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Cell degeneration is not a primary causer for Connexin26 (*GJB2*) deficiency associated hearing loss

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Abstract

Connexin 26 (Cx26, *GJB2*) mutations can induce congenital deafness and are responsible for ~50 % of nonsyndromic hearing loss in children. Mouse models show that Cx26 deficiency induces cochlear development disorder, hair cell loss, and spiral ganglion (SG) neuron degeneration. Hair cell loss and cell degeneration have been considered as a primary causer responsible for Cx26 deficiency associated hearing loss. In this study, by coincidental examination of cochlear postnatal development with recording of auditory brainstem response (ABR) and hair cell function, we found that occurrence of hearing loss in Cx26 knockout (KO) mice was ahead of hair cell loss and cochlear cell degeneration. ABR was absent at any frequencies (8 – 40 kHz) after birth. However, cochlear cells including SG neurons had no significant degeneration throughout postnatal development. Severe cochlear hair cell loss and SG neuron degeneration were only visible in middle and basal turns, i.e., in middle and high frequency regions, in the adult Cx26 KO mouse cochlea. Functional tests show that hair cells in Cx26 KO mice functioned normally; outer hair cells retained electromotility. These data suggest that cell degeneration is not a primary causer of Cx26 deficiency associated hearing loss. Some mechanisms other than cell degeneration, such as cochlear development disorders, may play an essential role in this common hereditary deafness.

Keywords

GJB2; gap junction; connexin; hair cell loss; deafness; inner ear

Introduction

Connexin 26 (Cx26, *GJB2*) mutations are a common genetic cause for nonsyndromic hearing loss and are responsible for ~50% of nonsyndromic hearing loss in children. In the clinic, Cx26 mutations can cause congenital deafness, resulting in a mild-moderate to profound sensorineural hearing loss [1, 2, 12, 21]. However, little is known about the pathology of this common nonsyndromic hearing loss.

Genetic-deficient mouse models provide a powerful tool to delineate the pathogenesis of human hearing disorders [4]. It has been reported that deletion of Cx26 in the cochlea can induce hearing loss; the cochlea has developmental disorders, severe hair cell loss, spiral ganglion (SG) neuron degeneration, and endocochlear potential (EP) reduction [3, 14, 17].

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These studies provide invaluable information about Cx26 mutation induced hearing loss. Hair cell loss and SG neuron degeneration have been considered to be mainly responsible for deafness [3, 14, 17]. However, there is neither connexin expression nor gap junctional coupling between hair cells [7, 19, 20, 22, 23]. In this study, we examined the postnatal cochlear development in Cx26 KO mice with coincidentally recording auditory brainstem response (ABR). We found that hearing loss in Cx26 KO mice is congenital and occurs ahead of cochlear cell degeneration, indicating that the cell degeneration is not a primary cause of deafness. This study provides important information not only for understanding the mechanism underlying Cx26 deficiency associated deafness but also for guiding treatment of this common hereditary deafness in the clinic.

Experimental Procedure

Cx26 KO mouse generation and genotyping

Cx26 KO mice were generated by crossing Cx26^{loxP/loxP} mice [3] with the *Pax2-Cre* mouse line [11]. Cx26^{loxP/loxP} and *Pax2-Cre* transgenic mice were purchased from EMMA (European Mouse Mutant Archive, EM00245) and MMRC (the Mutation Mouse Regional Center, Chapel Hill, NC), respectively. Immunofluorescent staining shows that Cx26 expression in the organ of Corti was deleted (Fig. S1). All experimental procedures were conducted in accordance with the policies of the University of Kentucky Animal Care & Use Committee.

ABR and cochlear microphonics (CM) measurements

ABR and CM recordings were performed in a double-wall sound isolated chamber using a Tucker-Davis ABR workstation (Tucker-Davis Tech. Alachua, FL). Mice were anesthetized by intraperitoneal injection with a mixture of ketamine and xylazine (8.5 ml saline+1 ml Ketamine+0.55 ml Xylazine, 0.1 ml/10 g). Body temperature was maintained at 37–38°C by placing anesthetized mice on an isothermal pad. Two subdermal needle electrodes were inserted at the vertex (active) and ventrolaterally to the right or left ear (reference). The ground needle electrode was inserted to the right leg. ABR was measured by clicks in alternative polarity and tone bursts (8 – 40 kHz) from 80 to 10 dB SPL in a 5 dB step. The signal was amplified (50,000x), filtered (300 – 3,000 Hz), and averaged by 500 times. The ABR threshold was determined by the lowest level at which an ABR can be recognized. If mice had severe hearing loss, the ABR test at the intensity range of 110–70 dB SPL was added. CM was evoked by the same tone bursts. The signal of response was amplified (50,000x), filtered (3 – 50 kHz), and averaged by 250 times.

Cochlear tissue preparation and morphological examination

Details of experimental protocols for cochlear tissue preparation and sectioning have been described in our previous reports [9, 16]. For cryostat sectioning, the cochlea was embedded in the OCT (Cat. 4583, Sakura Finetek USA Inc. CA) and cut 10 µm thickness at –22 to –24 °C by a cryostat (Thermo Electron Corp. Waltham, MA). For paraffin sections, the cochlea after decalcification was dehydrated sequentially in graded alcohol, embedded in paraffin, sectioned at 5 µm thickness, and stained with toluidine blue by using the conventional protocols. Condensed nuclear staining with toluidine blue is a sign of degeneration [6]. To count SG neurons, the area of Rosenthal's canal in the cochlear sections was measured by NIH image software (Bethesda, MD) [23], and the density of SGs was calculated by the number of SGs divided by the area of Rosenthal's canal.

Patch-clamp recording and nonlinear capacitance measurement

Outer hair cells (OHCs) were freshly isolated from the cochlea and perfused with an extracellular ionic blocking solution (100 NaCl, 20 TEA, 20 CsCl, 2 CoCl₂, 1.47 MgCl₂, 2 CaCl₂, 10 HEPES in mM; 305 mOsm and pH 7.2). The classical patch clamp recording was performed under the whole-cell configuration by using an Axopatch 200B patch clamp amplifier with a Digidata 1322A (Molecular Devices, CA) and jClamp (Scisft, New Haven, CT) [18]. The patch pipette was filled with an intracellular ionic blocking solution (140 CsCl, 10 EGTA, 2 MgCl₂, 10 HEPES in mM; 305 mOsm, and pH 7.2). OHC electromotility associated nonlinear capacitance (NLC) was measured by a two-sinusoidal method and fitted to the first derivative of a two-state Boltzmann function [13, 18]:

$$C_m = NLC + C_{lin} = Q_{max} \frac{ze}{kT} \frac{\exp\left(\frac{-ze(V_m - V_{pk})}{kT}\right)}{\left(1 + \exp\left(\frac{-ze(V_m - V_{pk})}{kT}\right)\right)^2} + C_{lin} \quad (1)$$

where Q_{max} is the maximum charge transferred, V_{pk} is the potential corresponding to the peak of NLC, z is the number of elementary charge (e), k is Boltzmann's constant, T is the absolute temperature, and C_{lin} is the cell membrane capacitance. Membrane potential (V_m) was corrected for electrode access resistance (R_s).

Results

ABR recordings in Cx26 KO mice show congenital deafness (Fig. 1). During postnatal development in WT mice, ABR is recordable at postnatal day 14 (P14) (Fig. 1B&D). At P16, the mouse hearing function was matured, and the ABR threshold decreased to the normal level at ~30 dB SPL. However, there was no ABR detectable in Cx26 KO mice at P14 (Fig. 1B&D). The ABR thresholds in Cx26 KO mice remained high around 100 dB SPL throughout postnatal development, demonstrating a congenital hearing loss (Fig. 1B). Hearing loss was over a whole-frequency range (Fig. 1C). ABR thresholds were high at whole-testing frequencies (8 – 40 kHz) (Fig. 1C&D).

However, there was no apparent cell degeneration in the Cx26 KO mouse cochlea during postnatal development. Fig. 2 shows that there is no significant cell loss in the organ of Corti of Cx26 KO mice throughout the postnatal development. An arrow in Fig. 2D indicates that the cochlear tunnel in the Cx26 KO mice was collapsed. However, in the adult Cx26 KO mice, the organ of Corti had severe hair cell and supporting cell degenerations. Fig. 2E shows that almost no hair cells and supporting cells are visible in the middle turn of the organ of Corti in the Cx26 KO mice at P60.

SG neurons also had no apparent degeneration in the Cx26 KO mouse during the postnatal development (Fig. 3). SG neurons appeared normal shape and no soma shrinkage is visible (Fig. 3A). Quantitative analysis shows that SG neurons in the Cx26 KO mice remained at the normal level throughout the postnatal development (Fig. 3B). The densities of SG neurons in the apical, middle, and basal turns of Cx26 KO mice were not reduced. In the adult Cx26 KO mouse cochlea, SG neurons show no significant degeneration in the apical turn but had severe degeneration in the middle and basal turns (indicated by empty arrows in middle column of Fig. 3A). The densities of SGs in the middle and basal turns were significantly reduced in comparison with WT mice (Fig. 3B).

Functional tests further demonstrate that hair cells in Cx26 KO mice retained normal functional capability. CM in Cx26 KO mice was reduced by 50–70% but was still recordable and had the same change pattern as WT mice during the postnatal development (Fig. 4A&B). OHCs in Cx26 KO mice also retained electromotility. NLC demonstrated a

normal bell-shape (Fig. 4D). The averages of fitting parameters were: $Q_{\max}=0.75\pm0.02$ pC, $z=0.82\pm0.02$, $V_{pk}=-79.7\pm3.5$ mV, and $C_{lin}=5.94\pm0.32$ pF ($n=10$).

Discussion

In this experiment, we coordinately examined the morphological changes and hearing function during postnatal development in Cx26 KO mice. We found that hearing loss is congenital. No ABR was detectable in Cx26 KO mice after the onset of hearing (Fig. 1). However, cochlear cells, including SG neurons, had no apparent degeneration in the postnatal development (Figs. 2–3). Functional study further shows that hair cells retain functional capability in Cx26 KO mice (Fig. 4). These findings indicate that cell degeneration is not a primary cause of Cx26 deficiency associated hearing loss in Cx26 KO mice.

In the experiments, we also found that hearing loss in Cx26 KO mice occurred in the whole-frequency range (Fig. 1C&D). However, inconsistent with whole-frequency hearing loss, significant cochlear cell loss and SG neuron degeneration were only visible in the middle and basal turns in the adult Cx26 KO mouse cochlea (Figs. 2–3, also see ref. 14, 17). This inconsistency further supports the concept that cell degeneration is not a primary causer for Cx26 deficiency induced hearing loss. As previously reported [14, 17], we found that Cx26 deficiency can arrest the cochlear tunnel development; the tunnel is not open in the organ of Corti in Cx26 KO mice (Fig. 2). The cochlear tunnel plays a critical role in the cochlear function. It has been found that dominant-negative Cx26 mutation R75W+ transgenic mice have congenital hearing loss [5, 8]. Hair cells have normal development and function [10], but the tunnel is closed.

Cx26 deficiency can also result in EP reduction [3]. EP is a driving force for hair cell transduction process. Reduction of EP can induce hearing loss. We found hearing loss in Cx26 KO mice over whole-frequency region (Fig. 1C&D), which is consistent with characteristics of EP reduction induced hearing loss [15]. CM in Cx26 KO mice is also recordable but reduced (Fig. 4A&B). Taken together, these data indicate that Cx26 deficiency associated hearing loss may primarily result from cochlear development disorder and EP reduction rather than cell degeneration. These new findings also provide an insight on the development of new therapeutic strategies and approaches for protection and treatment of this hereditary hearing loss.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We coincidentally examined cochlear development and hearing loss in Cx26 KO mice.
- Deafness is congenital and occurs at all frequencies before cell degeneration
- Degeneration is only visible in middle-high frequency regions in the adult cochlea
- Cx26 deficiency associated deafness is not resulted from cell degeneration.

Fig. 1.

Congenital hearing loss in Cx26 KO mice. A: ABR waveforms evoked by click and tone-bursts in Cx26 KO and WT mice at P18. WT littermates served as control. B: Postnatal development changes of ABR threshold in Cx26 KO and WT mice. ABR was evoked by click stimulation. C: ABR thresholds evoked by tone-bursts in Cx26 KO and WT mice. D: Postnatal development changes of ABR frequency-thresholds in Cx26 KO and WT mice. ABR thresholds in whole-testing frequency range (8 – 40 kHz) are elevated in Cx26 KO mice during postnatal development.

Fig. 2. No significant cell loss in the Cx26 KO mouse cochlea during postnatal development. A-D: Cross-sections of the Cx26 KO mouse cochlea in the middle turn from P17 to P28. A red arrow in panel D indicates that the cochlear tunnel in Cx26 KO mice is still closed at P28. E: The cross-section of the Cx26 KO mouse cochlea in the middle turn at P60. Arrows indicate complete hair cell and supporting cell loss in the organ of Corti. F: The cross-section of the middle turn of the WT mouse cochlea at P60. An arrow indicates the open cochlear tunnel. Scale bar = 30 μ m.

Fig. 3.

Cx26 KO mice have no significant SG neuron degeneration throughout the postnatal development. **A:** Cross-sections of Rosenthal's canal in the apical, middle, and basal turns in Cx26 KO and WT mice at P17, P28, and adult (P60). Empty arrows indicate severe SG neuron degeneration in the middle and basal turns in the adult Cx26 KO mice. Scale bar = 30 μ m. **B:** Quantitative analysis of SG density in the apical, middle, and basal turns of Cx26 KO and WT mice. A circle indicates that the densities of SG neurons in the middle and basal turns in the adult (P60) Cx26 KO mice have a significant reduction (t-test: $p < 0.01$).

Fig. 4. Hair cell function tests in the Cx26 KO mice. A: CM recorded at P17 by 8 kHz tone-bursts. B: Postnatal changes of CM in Cx26 KO and WT mice. C&D: Patch clamp recording of OHC electromotility associated nonlinear capacitance from Cx26 KO mice at P22. A smooth line in panel D represents Boltzmann fitting.