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CONNEXIN26 (*GJB2*) DEFICIENCY REDUCES ACTIVE COCHLEAR AMPLIFICATION LEADING TO LATE-ONSET HEARING LOSS

Y. ZHU,[†] J. CHEN,[†] C. LIANG,[†] L. ZONG, J. CHEN, R. O. JONES AND H.-B. ZHAO[*]

Dept. of Otolaryngology, University of Kentucky Medical School, Lexington, KY 40536, United States

Abstract—Connexin26 (Cx26, *GJB2*) mutations account for >50% of nonsyndromic hearing loss. The deafness is not always congenital. A large group of these patients (~30%) demonstrate a late-onset hearing loss, starting in childhood. They have normal hearing early in life and are therefore good candidates for applying protective and therapeutic interventions. However, the underlying deafness mechanism is unclear. In this study, we used a time-controlled, inducible gene knockout technique to knockout Cx26 expression in the cochlea after birth. We found that deletion of Cx26 after postnatal day 5 (P5) in mice could lead to late-onset hearing loss. Similar to clinical observations, the mice demonstrated progressive, mild to moderate hearing loss. The hearing loss initiated at high frequencies and then extended to the middle- and low-frequency range. The cochlea showed normal development and had no apparent hair cell loss. However, distortion product otoacoustic emission (DPOAE) was reduced. The reduction was also progressive and large at high-frequencies. Consistent with DPOAE reduction, we found that outer hair cell electromotility-associated nonlinear capacitance was shifted to the right and the slope of voltage dependence was reduced. The endocochlear potential was reduced in Cx26 conditional knockout (cKO) mice but the reduction was not associated with progressive hearing loss. These data suggest that Cx26 deficiency may impair active cochlear amplification leading to late-onset hearing loss. Our study also helps develop newer protective and therapeutic interventions to this common nonsyndromic hearing loss.

Key words: gap junction, connexin, Cx26, deafness, inner ear, active cochlear amplification.

INTRODUCTION

Connexin26 (Cx26, *GJB2*) mutations are a common genetic cause for nonsyndromic hearing loss, responsible for >50% of nonsyndromic hearing loss in children. Clinically, the majority of Cx26 deafness mutations are recessive but a few are dominant, resulting in DFNB1 (recessive) and DFNA3 (dominant) nonsyndromic hearing loss (Zhao et al., 2006; Castillo and Castillo, 2011; Chan and Chang, 2014). The hearing loss, especially caused by p.M34T and p.V37I mutations, is not always congenital. A significant minority of these patients (~30%) demonstrate a progressive, late-onset hearing loss. The hearing loss starts or occurs in childhood, is mild to moderate, and usually appears severe at high-frequencies (Cohn et al., 1999; Murgia et al., 1999; Wilcox et al., 2000; Kenneson et al., 2002; Engel-Yeger et al., 2003; Pollak et al., 2007; Orzan and Murgia, 2007; Gopalarao et al., 2008; Kenna et al., 2010; Chan et al., 2010). These individuals have normal hearing early in life and are good candidates for applying protective and therapeutic interventions. However, little is known about the underlying deafness mechanism.

Previous studies show that knockout (KO) of Cx26 in the cochlea can cause congenital deafness with cochlear development disorders, cell degeneration, and endocochlear potential (EP) reduction (Cohen-Salmon et al., 2002; Sun et al., 2009; Wang et al., 2009; Liang et al., 2012; Chen et al., 2014). We further found that hair cell degeneration and EP reduction are not primary causes for congenital deafness; the congenital deafness is associated with cochlear development disorders (Liang et al., 2012; Chen et al., 2014). These studies provide invaluable information about Cx26 mutation-induced congenital hearing loss.

Recently, we also found that Cx26 deficiency can reduce active cochlear amplification (Zhu et al., 2013), even though there is no gap junction and connexin expression in hair cells (Kikuchi et al., 1995; Zhao and Santos-Sacchi, 1999; Zhao, 2000; Zhao and Yu, 2006; Yu and Zhao, 2009). Active cochlear amplification is required for normal mammalian hearing to amplify acoustic stimulation increasing hearing sensitivity and frequency selectivity (Dallos, 2008; Hudspeth, 2008). In this study, we used a time-controlled, inducible gene-knockout technique to delete Cx26 expression in the cochlea after birth. We found that Cx26 deficiency could reduce active cochlear amplification leading to late-onset, progressive hearing loss.

*Correspondence to: H.-B. Zhao, Dept. of Otolaryngology, University of Kentucky Medical Center, 800 Rose Street, Lexington, KY 40536-0293, USA. Tel: +1-859-257-5097x82138; fax: +1-859-257-5096.
E-mail address: hzhao2@uky.edu (H.-B. Zhao).
[†] Equal contributors.

Abbreviations: 4-HTMX, 4-hydroxytamoxifen; ABR, auditory brainstem response; cKO, conditional knockout; DPOAE, distortion product otoacoustic emission; EP, endocochlear potential; KO, knockout; NLC, nonlinear capacitance; OHC, outer hair cells.

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EXPERIMENTAL PROCEDURES

Cx26 conditional KO mice and genotyping

As we previously reported (Chen et al., 2014), *Cx26*$^{loxP/loxP}$ transgenic mice (European Mouse Mutant Archive, E00245) were crossed with the *ROSA26*$^{Cre/Esr1}$ mouse line (Jackson Lab, stock No. 004847), which has a tamoxifen-inducible Cre-mediated recombination system driven by *Gt(ROSA)26Sor* promoter and can be excised by tamoxifen. The *ROSA26*^{CreEsr1} transgene and the Cx26 floxed allele were detected on tail genomic DNA by PCR amplification using the following primers: CreF: 5′-GCG GTC TGG CAG TAA AAA CTA TC-3′ and CreR: 5′-GTG AAA CAG CAT TGC TGT CAC TT-3′ for the *CreEsr1* transgene; Cx26F: 5′-CTT TCC AAT GCT GGT GGAGTG-3′ and Cx26R: 5′-ACA GAA ATG TGT TGG TGA TGG-3′ for the Cx26 floxed allele (Cohen-Salmon et al., 2002). *Cx26*$^{loxP/loxP}$ and WT mice generated 400 and 300 bps bands, respectively. The bands of ROSA26-Esr1/Cre positive and WT mice were located at 102 and 400 bps, respectively.

Mice were intraperitoneally injected with 4-hydroxytamoxifen (4-HTMX, H7904, Sigma–Aldrich, St. Louis, MO) with 0.5 mg/10 g per day for three days to activate Cre expression to delete Cx26. The injection was performed at postnatal day 10 (P10), unless otherwise indicated in text. This injection procedure ensured that Cx26 expression in the cochlea could be deleted but the mice have no congenital hearing loss (Chen et al., 2014). All experimental procedures were conducted in accordance with the policies of University of the Kentucky Animal Care & Use Committee.

Auditory brainstem response and distortion product otoacoustic emission measurements

Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) were recorded in a double-wall sound isolated chamber by use of a Tucker-Davis ABR workstation (Tucker-Davis Tech., Alachua, FL). As we previously reported (Liang et al., 2012; Zhu et al., 2013; Chen et al., 2014), mice were anesthetized by intraperitoneal injection with a mixture of Ketamine and Xylazine (a stock solution: 8.5-ml saline + 1-ml Ketamine + 0.55-ml Xylazine) given at a dose of 0.1 ml/10 g body weight. Body temperature was maintained at 37–38 °C by placing anesthetized mice on an isothermal pad (Deltaphase, model 39dp, Braintree Scientific Inc., Massachusetts). Subdermal needle electrodes were inserted at the vortex (an active electrode) and ventrolaterally to the right ear (a reference electrode) and to the left ear (a ground electrode). ABR was measured by both clicks and series of tone bursts (4–40 kHz, 10–80 dB SPL, a 5 dB-step). 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 70–110 dB SPL was used.

DPOAE was recorded as described by our previous reports (Zhu et al., 2013). Two plastic tubes were inserted into the external ear canal and sealed with an earplug. Two pure tones (f_1 and f_2) were simultaneously delivered into the ear. The ratio of f_2 versus f_1 (f_2/f_1) was 1.22. 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 to 20 kHz. The intensity of f_1 (L_1) was set at 5 dB SPL higher than that of f_2 (L_2). The distortion product was recorded from the L_1/L_2 level of 25/20 –65/60 dB SPL. One hundred fifty responses were averaged. A cubic distortion component of $2f_1–f_2$ in DPOAEs was measured.

Immunofluorescent staining

The cochlear tissue preparation and immunofluorescent staining were performed as we previously reported (Zhao and Yu, 2006; Liu and Zhao, 2008). The cochlear cross-section or isolated sensory epithelia were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 30 min. After being washed with PBS (0.1 M) 3 times, the tissue was incubated in a blocking solution (10% goat serum and 1% BSA in the PBS) with 0.1% Triton X-100 for 30 min at room temperature. The tissue then was incubated with primary antibody in the blocking solution at 4 °C overnight. Monoclonal mouse anti-Cx26 (Cat# 33-5800), and polyclonal rabbit anti-Cx30 (Cat# 71-2200, Invitrogen Corp, Carlsbad, CA) were used. After completely washing out the primary antibodies with PBS, the reaction to a 1:600 dilution of secondary Alexa Fluor® 488- or 568-conjugated antibodies (Molecular Probes) in the blocking solution followed at room temperature for 1 h. After completely washing out, the section was mounted with a fluorescence mounting medium (H-1000, Vector Lab, CA) and observed under a Leica confocal microscope (Leica TCS SP2).

For quantitative analysis of Cx30 expression in the cochlea, immunofluorescent staining of the cochlear sections of WT mice and Cx26 cKO mice were performed in parallel at the same time and observed under the same confocal settings as we previously reported (Zhao and Yu, 2006). Serial sections of the confocal image were taken along the *Z*-axis. The staining intensity of Cx30 was measured by use of ImageJ software (NIH, Bethesda, USA) (Zhao and Yu, 2006; Yu et al., 2008).

Patch-clamp recording and nonlinear capacitance measurement

Outer hair cells (OHCs) were freshly isolated from the cochlea (Yu and Zhao, 2008; Zhu et al., 2013). The classical patch-clamp recording was performed under the whole-cell configuration by using an Axopatch 200B patch-clamp amplifier (Molecular Devices, CA) with jClamp (Scisft, New Haven, CT) (Yu and Zhao, 2008). The patch pipette was filled with an intracellular ionic blocking solution (140 CsCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES in mM; 310 mOsm and pH 7.2) and had initial resistance of 2.5–3.5 MΩ in bath solution (142 NaCl, 5.37 KCl, 1.47 $MgCl_2$, 2 $CaCl_2$, 10 HEPES in mM, 310 mOsm and pH 7.2). The OHC electromotility-associated nonlinear capacitance (NLC) was measured by a two-sinusoidal method (Santos-Sacchi et al., 1998; Yu and Zhao, 2008). The signal was filtered by a 4-pole low-pass Bessel filter with a cut-off frequency of 10 kHz