Usher syndrome IIIA gene clarin-1 is essential for hair cell function and associated neural activation†

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Usher syndrome 3A (USH3A) is an autosomal recessive disorder characterized by progressive loss of hearing and vision due to mutation in the clarin-1 (CLRN1) gene. Lack of an animal model has hindered our ability to understand the function of CLRN1 and the pathophysiology associated with USH3A. Here we report for the first time a mouse model for ear disease in USH3A. Detailed evaluation of inner ear phenotype in the Clrn1 knockout mouse (Clrn1<sup>−/−</sup>) coupled with expression pattern of Clrn1 in the inner ear are presented here. Clrn1 was expressed as early as embryonic day 16.5 in the auditory and vestibular hair cells and associated ganglionic neurons, with its expression being higher in outer hair cells (OHCs) than inner hair cells. Clrn1<sup>−/−</sup> mice showed early onset hearing loss that rapidly progressed to severe levels. Two to three weeks after birth (P14–P21), Clrn1<sup>−/−</sup> mice showed elevated auditory-evoked brainstem response (ABR) thresholds and prolonged peak and interpeak latencies. By P21, ~70% of Clrn1<sup>−/−</sup> mice had no detectable ABR and by P30 these mice were deaf. Distortion product otoacoustic emissions were not recordable from Clrn1<sup>−/−</sup> mice. Vestibular function in Clrn1<sup>−/−</sup> mice mirrored the cochlear phenotype, although it deteriorated more gradually than cochlear function. Disorganization of OHC stereocilia was seen as early as P2 and by P21 OHC loss was observed. In sum, hair cell dysfunction and prolonged peak latencies in vestibular and cochlear evoked potentials in Clrn1<sup>−/−</sup> mice strongly indicate that Clrn1 is necessary for hair cell function and associated neural activation.

INTRODUCTION

Usher syndrome is the most common cause of sensory impairment wherein deafness and blindness occur together. It is clinically subdivided into three types based on the degree of deafness and the presence of vestibular dysfunction (1). USH1 is the most severe form and is characterized by profound congenital hearing loss and vestibular dysfunction combined with pre-pubertal onset of retinitis pigmentosa (RP). In USH2 hearing loss is milder, the onset of RP is after puberty and vestibular function is unaffected. USH3 patients show progressive hearing loss and variable degrees of vestibular dysfunction.

At least 13 loci have been linked to the three types of Usher syndrome, including one locus linked to USH3 (http://webh01.ua.ac.be/hhh/). Genes associated with many of these loci have been identified and they encode proteins that belong to diverse classes of proteins (2,3). CLRN1

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†This work is dedicated to Cindy Elden. We appreciate the vision, inspiration and generosity of the Elden family that made this work possible.
‡The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors and the last two authors should be regarded as joint Senior Authors.

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(USH3A), the only member of USH3, codes for a four transmembrane-domain protein (4) belonging to a large family of transmembrane proteins which include both the tetraspanin and the claudin families. Members of this family participate in a variety of functions including regulating cell morphology, motility, invasion, fusion and signaling (5–7). Tetraspanins are known to form homo-multimers leading to the assembly of microdomains that interact and nucleate the congregation of other non-tetraspanin membrane proteins. CLRN1 shares some of the features common to tetraspanin proteins, including the predicted four transmembrane domain topology, and very short intracellular loops. This protein may play a vital role in creating and assembling membrane microdomains involved in adhesion strengthening and signaling (7). However, the precise function of CLRN1 in the inner ear is not known.

Several different mutations of human CLRN1 have been found that cause progressive hearing loss with variable penetrance linked to the N48K mutation, or profound hearing impairment linked to the Y176 stop mutation (4). Similarities between CLRN11 and the calcium channel gamma subunit protein 2 (CACNG2, stargazin) have been proposed (4). Stargazin has been shown to play a key role in the shaping and maintenance of cerebellar synapses (8). However, in vivo studies are needed to reveal the molecular mechanism that underlies CLRN1 function. USH3 is inherited in a recessive pattern, suggesting that the loss of function is the cause of the disease. Therefore, studies of the Clrn1-null mouse should provide insights into the involved pathogenic mechanisms. Here we report a detailed analysis of the Clrn1−/− mouse inner ear phenotype and describe the expression pattern of Clrn1 in the vestibular and cochlear neuroepithelia. Our results suggest that Clrn1 plays a novel role in hair cell development and function.

RESULTS
Clarin-1 is expressed in hair cells and ganglion cells of the inner ear
To determine the expression pattern of Clrn1 in the cochlear and vestibular hair cells, we carried out mRNA in situ hybridization at embryonic (E) stages 16.5, 18.5 and postnatal (P) day 0, 3 and 5. Clrn1 was found to be expressed as early as E16.5 in the inner ear, in hair cells of the auditory and vestibular sensory epithelia and in the spiral ganglion neurons. Expression was most apparent in the spiral ganglion cells (SGCs) and in the hair cells of the basal turn of the cochlea compared with apical turns at early stages, suggesting the time and location of the onset of Clrn1 expression (Fig. 1). Hair cell-specific genes typically are initially expressed in the more mature hair cells in the basal cochlea and spread to the apical hair cells with continued development, paralleling the gradient in hair cell maturation. By E18.5, all cochlear hair cells expressed Clrn1, with a higher level of expression in the outer hair cells (OHCs) as compared to inner hair cells (IHCs) (Fig. 1). Expression of Clrn1 was detected by in situ hybridization in the inner ear of all stages analyzed, i.e. from both E16.5 to P5, confirming previously reported in situ data (4), and from P30 and P60 by RT–PCR (Fig. 3D).

Clrn1 was also expressed in the vestibular hair cells and Scarpa’s ganglion cells. Closer examination of the Clrn1 labeling in the embryonic saccule revealed strong expression in the hair cells and a much weaker expression in Scarpa’s ganglion cells (Fig. 2).

Generation of Clrn1−/− mice
To study the function of Clrn1, a transgenic mouse lacking the first coding exon (exon 1) of this gene was produced by homologous recombination (Fig. 3A and B). Normal Mendelian segregation of the Clrn1 exon 1 deleted allele (wild-type Clrn1+/+, heterozygous Clrn1+/- and homozygous Clrn1−/−) was observed. Lack of Clrn1 expression in the inner ears of Clrn1−/− mice was confirmed by RT–PCR at various time points from birth well into adulthood (P0–P60) (data from time points P30 and P60 are shown in Fig. 3C and D). In situ hybridization of cochlear duct sections from Clrn1−/− mice confirmed absence of Clrn1 expression in hair cells and SGCs (Fig. 3E). These results demonstrate that Clrn1 mRNA is not expressed in the inner ear of the Clrn1−/− mouse.

We also confirmed the lack of full-length Clrn1 expression in the retina of the Clrn1−/− mouse (data not shown). In Clrn1−/− mice, though, we failed to discover the deficiencies in structural abnormality of photoreceptors and other neurons at the age of 4 months (data not shown). Furthermore, electroretinograms (ERG) analyses did not reveal the sign of photoreceptor degeneration up to the age of 16 months, as exemplified by the lack of significant differences in the a-wave amplitudes at various light conditions (Fig. 4).

Clrn1−/− mice show progressive hearing loss
To assess hearing function in Clrn1−/− mice, we performed auditory brainstem response (ABR) tests on mice of different ages starting at P21, the age at which the auditory system in mice becomes fully mature. ABR tests reflect the electrical responses of both the cochlear ganglion neurons and the nuclei of the central auditory pathway to sound stimulation. About ~30% of Clrn1−/− mice showed elevated ABR thresholds (Fig. 5A), but the majority (~70%) failed to produce detectable ABR responses at P21. As expected, Clrn1+/+ littermates produced characteristic ABR waveforms (peaks 1–4) at thresholds from 25 to 45 dB peSPL (decibel peak equivalent Sound Pressure Level) for pure tones 8–32 kHz (Fig. 5A); Clrn1−/− mice showed similar results (data not shown). Interestingly, in the recordings from mutant mice, response peak latencies were significantly prolonged compared with controls for all four peaks. For example, at 8 kHz the initial response peak (peak 1) occurred at ~2.0 ms in Clrn1+/+ mice, but it was close to 3 ms in the Clrn1−/− mice (Fig. 5B). The interpeak latencies P1−P2 and P1−P3 were also prolonged in the mutants compared with control siblings (Table 1). By P30, hearing function was not detectable in any Clrn1−/− mice tested (data not shown). These results suggest that Clrn1−/− mice have some auditory function at young ages but lose it rapidly after P21. This prompted us to test mutants at time points earlier than P21. All of six Clrn1−/− mice tested at P14 and P20 showed...
some hearing function; however, thresholds were significantly elevated and peak latencies prolonged (Table 2). The increased intensity of sound needed to elicit a response from mutant ears at P14–21 suggests diminished hair cell function early in the life of \( Clrn1^{2/2} \) mice and the prolonged P1 latency implies a delay in neural activation.

Loss of OHC function in \( Clrn1^{2/2} \) mice

To determine whether the hearing impairment in \( Clrn1^{2/2} \) mice specifically involved an OHC defect, we recorded distortion product otoacoustic emissions (DPOAEs) which are indicative of OHC amplification activity. At P21, \( Clrn1^{2/2} \) mice produced no detectable DPOAEs above the noise floor (NF) (Fig. 5C and D). Although, more than 10 \( Clrn1^{2/2} \) mice with high threshold ABR, similar to those tested in Figure 5A and B, were tested at P21, we were unable to record any DPOAEs from these mutants. In contrast, wild-type controls showed normal DPOAE responses (Fig. 5C and D). Absence of DPOAEs at a very young age clearly indicates lack of OHC function in \( Clrn1^{2/2} \) mice.

In summary, our hearing assessments are consistent with a cochlear lesion site and a sensorineural hearing loss. Further, auditory results show that \( Clrn1^{2/2} \) mutation affects hair cell function, and either hair cell to afferent nerve communication or primary afferent neural activation.

\( Clrn1^{2/2} \) mice show progressive loss of balance function

In \( Clrn1^{2/2} \) mice, balance function was not overtly/severely affected by P30. In contrast, circling and head bobbing activity in the Usher 1F model is evident as early as P12 and becomes more obvious with age (9,10). The head bobbing phenotype in \( Clrn1^{2/2} \) mice is mild and variable at young ages (P21–40) with some mutants indistinguishable from their wild-type siblings. However, vestibular dysfunction became more apparent with age such that some \( Clrn1^{2/2} \) mice evidenced clearer signs of head bobbing than others by 6 months of age. Young adult (P21–P90) mutants were subjected to swim tests along with controls. Generally, \( Clrn1^{2/2} \) mice appeared less stable in the water compared with controls (\( Clrn1^{+/+} \) or \( Clrn1^{+/2} \)), tending to roll from one side to the other, even though they used their tails effectively to remain prone in
the water. But abnormal swimming behavior was not discernable in all tested \textit{Clrn1}^{-/-} mice which prompted us to quantify vestibular function in these knockout (KO) animals.

To quantitatively examine vestibular function in \textit{Clrn1}^{-/-} mice, we recorded vestibular evoked potentials (VsEP) of \textit{Clrn1}^{-/-} and \textit{Clrn1}^{+/+} mice at P21–P30. It should be noted that VsEP recordings with linear stimulation specifically assess the otoconial organs (utricle and saccule). On average, VsEP thresholds of \textit{Clrn1}^{-/-} mice ($\pm 5.5 \pm 3.6 \text{ dB re: 1.0 g/ms}$) were significantly higher than those of \textit{Clrn1}^{+/+} controls ($\pm 11.0 \pm 1.2 \text{ dB re: 1.0 g/ms}$), although the \textit{Clrn1}^{-/-} mice showed greater variability in their response thresholds ($t = -3.5$, $P = 0.012$). Some \textit{Clrn1}^{-/-} mice had normal VsEP thresholds, but all evidenced abnormalities in response peak latencies (Fig. 6A). P1 peak latencies were significantly prolonged ($t = 2.26$, $P = 0.00003$) (Fig. 6B and Table 2).

In short, albeit more gradually apparent, the overall characteristics of the vestibular phenotype are similar to those observed in the auditory system of the \textit{Clrn1}^{-/-} mice, thus confirming that the organ of Corti and the saccular and utricular sensory receptors of the inner ear are affected. This strengthens our conclusion that disabling mutations in \textit{Clrn1} affect hair cell function and either hair cell to afferent nerve communication or primary afferent neural activation. However, no significant change in the vestibular hair cell morphology was observed within P21–30 (data not shown). Changes in the vestibular system tend to progress much more slowly than in the cochlea as noted above. Therefore, it will be necessary to examine older (>P30) \textit{Clrn1}^{-/-} mice carefully before we come to any conclusion about the effect of \textit{Clrn1} mutation on vestibular hair cells morphology.

**The stereocilia of OHCs are defective in \textit{Clrn1}^{-/-} mice**

To better understand the structure–function relationship in \textit{Clrn1}^{-/-} mice, we examined organs of Corti from young animals by scanning electron microscopy (SEM). Stereocilia display a ‘V’ shaped configuration on the OHCs and a crescent shaped configuration on the IHCs in normal mice by P10. There were obvious abnormalities in the arrangement of stereocilia in \textit{Clrn1}^{-/-} mice as compared to controls as early as P2 (Fig. 7A and B). Abnormalities similar to those observed at P2 were also noted in OHC stereocilia at P6 (Fig. 7C and D). By P10, the derangement of the stereocilia was more apparent compared with the well organized, mature stereocilia bundles typically observed in the control

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**Figure 2.** Expression of \textit{Clrn1} in the mouse saccule detected using in situ hybridization. (A and A') \textit{Clrn1} is expressed in vestibular hair cells (arrowhead) and ganglion neurons, as shown in (A') using post-in situ immunolabeling with anti-TuJ1 antibody (arrow). (B and B') Higher magnification view of \textit{Clrn1} expression in vestibular hair cells co-labeled with myosin VI. (B' and B'') Arrowhead points to an example of a double-labeled cell.
Figure 3. Generation *Clrn1* transgenic knockout. (A) Map of the targeted exon, targeting construct and excision of exon 1 after exposure to cre recombinase. (B) PCR-based genotyping to identify mice heterozygous (+/−) or homozygous (−/−) for the KO allele. PCR products amplified using C1/P4 or P3/P4 primers resolved on agarose gel. (C) Exon-intron map of *Clrn-1* and location of RT–PCR primers. (D) RT–PCR analysis of *Clrn-1* expression in wild-type and KO cochlea at P30 and 60. (E) shows *in situ* hybridization of *Clrn1* mRNA in wild-type or *Clrn1*−/− mouse cochlea. Antisense probe used to localize *Clrn1* in the middle turn of the cochlea at P1. Arrows point to the expression in the outer hair cells (OHCs), while arrowheads point to expression in the spiral ganglion (SG) cells. *Clrn1* expression is absent in the KO mouse.
specimen at P10 (Fig. 7E and F). In addition to general disorganization of the bundles, circular clusters of abnormal stereocilia were also seen on some OHC (Fig. 7F). Stereocilia defects of the same type and approximate severity also were present at P15 and the basal and upper cochlear turns appeared to be similarly affected (Fig. 7G and H). There was little progression in the severity of stereocilia defects during the period from P2 to P15, suggesting that progression of this pathology is relatively slow during that interval. In all cases, the stereocilia of IHCs appeared normal or only mildly affected. The severity of stereocilia defects in OHCs varied among $Clrn1^{2/2}$ mice, consistent with the variable severity of hearing loss in different animals.

**DISCUSSION**

The use of gene knockout technology in the mouse is a powerful approach for study of diseases such as USH3A. One of the most common mutations in USH3A patients is the Y176X mutation in $CLRN1$. The premature stop codon present in Y176X would result in a small truncated protein that is most likely functionally inactive or null. Therefore, we hypothesized that the inner ear phenotype in $Clrn1$-null mouse would be similar to the clinical presentation of patients harboring the Y176X mutation. Our results show that $Clrn1^{-/-}$ mice display early onset hearing loss that rapidly progresses to a profound loss by ~P30. In contrast, vestibular dysfunction was relatively mild in young animals, progressing slowly (relative to hearing loss) to a severe deficit with age. The overall inner ear phenotype in the $Clrn1^{-/-}$ mouse is similar to the inner ear dysfunction observed in USH3A patients with a presumptive null mutation in $CLRN1$. As with other Usher mouse models, we failed to detect any obvious retinal dysfunctions in $Clrn1^{-/-}$ mice. Therefore, the KO mouse reported here should be a good model for the ear disease occurring in human USH3A patients.

$Clrn1^{-/-}$ mice showed early onset (P14–21) hearing loss with elevated ABR thresholds of 85–95 dB peSPL. In addition, the absolute latencies of all ABR waves and the supporting cells were also degenerated in the basal turn, leading to collapse of the organ of Corti.
interpeak latencies between the waves were significantly delayed, suggesting a neural deficit in addition to a hair cell function deficiency. In the vestibular apparatus, gravity receptor function declined more gradually, but the overall profile was in agreement with the cochlear phenotype. Similar to the delayed latency in ABR peaks, prolonged VsEP peak latencies were observed in all Clrn1<sup>2/2</sup> mice tested, suggesting a defect in gravity receptor hair cell function and associated neural activation. Prolonged peak latencies seen in Clrn1<sup>2/2</sup> mice are reminiscent of mutants with demyelinating disorders (11,12). These findings suggest that the auditory and vestibular deficits in Clrn1<sup>2/2</sup> mice are caused by peripheral defects that affect sensory transduction, the communication of hair cells with afferent neurons and/or signal propagation along the eighth nerve. It has been predicted that CLRN1 might play a role in ribbon synapses based on sequence similarities between the CLRN1-specific motif and stargazin, a cerebellum synapse protein (4). The expression of Clrn1 in hair cells and the functional deficits observed in Clrn1<sup>2/2</sup> mice support a possible role for CLRN1 in ribbon synapses. The progressive deterioration in cochlear and vestibular function observed in Clrn1<sup>2/2</sup> mice is reminiscent of the clinical ear disorder in USH3A (13,14).

In situ mRNA hybridization results confirm the specific expression of Clrn1 in hair cells and ganglion cells of the cochlea and saccule as early as E16.5 and indicate that this expression continues during the postnatal period. While stereocilia bundle morphogenesis is still underway at E16.5 (15), this timing also coincides with the onset of mechanotransduction in embryonic hair cells of mice (16). In the cochlea, mRNA in situ hybridization shows stronger expression of Clrn1 in OHCs as compared to IHCs, consistent with the fact that DPOAEs are absent in hearing Clrn1<sup>2/2</sup> mice. SEM studies also provide strong support for early onset OHC defects in Clrn1<sup>2/2</sup> mice. Our results indicate that the auditory phenotype is caused at least in part by hair cell defects and that Clrn1 is required during hair cell development.

Distinct features of the inner ear phenotype in Clrn1<sup>2/2</sup> mice as compared to the phenotype reported in Usher type I and II mouse models suggests a novel inner ear function for CLRN1. Mouse mutants harboring presumptive null mutations in Usher type I genes exhibit severe disorganization of stereocilia during the early stages of IHC and OHC development (17–19), and the progression of stereocilia pathology from P0 to P15 is quite rapid, as exemplified in Usher type 1F models (20,21). In contrast, mice carrying a null mutation in Clrn1 display relatively less severe stereociliary defects on OHCs in all turns of the cochlea at early postnatal stages, but the progression of severity seems slow from P2 to P15; the stereociliary defects in IHCs are barely detectable at these stages.
Table 1. Peak latencies and interpeak latencies of ABR response for Clrn1+/+ and Clrn1−/− mice, aged 25 days. Thresholds were measured in dB peSPL for ABR and dB re: 1.0 g/ ms for VsEP. Latency was measured in ms and amplitude in µV at equal sensation levels (12 dBSL for ABR and 9 dBSL for VsEPs). The number in parentheses indicates the number of animals tested for each measure. Data in this table were generated in the laboratory of SMJ at ECU.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age</th>
<th>ABR (8 kHz)</th>
<th>VsEP</th>
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<tbody>
<tr>
<td></td>
<td>Clrn1+/+</td>
<td>Clrn1−/−</td>
<td></td>
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<tr>
<td>Threshold</td>
<td>14 days</td>
<td>42.2 ± 8.4 (4)</td>
<td>a 6.5 ± 6.1 (6)</td>
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<tr>
<td></td>
<td>20 days</td>
<td>41.0 ± 8.5 (2)</td>
<td>b 7.0 ± 5.0 (6)</td>
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<tr>
<td></td>
<td>25 days</td>
<td>33.6 ± 1.2 (4)</td>
<td>b 3.2 ± 0.11 (6)</td>
</tr>
<tr>
<td>P1 latency</td>
<td>14 days</td>
<td>2.04 ± 0.8 (4)</td>
<td>b 3.26 ± 0.18 (6)</td>
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<td></td>
<td>20 days</td>
<td>2.21 ± 0.10 (2)</td>
<td>b NR</td>
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<tr>
<td></td>
<td>25 days</td>
<td>a NR</td>
<td>a 1.36 ± 0.05 (3)</td>
</tr>
<tr>
<td>P1-N1</td>
<td>14 days</td>
<td>3.16 ± 1.23 (4)</td>
<td>b 3.12 ± 0.24 (6)</td>
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<tr>
<td>Amplitude</td>
<td>14 days</td>
<td>0.96 ± 0.07 (2)</td>
<td>b 0.70 ± 0.11 (4)</td>
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<tr>
<td></td>
<td>25 days</td>
<td>a NR</td>
<td>a 3.00 ± 0.11 (4)</td>
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<tr>
<td>IPL P1−P2</td>
<td>20 days</td>
<td>0.96 ± 0.01 (2)</td>
<td>b 1.07 ± 0.06 (2)</td>
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<tr>
<td>IPL P1−P3</td>
<td>20 days</td>
<td>2.02 ± 0.01 (2)</td>
<td>b 2.23 ± 0.11 (2)</td>
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Table 2. ABR and VsEP response parameters for Clrn1+/+ and Clrn1−/− mice tested at various ages. Thresholds were measured in dB peSPL for ABR and dB re: 1.0 g/ ms for VsEP. Latency was measured in ms and amplitude in µV at equal sensation levels (12 dBSL for ABR and 9 dBSL for VsEPs). The number in parentheses indicates the number of animals tested for each measure. Data in this table were generated in the laboratory of SMJ at ECU.

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IPL, interpeak latencies. a not recorded; b No ABR at 100 dB peSPL; c significantly different (P < 0.05).
Prolonged peak latencies in vestibular and cochlear evoked potentials strongly suggest that \textit{Clrn1} is necessary for normal sensory transduction, hair cell to afferent communication and/or primary afferent neural activation.

**MATERIALS AND METHODS**

**Transgenic targeting of the \textit{Clrn1} gene**

A \textit{Clrn1} KO mouse was generated by IngenKO, Pty. Ltd. (Clayton, Victoria, Australia). Briefly, the targeting construct was produced by using the ET cloning system (29) with C57BL/6J genomic DNA. The construct was designed such that loxP sites flanked part of the \textit{Clrn1} upstream promoter, the 5'-UTR, the coding part of the first exon (exon 1), 269 bp of the first intron and a neomycin resistance gene (NeoR). Standard protocol was used to generate cre-mediated targeted deletion of exon 1. The targeting construct was subsequently electroporated into C57BL/6J embryonic stem (ES) cells and recombinant clones were selected using G418. Selected clones were transfected with Cre recombinase and screened by PCR for removal of the \textit{Clrn1} gene fragment noted above. Recombined ES cells were microinjected into BALB/c blastocysts and implanted into pseudo-pregnant mothers. Chimeric progeny were obtained, and highly chimeric animals were subsequently mated with C57BL/6J mice to identify germ-line transmission of the \textit{Clrn1} deletion. Founder animals were identified, and heterozygous progeny were delivered to the University of California, Berkeley (UCB). \textit{Clrn1}⁻/⁻ mice derived from mating \textit{Clrn1}⁺/+ mice were sent to Case Western Reserve University (CWRU) for further evaluation. The new allele described in this report was maintained by crossing it to the C57BL/6J (B6) strain, which was used in all parts of this study. The Animal Care and Use Committee at CWRU approved the care and use of the mice included in this investigation.

**Genotyping**

A PCR-based protocol was used to identify \textit{Clrn1}⁺/+, \textit{Clrn1}⁺/- and \textit{Clrn1}⁻/⁻ mice. Genomic DNA was isolated from mouse tails by using the Qiagen DNaeasy Blood & Tissue Kit. The primers used for genotyping were C1: 5'-TTTACCGAAGCCCTTTTCTCG-3'; P3: 5'-GGAGTAAGAGTAGTCAACGG-3'; and P4: 5'-GCATTTCAGCAG ATCAC. PCR was carried out at an annealing temperature of 55°C for 35 cycles. PCR products were resolved in 1.5% agarose gels. The genotypes of \textit{Clrn1}⁺/+, \textit{Clrn1}⁺/- and \textit{Clrn1}⁻/⁻ mice were distinguished by PCR (Fig. 3B). In PCR reactions with P3/P4 primers, only a 782 bp band was detected in KO mice, while a 2 kb band was the only band present in \textit{Clrn1}⁺/+ mice; the genotype was further confirmed by PCR with C1/P4 primers. Since the C1 primer sequence is located in the first exon, a 1 kb band present in samples from \textit{Clrn1}⁺/+ and \textit{Clrn1}⁺/- was not detected in \textit{Clrn1}⁻/⁻ mice.

**Anatomical analyses**

For morphological studies of the sequence of postnatal degenerative changes occurring in the inner ears of \textit{Clrn1}⁻/⁻ mutants, mice were examined at each of five time points (2, 5, 10, 20 and 30 days of age). Five \textit{Clrn1}⁻/⁻ mice plus at least two \textit{Clrn1}⁺/+ controls per time point were processed for histological analysis. In all cases, inner ear tissues were processed using standard procedure (10).

Methods used for SEM have been described elsewhere (20,21). \textit{Clrn1}⁻/⁻ mice with age-matched control specimens (heterozygous littersmates) were studied by SEM at each of four time points (P2, P5, P10 and P15).

**Electroretinograms**

ERGs were recorded as described previously (30).
ABR recording was conducted as previously described (31). To test hearing function, Clrn1+/− mice were presented with broadband clicks or pure tone stimuli at 8, 16 or 32 kHz. Clrn1+/+ [number (n) of animals with this genotype tested = 20], Clrn1+/− (n = 10) and Clrn1−/− (n = 60) mice were analyzed. Click stimuli of 100 μs duration were presented for at least 500 sweeps to both the left and right ears (one at a time) through high frequency transducers (a closed system). ABR thresholds were reported as dB peSPL.

Figure 7. Scanning electron micrographs showing surface views of the organ of Corti in Clrn1+/+ and Clrn1−/− mice aged P2 through P15. Relative to Clrn+/+ (shown in left column) the OHCs from the Clrn−/− animals (right column) show abnormally arranged stereocilia. (A and B) P2; (C and D) P6; (E and F) P10; (G and H) P15. All micrographs taken from basal cochlear turn, except (G and H) which show mid-basal and lower apical turns, respectively. The arrow in (F) indicates a circular cluster of stereocilia (a feature occasionally seen on OHC in the mutants). Scale bars in (A–F) indicate 5 μm; scales bars in (G) and (H) indicate 10 μm.
were obtained from both ears for each animal by reducing the stimulus intensity from 100 dB peSPL in 10 dB steps; this sequence was repeated in 5 dB steps until the lowest intensity that evoked a reproducible ABR pattern was detected.

DPOAE measurements

The basic protocol used for DPOAE recording has been described elsewhere (32,33). The main functional measure used in this study was the 2f1–f2 DPOAE. Briefly, the f1 and f2 primary tones were generated by a synthesizer [Tucker-Davis Technologies (TDT, FL)] and attenuated under computer control by using TDT software. The f1 and f2 primaries (f1/f2 = 1.2) were then presented over two separate transducers with a 10 dB difference in intensity, f1 being 10 dB higher than f2, and delivered to the outer ear canal through an acoustic probe (Etymotic Research, ER-10B+, Elk Grove Village, IL), where they were allowed to acoustically mix. Ear-canal sound pressure levels were measured by the ER-10B+ emissions microphone assembly embedded in the probe. Corresponding NFs were computed by averaging the levels of the ear-canal sound pressure for five frequency bins above and below the 2f1–f2 DPOAE frequency bin (i.e. ±54 Hz). DPOAEs, considered present when they were at least 3 dB above the NF, are represented as input/output (I/O) functions: the input at the f1 level (x-axis) is plotted against the output, represented as the mean (±SD) DPOAE levels (y-axis), at these two frequency pairs, i.e. 16–13.3 and 8–6.6 kHz. For I/O testing, data were averaged from 5+/− and 5−/− mice; results then were compared with the averaged NF from five trials (n = 5).

Vestibular evoked potentials

The use of animals for VsEPs was approved at East Carolina University. Mice were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) solution and core body temperature was maintained at 37.0 ± 0.1°C with a homeothermic heating blanket system (FHC, Inc.). Recording electrodes were placed subcutaneously at the nuchal crest (non-inverting), the pinna (inverting) and the hip (ground). VsEPs were obtained at P14 (WT, n = 4; KO, n = 1), P21 (WT, n = 2; KO, n = 7), P25 (KO, n = 3; WT, n = 3), P30 (KO, n = 3; WT, n = 3) and at 3–6 months of age (KO, n = 3). VsEP recording procedures followed methods described previously (34–36). Here we used a non-invasive spring clip to couple the head to a voltage-controlled mechanical shaker that delivered stimuli to the head. Linear acceleration pulses (2 ms duration, 17 pulses/s) were presented to the cranium.
in the naso-occipital axis by using two stimulus polarities, normal (upward) and inverted (initial downward movement). Stimulus amplitude ranged from +6 to −18 dB re: 1.0 g/ms (where 1.0 g = 9.8 m/s²) adjusted in 3 dB steps. Ongoing electrophysiological activity was amplified (200 000 ×), filtered (300–3000 Hz, −6 dB amplitude points) and digitized (1024 points, 10 µs/point). Primary responses (256) were averaged and replicated for each VsEP waveform. VsEP recordings began at the maximum stimulus intensity (i.e. +6 dB re: 1.0 g/ms) with and without acoustic masking (broadband forward masker 50–50 000 Hz at 97 dB SPL), then the intensity was dropped to −18 dB and subsequently raised in 3 dB steps to complete an intensity profile. The masker was used to verify the absence of auditory components in the VsEP waveform. The first three positive and negative response peaks were scored. Peak latencies (measured in ms), peak-to-peak amplitudes (measured in µV) and thresholds (measured in dB re: 1.0 g/ms) were quantified. Descriptive statistics were generated and the independent samples t-test (assuming unequal variances) was used to compare VsEP response parameters between Clrn1−/− and Clrn1+/+ mice.

Reverse transcriptase-polymerase chain reaction

RT–PCR was used to screen for Clrn1 mRNA in the inner ears of the Clrn1−/− and Clrn1+/+ mice. RT–PCR protocol was carried out as previously described (9). RT–PCR was used to amplify various exon combinations of Clrn1 (Fig. 3D). Primers used for this work were C1: 5′-TTA CCGAAGCCTTTTTCCTG-3′; C2: 5′-TATGGACTTCCTTGG GCCAC-3′; C3: 5′- AGGTACTCTCTGTATGAGGACAA -3′; C4: 5′- TCTTCTCCATGATCTTCTGTC-3′. The following PCR conditions were used: 94°C for 2 min followed by 34 cycles of 30 s each, 55°C for 30 s and 72°C for 1 min. All PCR products were resolved on 3% low range ultra agarose gels (BIO-RAD) and stained with 5% ethidium bromide. Three bands of the following sizes were expected: 834, 780 and 650 bp. Sequences of RT–PCR products were determined with BigDye Terminator Cycle sequencing reagents and protocols (Applied Biosystems, CA). The ABI Prism 377 DNA sequencer (Applied Biosystems) was used to analyze and display the resulting sequence data.

In situ hybridization

Animals were housed in the Department of Comparative Medicine at the University of Washington and were euthanized according to approved protocols. Timed pregnant female mice were sacrificed and embryos removed at E16.5 and E18.5. Embryos were fixed in a modified Carnoy’s solution (60% ethanol:4% formaldehyde:10% acetic acid) overnight at 4°C. Specimens were washed and dehydrated in 100% ethanol overnight at 4°C and then embedded in paraffin; 6 µm sections were subsequently cut and collected. For the postnatal mice, we assigned the day of birth as postnatal day 0 (P0) and sacrificed pups at P0, P3 and P5, according to approved protocols. We then dissected the cochleas from these animals, fixed them and processed them for paraffin sectioning as described above. At least three animals were examined for each time point. Mouse Clrn1 cDNA (clone ID: 40130533) was obtained from Open Biosystems Inc. A full-length clone containing exons 1 and 4 with 3′ and 5′-UTRs was used as the template to generate Digoxigenin (DIG)-labeled probes, which were prepared according to the manufacturer's manual for DIG-11-UTP (Roche, Indianapolis, IN); hybridizations were then carried out according to Hayashi et al. (37). In situ products were visualized by using anti-DIG alkaline phosphatase-conjugated secondary antibody (Roche) and the NBT/ BCIP liquid substrate system (Sigma, St Louis, MO). After in situ hybridization, slides were fixed with 4% paraformaldehyde for 1 h and washed in PBS. Slides then were incubated with 10% fetal bovine serum and 2% non-fat dry milk in PBS/0.1% Triton X-100 (PBST) for 30 min. After overnight incubation with the primary antibody [rabbit anti-Myosin6 (Myo6, Proteus Biosystems)] at 1:2000 dilution, or rabbit anti β-tubulin III (TuJ1, Covance, Austin, TX) at 1:1000 dilution, slides were washed and incubated in fluorescent-conjugated secondary antibody, rinsed with PBST and cover-slipped in Fluoromount G (Southern Biotechnology, Birmingham, AL). Images were captured by a Zeiss Axiopt microscope with a SPOT CCD camera and processed by using Adobe Photoshop.

AUTHORS’ CONTRIBUTIONS


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