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Progressive age-dependence and frequency difference in the effect of gap junctions on active cochlear amplification and hearing

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ABSTRACT

Mutations of Connexin 26 (Cx26, *GJB2*), which is a predominant gap junction isoform in the cochlea, can induce high incidence of nonsyndromic hearing loss. We previously found that targeted-deletion of Cx26 in supporting Deiters cells and outer pillar cells in the cochlea can influence outer hair cell (OHC) electromotility and reduce active cochlear amplification leading to hearing loss, even though there are no gap junction connexin expressions in the auditory sensory hair cells. Here, we further report that hearing loss and the reduction of active amplification in the Cx26 targeted-deletion mice are progressive and different at high and low frequency regions, first occurring in the high frequency region and then progressively extending to the middle and low frequency regions with mouse age increased. The speed of hearing loss extending was fast in the basal high frequency region and slow in the apical low frequency region, showing a logarithmic function with mouse age. Before postnatal day 25, there were no significant hearing loss and the reduction of active cochlear amplification in the low frequency region. Hearing loss and the reduction of active cochlear amplification also had frequency difference, severe and large in the high frequency regions. These new data indicate that the effect of gap junction on active cochlear amplification is progressive, but, consistent with our previous report, exists in both high and low frequency regions in adulthood. These new data also suggest that cochlear gap junctions may have an important role in age-related hearing loss.

1. Introduction

In the mammalian cochlea, outer hair cells (OHCs) can be motile under acoustic/electronic stimulation [1]. This OHC electromotility acts as an active cochlear amplifier to burst the basilar membrane vibration increasing hearing sensitivity and frequency selectivity [1,2]. This active cochlear amplification is required for normal hearing in mammalians. Knockout of prestin, which is the motor protein of OHC electromotility, induces hearing loss [3,4].

In vivo, OHCs are surrounded by supporting cells. The cochlear supporting cells are well-coupled by gap junctions [5,6]. Connexin26 (Cx26) and Cx30 are predominant connexin gap junction isoforms in the inner ear [6–10]. Cx26 (*GJB2*) mutations can induce hearing loss and account for more than 50% cases of nonsyndromic hearing loss [6,11–13]. Connexin expression and gap junctions in the cochlea only exist in non-sensory supporting cells but not in sensory hair cells including OHCs [7,9,14–16]. However, our previous studies demonstrated that gap junctional coupling between cochlear supporting cells can influence OHC electromotility and active cochlear amplification [16], indicating that connexin gap junctions in the cochlea play more broad and important functions in hearing. Our recent studies further demonstrated that knockout of Cx26 expression in the cochlear supporting cells can affect OHC electromotility leading to hearing loss [17,18]. Target-deletion of C26 in the cochlear supporting Deiters cells (DCs) and outer pillar cells (OPCs), which constrain OHCs standing on the basilar membrane, can reduce OHC electromotility and active cochlear amplification and cause hearing loss [17]. In this study, we further found that the hearing loss and reduction of active cochlear amplification are progressive, extending from high-frequency region to low-frequency region with mouse age, and also have difference in high and low frequency regions. These new data demonstrate that

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gap junction can regulate active cochlear amplification in apical low-frequency region as well as the basal high-frequency region but the regulation in the high-frequency region are more susceptible and vulnerable to be impaired. Our new findings provide a light on why hearing function in high-frequency range is vulnerable to be compromised with aging and noise stress.

2. Materials and methods

2.1. Cx26 conditional KO mouse generation and genotyping

As described in our previous report [17], *Prox1-CreER$^{T2(+/+)}$;Cx26$^{LoxP(\pm)}$* mice were generated by crossing of *Cx26$^{loxP/loxP}$* transgenic mice (EMMA, EM00245) [19,20] with *Prox1-CreER^{T2}* mouse line (Stock No. 022075, Jackson Laboratory, USA) and used for breeding. Mouse genotyping was identified by PCR amplification of tail genomic DNA. Tamoxifen (T5648, Sigma-Aldrich, St. Louis, MO) was administrated to all litters at postnatal day 0 (P0) by intraperitoneal injection (0.5 mg/10 g × 3 days) [17]. *Prox1-CreER$^{T2(+/+)}$;Cx26$^{LoxP(+/+)}$* mice were used as Cx26-Preox1 conditional knockout (cKO) mice and littermates of *Prox1-CreER$^{T2(+/+)}$;Cx26$^{LoxP(-/-)}$* were used as wild-type (WT) control. All experimental procedures were conducted in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the policies of University of Kentucky Animal Care & Use Committee.

2.2. Auditory brainstem response and distortion product otoacoustic emission measurements

As described in our previous reports [17,18,20], auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) were recorded by use of a Tucker-Davis ABR & DPOAE workstation with ES-1 high frequency speaker (Tucker-Davis Tech. Alachua, FL). ABR was recorded by stimulation with clicks in alternative polarity and tone bursts (4–40 kHz) in a 5 dB step and was averaged by 500 times. The ABR threshold was determined by the lowest level at which an ABR can be recognized. For DPOAE recording, the test frequencies were presented by a geometric mean of f_1 and f_2 [$f_0 = (f_1 \times f_2)^{1/2}$] from f_0 = 4–20 kHz. The distortion product was recorded from the L_1/L_2 level of 60/55 to 25/20 dB SPL with average of 150 times. A cubic distortion component of $2f_1$-f_2 was measured.

2.3. Immunofluorescent staining and confocal microscopy

The cochlear tissue preparation and immunofluorescent staining were performed as previously reported [9,10]. Monoclonal mouse anti-Cx26 (Cat# 33–5800), polyclonal rabbit anti-Cx30 (Cat#71–2200, Invitrogen Corp, Carlsbad, CA), and monoclonal mouse anti-prestin (a kind gift from Dr. Jing Zheng at Northwestern University) were used and visualized by secondary Alexa Fluor® 488 or 568 conjugated antibodies (Molecular Probes). The staining was observed under fluorescence or confocal laser-scanning microscopes.

2.4. Statistical analysis

Data were plotted by SigmaPlot and statistically analyzed by SPSS (SPSS Inc.; Chicago, IL). Error bars represent SEM.

3. Results

3.1. Deletion of Cx26 in the cochlea in Cx26-Prox1 cKO mice

Fig. 1 shows immunofluorescent staining for Cx26 in the cochlea of Cx26-Prox1 cKO mice. Cx26 labeling in DC and OPC area in Cx26 cKO mice was absent (Fig. 1C–F). However, Cx30 expression still remained in this region (Fig. 1E&F). Also, Cx26 deletion had no apparent difference along the cochlear sensory epithelium in whole-mounting preparation (Fig. 1D), indicating that there was no apparent difference in Cx26 deletion in low and high frequency region as demonstrated in our previous report [17].

3.2. Progress and frequency difference of hearing loss in Cx26-Prox1 cKO mice

Cx26-Prox1 cKO mice had hearing loss (Fig. 2) as demonstrated in our previous study [17]. However, hearing loss appears progressively age-dependent and difference in low and high frequencies (Figs. 2 and 3). At postnatal day 15 (P15), ABR thresholds in both Cx26 cKO and WT mice had no significant difference (Fig. 2A). At P25 (Fig. 2B), there was still no difference in the ABR thresholds between Cx26 cKO and WT mice at middle and low frequencies. However, at 40 kHz high-frequency, ABR threshold in Cx26 cKO mice was significantly increased to 64.5 ± 2.41 dB SPL, increasing about 21.7 dB SPL in comparison with 42.8 ± 3.32 dB SPL in WT mice (Fig. 2B). Then, hearing loss progressively extended to middle and low frequency regions. At P35, ABR threshold at 24 kHz in Cx26 cKO mice was significantly increased to 46.3 ± 3.37 dB SPL (Fig. 2C) and at P45 the ABR threshold at 16 kHz was significantly increased to 43.5 ± 1.30 dB SPL (Fig. 2D). Finally, at P60, the ABR thresholds in Cx26 cKO mice were significantly increased for 8 kHz tone and click stimulation (Fig. 2E).

The speed of hearing loss extending from high frequency to low frequency in Cx26 cKO mice was fast in the high frequency region and slow in the low frequency region (Fig. 3B). The first time of hearing loss occurrence at frequency could be described by a common logarithmic function of frequency with age: log y (kHz) = 2.087–0.0198 * x (day) (Fig. 3B). The estimated time of hearing loss occurring at 60 kHz, which is at the highest frequency region in mouse hearing, is expected at ~ P15.5, just after mouse hearing starting (P13-14) [20].

The increase of ABR threshold in Cx26 cKO mice is also different in low and high-frequency regions (Figs. 2 and 3A). In the frequency region >24 kHz, ABR threshold was increased largely (Fig. 3A). At P60, ABR threshold increase in >24 kHz frequency regions was about 40 dB SPL, whereas the increase of ABR threshold at <16 kHz frequency region was only about 10 dB SPL (Fig. 3A).

The degree of hearing loss was also progressively increased with mouse age (Fig. 3C). For example, at 24 kHz, ABR threshold was significantly increased by ~10, 20, and 40 dB SPL at P35, P45, and P60, respectively. At 16 kHz, the increase in ABR threshold at P60 in Cx26 cKO mice was 10.1 ± 3.77 dB SPL (Fig. 3C). However, the increase of ABR threshold at P80 was increased to 34.4 ± 7.15 dB SPL (Fig. 3C). The maximum of ABR threshold increase in Cx26 cKO mice was about 40 dB SPL (Figs. 2 and 3).

3.3. Progressive reduction of active cochlear amplification in Cx26 cKO mice

As mentioned above, mammalian hearing relies upon active cochlear amplification. Deficiency of active cochlear amplification can induce hearing loss. Fig. 4 shows that active cochlear amplification as measured by DPOAE in Cx26 cKO mice was reduced. The reduction was also progressive. Before P25, there was no any

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