore. These subunits form a heterotetramer.[29] There are multiple members of Shaker family potassium channels (KCNA1, 2, 3, and 6) expressed in rat cochlear nucleus.[25] In rats, KCNA1 (Kv1.1) and KCNA2 (Kv1.2) channels are expressed in bushy and octopus cells in the ventral cochlear nucleus, and in giant and pyramidal cells in the dorsal cochlear nucleus.[30] Our RNA-seq data confirms that *Kcna1, 2* and *6* are expressed in mouse cochlear nucleus. This study reveals that KCNAB2 is co-expressed in cochlear nucleus neurons with KCNA1 and KCNA2 which are known to interact with KCNAB2.[24] However, co-expression with KCNA6 was not detectable. Approximately one-third of KCNAB2+ cells are not accounted to be co-expressed with an α subunit. One possibility is that we under-estimated what we considered co-expression based on staining intensity. The other possibility is that KCNA1 and 2 expressions may be more restricted than KCNAB2 expressions.

What is the Significance of Decreased Kcnab2 *Expression in the Fmr1-KO Mouse?*

The *Kcnab2* gene is mapped to the 1p36 deletion syndrome which is associated with intellectual delay and epilepsy.[22,23] *Kcnab2*-KO mice have been shown to exhibit susceptibility to seizures.[23] Although auditory brainstem responses have been reported to be statistically no different from WT controls,[23] there is no further study on auditory effects (such as auditory brainstem response amplitudes or latencies and susceptibility to audiogenic seizures). It would be interesting to characterize these mice further.

One study found that KCNAB2 is able to shift the threshold of potassium channel activation by −10 mV without altering inactivation.[31] What would reduction of KCNAB2 abundance be expected to cause? We would expect a loss of KCNAB2 to result in other ß subunits such as KCNAB1 to interact with the Shaker potassium channels. KCNAB1, unlike KCNAB2, increases the threshold of channel activation to above the action potential and causes increased inactivation (due to its ball and chain domain)[31] (Fig. 6). The result is prolonged action potential and hyper-excitability due to reduced potassium efflux. Consistent with this, there are reports that deletion of the KCNAB2 subunit causes seizure susceptibility, memory problems, and amygdala hyperexcitability.[23]

How Does a Decrease in KCNAB2 Abundance in the Fmr1*-KO Cochlear Nucleus Fit with the Central Gain Enhancement Model?*

Although the pathophysiology of hyperacusis and tinnitus are still not well understood, there are findings that support the hypothesis that cochlear damage can lead to maladaptive central responses known as central gain.[20] It is known that there are enhanced responses at the level of the inferior colliculus[34] in response to salicylate treatment used to induce tinnitus. Higher up in the auditory cortex, cochlear damage has been shown to increase the amplitude of cortical response to suprathreshold sounds despite reduced neural input due

to cochlear damage.[35] Single unit recordings do indicate altered spontaneous activity in the cochlear nucleus after an intense tone stimulation.[36,37] Thus, the gain seen at the level of the inferior colliculus may be contributed also by changes in the cochlear nucleus. KCNAB2 expression is also found in other areas of the auditory pathway, including Type I (and to a lesser extent Type II) spiral ganglion neurons,[38,39] cochlear hair cells,[32] and superior olivary complex.[33] Thus, we cannot rule out the possibility that KCNAB2 reduction elsewhere along the auditory pathway could contribute to the auditory seizures.

Limitations and Potential Future Studies

Considering that the loss of FMRP results in many direct as well as indirect effects, auditory hypersensitivity and auditory seizures in the *Fmr1*-KO mouse are not likely due to the dysregulation of a single gene. While it remains an uncertainly, it is likely that KCNAB2 downregulation within the cochlear nucleus of *Fmr1*-KO mice contributes to their auditory hypersensitivity and auditory seizures. Although KCNAB2 is expressed in other cells along the auditory pathway, KCNAB2 in the cochlear nucleus is likely to contribute as well. Future experiments could include tissue-specific knock-down of *Kcnab2* mRNA in WT mice or restoration to normal levels of KCNAB2 in *Fmr1*-KO mice to test for a partial rescue of phenotype. Other experiments may include the characterization of neuronal activity by electrophysiology of KCNAB2-expressing neurons to determine whether there are measurable effects on cochlear nucleus activity in *Fmr1*-KO versus WT animals.

CONCLUSION

In summary, we have performed an initial comparison of the cochlear nucleus transcriptome in WT and *Fmr1*-KO mice, the latter of which manifest auditory hypersensitivity. Although FMRP is a known mRNA-binding protein, many of the altered genes produce mRNAs that are known not to bind FMRP, which can be explained by indirect effects (i.e., increased translation of FMRP targets causing downstream transcriptional changes). Furthermore, what are normally FMRP-bound mRNAs tended to be downregulated compared to non-FMRP-bound mRNAs in the *Fmr1*-KO mouse, suggesting that FMRP binding to an mRNA may help stabilize bound mRNA. Many of the upregulated genes are expressed in glial cells while many of the downregulated genes are involved in neuronal synaptic pathways. Furthermore, of the several genes whose expression is reduced in *Fmr1*-KO mice and that are involved in synaptic pathways, *Kcnab2* is a promising candidate for further studies in the mechanism of hyperacusis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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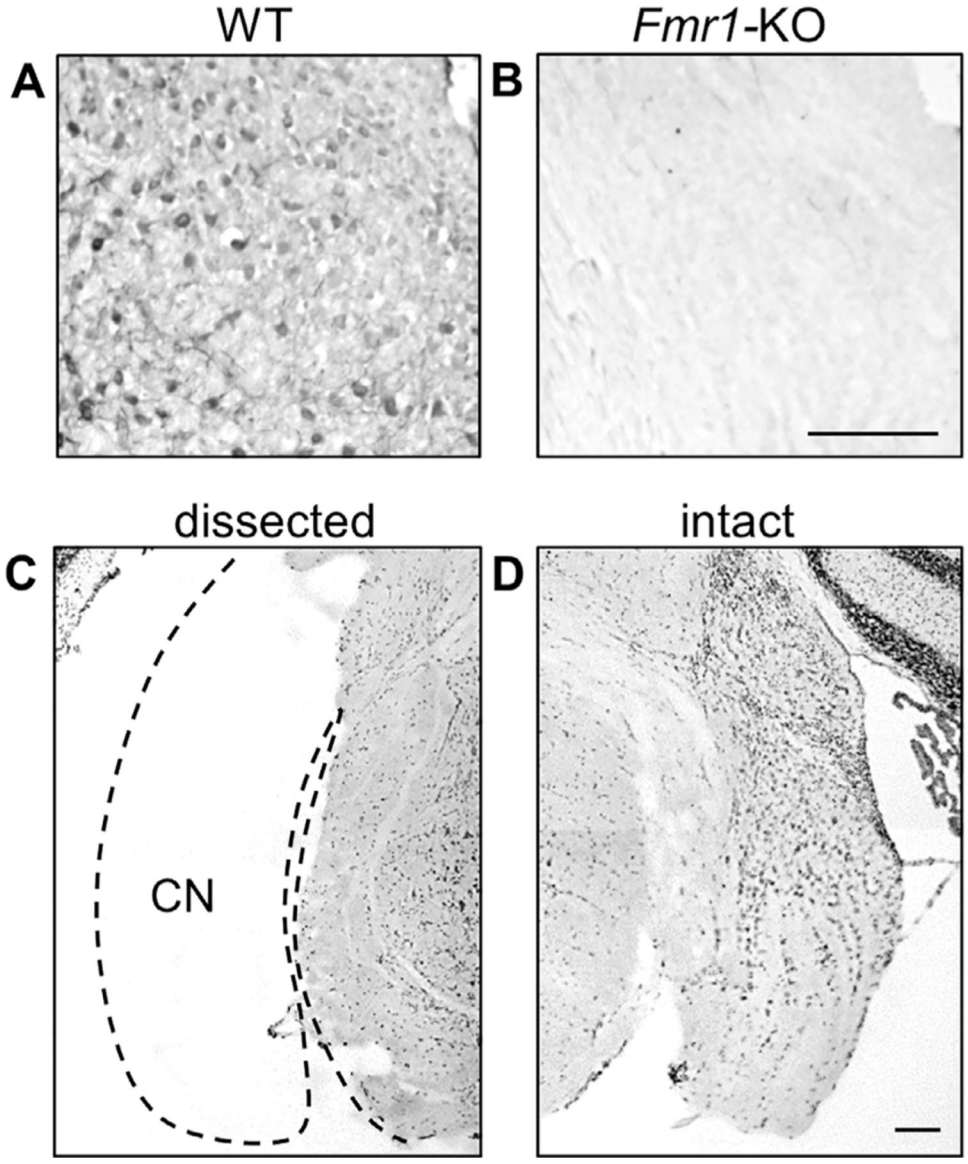

FIGURE 1.

Fragile X Protein (FMRP) immunostaining of cochlear nucleus. (A) Strong FMRP expression in WT cochlear nucleus that is (B) absent in the *Fmr1*-KO. (C) Delineation (dotted line) of a surgically excised cochlear nucleus (CN) from a coronal slice of brainstem with (D) intact side shown as a reference. Scale bars = 200 μm.

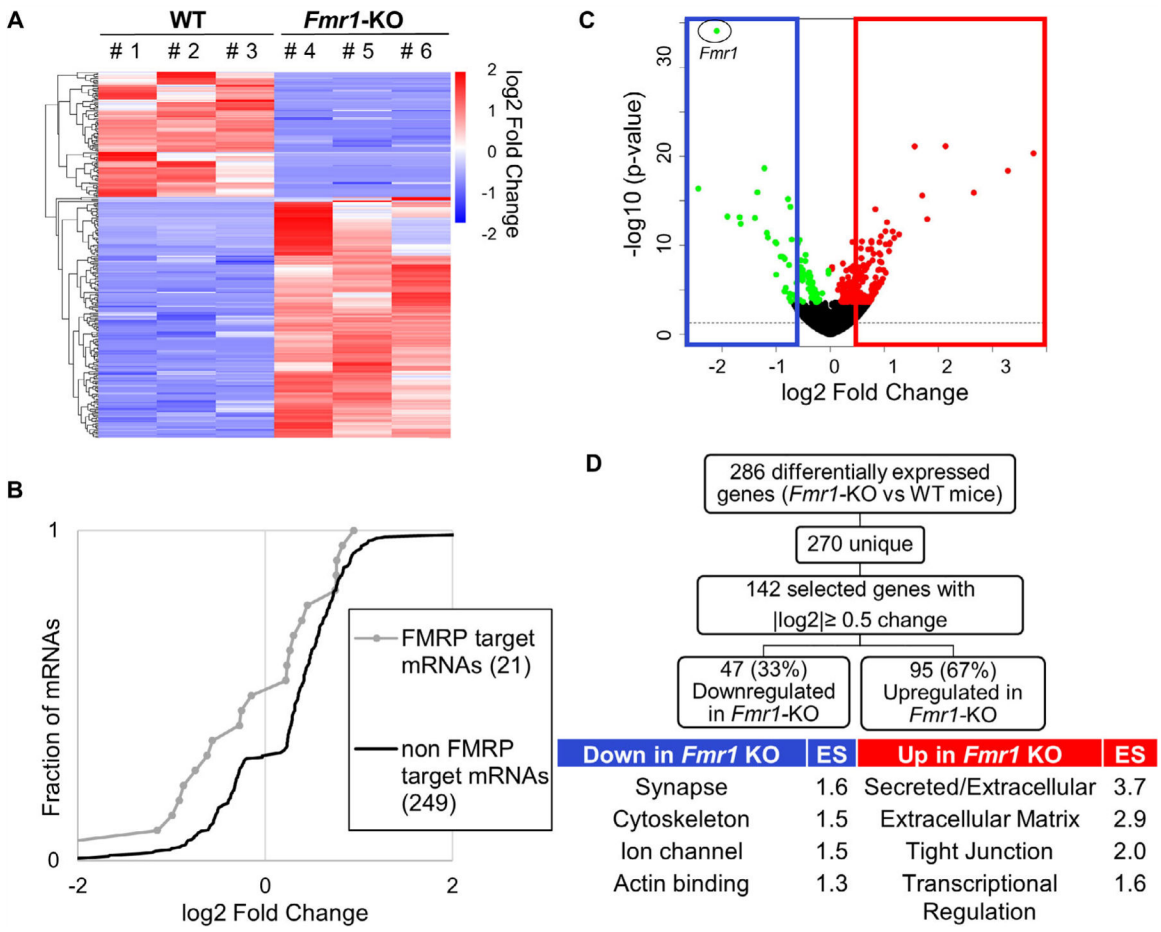

FIGURE 2.

Altered gene expression in *Fmr1*-KO compared to WT cochlear nucleus. (A) Heat map of cochlear nuclei transcripts that are either upregulated (red) or downregulated (blue) in *Fmr1*-KO (columns #4–6) compared to WT mice (columns #1–3). Three biological replicates, each generated from pooling the cochlear nuclei of two mice, were used for each genotype. (B) Distribution plot of the fraction of differentially expressed genes and their log2 fold differences between *Fmr1*-KO and WT for known targets of Fragile X Protein (FMRP (gray)) versus others (black). FMRP targets are downregulated overall compared to non-targets shown by a leftward shift in the distribution curve. (C) Volcano plot showing the logarithmic distribution of genes based on fold-difference versus p-value. Circle denotes *Fmr1*, whose partial transcript is detected in the *Fmr1*-KO mice. The boxed area represents genes with at least |log2| ≥ 0.5-fold difference that were selected for gene ontology analyses. (D) Gene ontology performed using DAVID program for enrichment analysis. (ES = enrichment score).

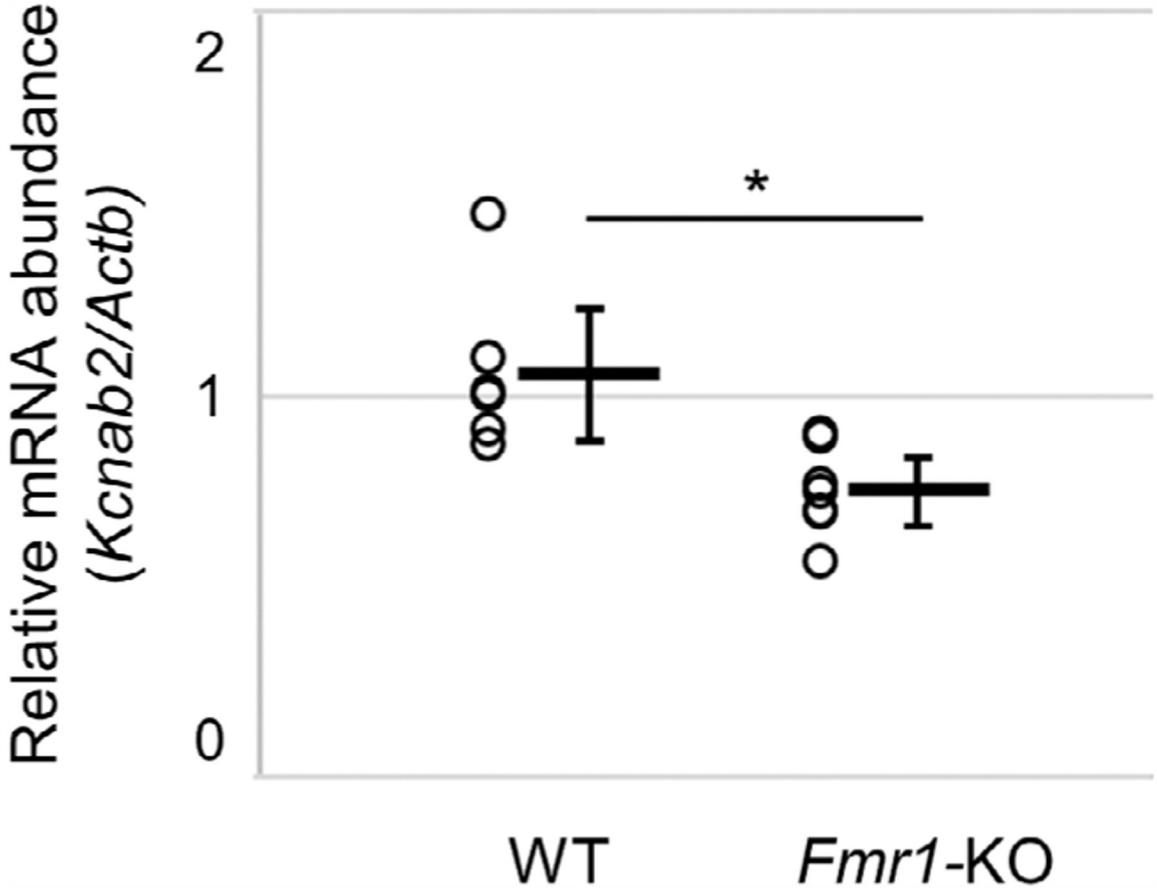

FIGURE 3.

Quantitation of *Kcnab2* mRNA by RT-qPCR. Comparison of WT and *Fmr1*-KO cochlear nuclei shows a statistically significant difference in *Kcnab2* mRNA abundance when normalized to the control *Actb*: 1.06 (95% CI [0.88,1.23], $n = 6$) versus 0.75 (95% CI [0.66,0.84], $n = 7$), respectively. Individual data represented by open circles, bar = averages, error bars = 95% confidence intervals, (*) $p < 0.01$, Student's t-test.

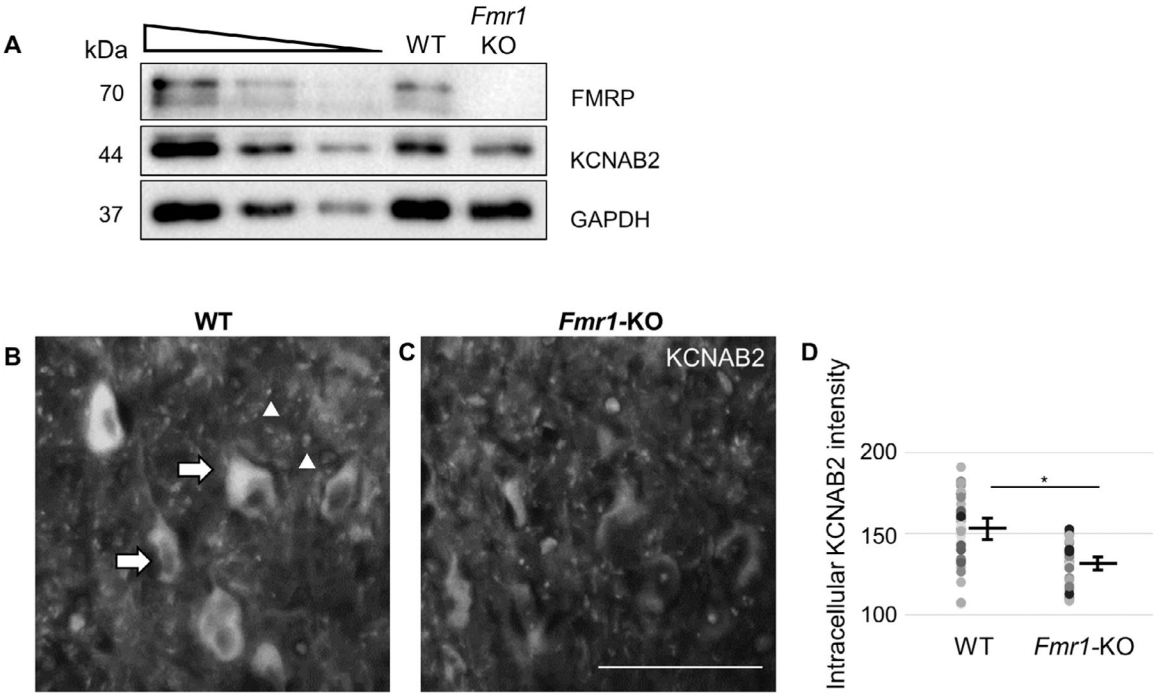

FIGURE 4.

KCNAB2 expression in the cochlear nucleus. (A) Western blotting of cochlear nucleus lysate for KCNAB2 and Fragile X Protein (FMRP). GAPDH shown as loading control. (B) Immunostaining for KCNAB2 in the WT cochlear nucleus is within a subset of cells (arrows). Punctate staining also seen in neurites (arrowhead). (C) KCNAB2 in the *Fmr1*-KO is reduced compared to that in WT. (D) Intensity of KCNAB2 staining within positive cells is reduced by 21 points in the *Fmr1*-KO mice (131.51, 95% CI [127.33,135.69], $n = 35$) compared to WT (152.95, 95% CI [146.24,159.66], $n = 41$). Individual cell data shown in circles, bar = averages, error bars = 95% confidence intervals, (*) $p < 0.01$, Student's t-test, scale bar, 100 μm.

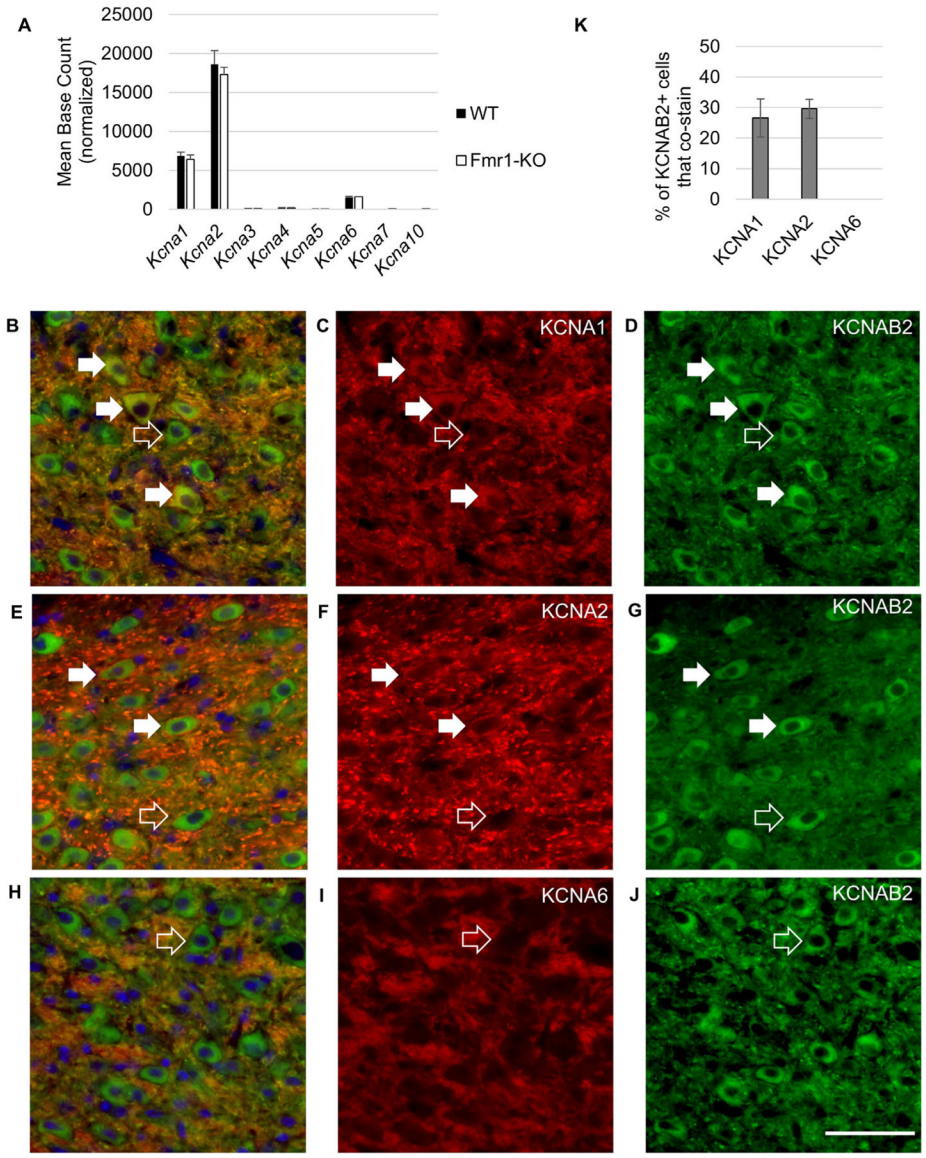

FIGURE 5.

KCNAB2 co-expression with KCNA1 and 2, but not 6. (A) mRNA levels of all Shaker α subunits from mouse cochlear nucleus using the RNA-seq normalized base count for WT (black) and *Fmr1*-KO (white) ($n = 3$ each). *Kcna1* and *Kcna2* are the most abundant while *Kcna6* barely detectable. There was no statistically significant difference between *Fmr1*-KO and WT by Student's t-test. (B–J) Ventral cochlear nuclei from adult WT mice were co-immunostained for KCNAB2 and KCNA1 (B–D), KCNA2 (E–G) or KCNA6 (H-J) ($n > 120$ cells for each, solid arrows indicate co-expression, empty arrows indicate no co-expression). Individual fluorescence channels for the α subunit are shown in the middle column and for the ß subunit are shown in the right column. KCNAB2 co-stained frequently with KCNA1 (solid arrows). KCNAB2 also co-stained with KCNA2. KCNA2 appeared to be localized along the plasma membrane. KCNAB2 did not co-stain with KCNA6 (empty arrows). (J) Quantitation of KCNAB2+ cells reveals that approximately one-third are KCNA1+ and

one-third are KCNA2+. KCNA6 co-staining was not detectable. Scale bar = 50 μm. Error bars = standard deviation.

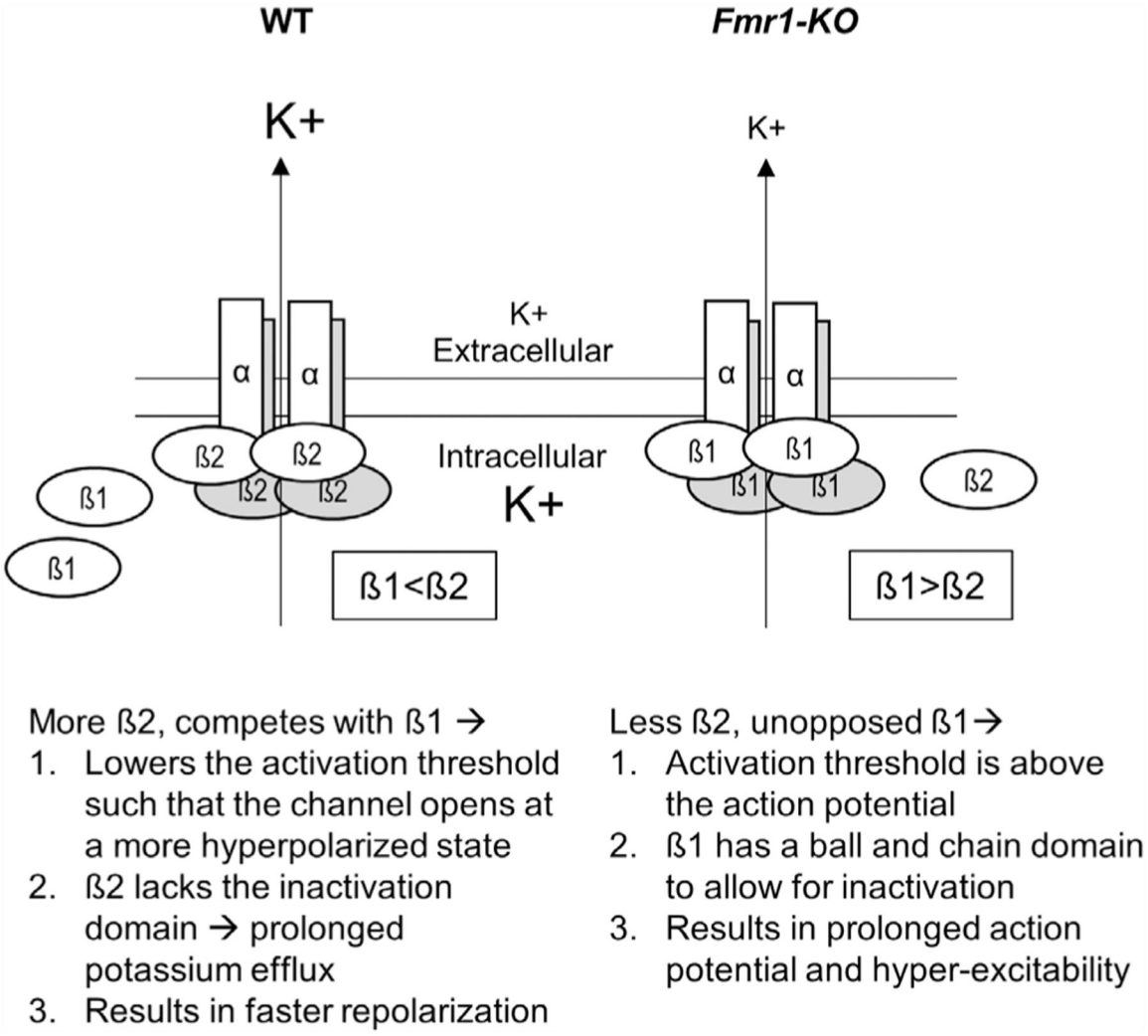

FIGURE 6.

Schematic of what may occur with an abnormally reduced abundance of the KCNAB2 subunit (ß2). KCNAB2 interacts with each of the pore-forming α subunits of the Shaker family voltage-gated potassium channels that form a heterotetramer (α). In the WT, KCNAB2 (ß2) competes with KCNAB1 (ß1) and lowers the activation threshold, and does not inactivate the channel, resulting in faster repolarization of the neuron. In the *Fmr1*-KO, a reduction in KCNAB2 allows unopposed KCNAB1 to increase the activation threshold and then inactivate the channel, resulting in prolonged action potential and hyper-excitability of the neuron.

TABLE I.

Antibodies Used in this Study.

Detects	Antigen	Host, Dilution	Catalogue	RRID
FMRP	Human FMRP	Rabbit, polyclonal	Abcam, ab17722	AB_2278530
KCNAB2	aa550-C-term Rat Kvß2 aa20–34	1:1000 (W), 1:500 (IHC) Rabbit polyclonal	Alomone Lab, APC-117	AB_2039961
GAPDH	Human GAPDH C-term	1:2000 (W), 1:450 (IHC) Rabbit monoclonal 14C10 1:200 (W)	Cell Signaling, 2118	AB_10693448

aa, amino acid; IHC, immunohistochemistry; W, western blotting.

TABLE II.

List of Synaptic Pathway-related Genes that were Abnormally Decreased in Expression in the Cochlear Nucleus of *Fmr1*-KO Mice.

Genes	Aliases	Description	Log_2	Base mean	Allen Brain Atlas	FMRP bound
Piezo1	DHS, FAM38A, Mib, LMPH3	Mechanosensitive ion channel	−1.657	55	Yes	No
Kcnip4	CALP, KCHIP4	Voltage-gated potassium channel interacting protein 4	−1.348	172	Yes	No
Gria3	GluR3, AMPAR3	AMPA receptor subunit	−1.001	311	Yes	No
Kcnab2	Kvß2, HKvbeta2	Voltage-gated, Shaker-related subfamily, beta member 2	−0.781	1998	Yes	No
Clcn7	Clc7, Opta2, Optb4	Voltage-gated chloride channel 7	−0.615	681	Yes	No
Pacsin1		PKC substrate regulates KCC2	−0.559	795	Yes	No

All genes were confirmed for expression in the adult mouse cochlear nucleus using the Allen Brain Atlas[21] which is an online database of in-situ hybridization of mouse and human brain. Fragile X Protein (FMRP) bound targets are defined by previous studies using FMRP cross-linking immunoprecipitation.[17,18]