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## Connexin26 (*GJB2*) deficiency reduces active cochlear amplification leading to late-onset hearing loss

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### 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.

### Keywords

gap junction; connexin; Cx26; deafness; inner ear; active cochlear amplification

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## 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 but not about Cx26 deficiency induced late-onset 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.

## Materials and Methods

### Cx26 conditional KO mice and genotyping

As we previously reported (Chen et al. 2014), *Cx26<sup>loxP/loxP</sup>* transgenic mice (European Mouse Mutant Archive, E00245) were crossed with the *ROSA26<sup>Cre/Esr1</sup>* 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<sup>Cre/Esr1</sup>* 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<sup>loxP/loxP</sup>* 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/10g 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 vertex (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 5dB-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<sub>2</sub>, 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<sub>2</sub>, 2 CaCl<sub>2</sub>, 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 and digitized utilizing a Digidata 1322A (Molecular Devices, CA). The capacitance was calculated by admittance analysis of the current response. All experimental procedures were performed at room temperature (23 °C).

The voltage-dependent NLC was fitted to the first derivative of a two-state Boltzmann function with jClamp and MATLAB (Yu and Zhao, 2008, 2009):

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

where *NLC* is the nonlinear capacitance component associated with OHC electromotility, *Q<sub>max</sub>* is the maximum charge transferred, *V<sub>pk</sub>* is the voltage at the peak of NLC, and *α* is the slope of voltage dependence. Membrane potential (*V<sub>m</sub>*) was corrected for electrode access resistance (*R<sub>s</sub>*).

### Data processing and statistical analysis

Data were plotted by SigmaPlot and statistically analyzed by SPSS v18.0 (SPSS Inc. Chicago, IL). Error bars represent s.e.m. and data were expressed as mean ± s.e.m. other than indicated in text. Data were plotted by SigmaPlot software (SPSS Inc., Chicago).

## Results

### Late-onset, progressive hearing loss in Cx26 conditional KO mice

Fig. 1 shows that deletion of Cx26 expression in the cochlea at P10 could cause late-onset, progressive hearing loss. ABR recording shows that Cx26 conditional KO (cKO) mice had normal hearing after maturation. The ABR threshold was at the same level as WT mice until P30 (Fig. 1a and Fig. 1b indicated by a green circle). Then, the ABR threshold in Cx26 cKO mice showed a progressive increase (Fig. 1b). At P45–60, the ABR threshold significantly increased to 45–50 dB SPL ( $P < 0.001$ , one-way ANOVA with a Bonferroni correction). At P90–110, the ABR threshold increased to  $> 70$  dB SPL. In comparison with WT mice, the ABR thresholds in Cx26 cKO mice at P90–110 were significantly increased by 30–40 dB SPL ( $P < 0.001$ , one-way ANOVA with a Bonferroni correction). However, the ABR threshold of WT littermates in the whole recording period was not substantially increased (Fig. 1b).

Hearing loss in Cx26 cKO mice appeared severe at high-frequencies (Fig. 2). At P60, the ABR thresholds in Cx26 cKO mice at 8, 16, 24, 32, and 40 kHz were  $66.3 \pm 2.25$ ,  $59.2 \pm 3.25$ ,  $74.6 \pm 4.14$ ,  $88.3 \pm 3.75$ , and  $92.7 \pm 2.47$  dB SPL, respectively (Fig. 2a). The ABR threshold demonstrated a large increase at high-frequencies. The hearing loss also extended to middle and low frequencies (Fig. 2b). At P45, the ABR threshold at 40 kHz was increased by  $33.3 \pm 2.55$  dB SPL and progressively increased by  $44.9 \pm 2.47$  dB at P60 (Fig. 2b). However, the ABR threshold at 8 kHz at P45 had no significant increase (Fig. 2b). At P60, the ABR thresholds at 8 and 16 kHz were increased by  $19.7 \pm 2.25$  and  $18.7 \pm 3.25$  dB SPL, respectively. The increments were significant ( $P < 0.001$ , one-way ANOVA with a Bonferroni correction).

### Deletion of Cx26 in the cochlea in Cx26 cKO mice

We examined Cx26 deletion in the cochlea in Cx26 cKO mice. Fig. 3 shows that Cx26 labeling was almost completely absent in the cochlea in Cx26 cKO mice following injection of 4-HTMX at P10 (Fig. 3). However, there were no detectable developmental disorders in Cx26 cKO mice. The cochlear tunnel in Cx26 cKO mice was open (Fig. 3a). In addition, co-expressed Cx30 in Cx26 cKO mice appeared normal in immunofluorescent staining (Fig. 3c&f). Quantitative analyses showed that there was no significant difference in Cx30 staining between WT and Cx26 cKO mice (Fig. 4).

### Reduction of DPOAE in Cx26 cKO mice

Acoustic emission reflects the activation of active amplification in the cochlea. Fig. 5 shows that Cx26 cKO mice had a significant reduction in DPOAE (Fig. 5a&b). The reduction was large at high intensity and could be up to 40 dB (Fig. 5b). The reduction of DPOAE in Cx26 cKO mice also appeared as late-onset and was progressive. Fig. 5c shows that the DPOAE in the Cx26 cKO mice was normal after maturation. At P30, there was no significant difference in DPOAE measurements between Cx26 cKO mice and WT mice. Then, the DPOAE in the Cx26 cKO mice was reduced. At P90–110, the DPOAE in Cx26 cKO mice was reduced to noise level and was almost undetectable. The reduction of DPOAE also demonstrated a frequency-dependence and first occurred at higher frequencies (Fig. 5d). The

decrease was large at higher frequencies and the reduction was progressively increased. DPOAEs at 16 kHz and 20 kHz in Cx26 cKO mice were  $21.8 \pm 5.29$  and  $16.7 \pm 3.56$  dB SPL, respectively, at P60, and  $14.9 \pm 2.09$  and  $4.71 \pm 1.39$  dB SPL, respectively, at P90 (Fig. 5d). After P90, the DPOAE was almost reduced to zero (Fig. 5c&d). The DPOAE in Cx26 cKO mice at P110 was  $2.22 \pm 1.17$  dB SPL at 20 kHz.

### No substantial hair cell loss in Cx26 cKO mice

However, there was no substantial hair cell loss in Cx26 cKO mice (Fig. 6). Hair cell loss at P90 in the Cx26 cKO mice was similar to that in WT mice. Only at the very high frequency region in the basal turn, hair cell loss in both WT and Cx26 cKO mice was visible but less than 10% (Fig. 6). Hair cells in Cx26 cKO mice also displayed normal shape; no apparent destruction of stereocilia is visible (Inset in Fig. 6).

### EP reduction in Cx26 cKO mice

EP is a driving force required for hair cells generating auditory receptor current and potential. EP reduction can induce hearing loss (Chen and Zhao, 2014). Fig. 7 shows that EP in Cx26 cKO mice was reduced but the reduction was not associated with progressive hearing loss. The recorded EP in WT and Cx26 cKO mice at P35 was  $99.6 \pm 1.57$  and  $52.3 \pm 4.77$  mV, respectively. The EP in Cx26 cKO mice was significantly reduced by 47 mV ( $P < 0.001$ , t-test). At P80, the EP in Cx26 cKO mice was  $48.6 \pm 5.27$  mV, similar to that at P35 (Fig. 7a). There was no significant difference in EP reduction between P35 and P80 ( $P = 0.31$ , t-test). However, in contrast to the non-significant changes in EP, ABR threshold was significantly increased from  $34.8 \pm 0.69$  dB SPL at P35 to  $66.1 \pm 6.40$  dB SPL at P80 ( $P < 0.001$ , t-test, Fig. 7b).

### Shift of outer hair cell NLC in Cx26 cKO mice

We further examined OHC electromotility, which serves as an active cochlear amplifier (Brownell et al., 1985; Ashmore, 2008), by NLC measurement. In Cx26 cKO mice, NLC retained a normal bell-shape but was shifted to the right in the depolarization direction (Fig. 8a). The voltage of peak capacitance ( $V_{pk}$ ) was significantly shifted from  $-100.5 \pm 3.11$  mV in WT mice to  $-66.7 \pm 2.28$  mV in Cx26 cKO mice ( $P < 0.001$ , t-test) (Fig. 8b). The slope ( $\alpha$ ) of voltage dependence was also significantly reduced from  $35.5 \pm 0.9$  mV in WT mice to  $30.1 \pm 0.7$  mV in Cx26 cKO mice (Fig. 8c,  $P < 0.001$ , t-test). However, there was no reduction in the maximum charge ( $Q_{max}$ ), which reflects the functional expression of prestin on the plasma membrane (Ashmore 2008), in Cx26 cKO mice (Fig. 8d).  $Q_{max}$  in WT and Cx26 cKO mice was  $0.655 \pm 0.026$  and  $0.686 \pm 0.013$  pC, respectively ( $P = 0.15$ , t-test). Moreover, there was no significant difference in linear capacitance ( $C_{lin}$ ) of recorded OHCs between WT and Cx26 cKO groups (Fig. 8e). The recorded  $C_{lin}$  in WT and Cx26 cKO mice was  $5.34 \pm 0.17$  and  $5.55 \pm 0.19$  pF, respectively ( $P = 0.21$ , t-test), indicating that the recorded OHCs in both WT and Cx26 cKO groups had similar cell length.

### Hearing loss following deletion of Cx26 at different postnatal developmental stages

We further tested the effect of Cx26 deletion time on hearing function (Fig. 9). As we previously reported (Chen et al., 2014), mice showed normal hearing at young ages



following deletion of Cx26 after P5 (Fig. 9a). At P30, ABR thresholds were  $68.1 \pm 10.9$ ,  $34.2 \pm 3.27$ ,  $34.8 \pm 0.69$ ,  $27.5 \pm 1.44$ , and  $30.0 \pm 1.89$  dB SPL following deletion of Cx26 at P5, P8, P10, P15, and P20, respectively. In comparison with WT mice, there were no significant increments in ABR thresholds except that at P5. However, at P60, the ABR thresholds were  $75.0 \pm 8.66$ ,  $51.7 \pm 3.90$ , and  $36.3 \pm 2.26$  dB SPL with deletion of Cx26 at P5, 10, and 20, respectively (Fig. 9b). Hearing loss was apparent in Cx26 cKO mice, with earlier deletion of Cx26 in the cochlea producing more severe hearing loss.

## Discussion

In this study, we used an inducible gene knockout technique to delete Cx26 expression in the cochlea after birth. In mouse postnatal development, the cochlea shows normal morphology and the cochlear tunnel starts to open at P5. By P10, the EP and  $K^+$  concentration in the endolymph arise to high levels (Ehret, 1976; Kraus and Aulbach-Kraus, 1981). Hearing starts around P14 and matures at P19–20 (Liang et al., 2012). We found that the mice retained normal hearing before P30 following deletion of Cx26 after P5 (Figs. 1, 2, 9). Then, hearing loss became apparent, severe at high-frequencies (Figs. 1, 2). This progression of hearing loss is similar to late-onset hearing loss observed in Cx26 mutation-induced DFNA3 and DFNB1 nonsyndromic deafness patients, who have normal hearing in early life; the hearing loss starts in childhood (Pollak et al., 2007; Orzan and Murgia, 2007; Gopal Rao et al., 2008). Moreover, consistent with DPOAE reduction observed in patients (Engel-Yeger et al., 2002, 2003; Santarelli et al., 2007), the mouse model also demonstrated a progressive DPOAE reduction, severe at high frequencies (Fig. 5). These data indicate that late-onset hearing loss induced by Cx26 deficiency may result from impairment in active cochlear amplification.

Normal mammalian hearing relies upon active cochlear amplification to amplify acoustic stimulation increasing hearing sensitivity and frequency selectivity (Dallos, 2008; Hudspeth, 2008). Defects of active cochlear amplification can cause up to 40 dB SPL hearing loss (Ashmore 2008; Dallos, 2008), which we found in the experiments (Figs. 1, 2, 9). The hearing loss in Cx26 cKO mice was mild to moderate. The ABR threshold was increased up to 40 dB (Figs. 1, 2, 9). Moreover, we found that OHC NLC in Cx26 cKO mice was shifted to the right in the depolarization direction and the slope of voltage dependence was reduced (Fig. 8). OHC electromotility serves as a major basis of active cochlear amplifier in mammals (Brownell et al., 1985; Zheng et al., 2000; Ashmore 2008). Our previous studies demonstrated that Cx26 deficiency can affect OHC electromotility to reduce active amplification in the cochlea leading to hearing loss (Yu and Zhao, 2009; Zhu et al., 2013), even though hair cells have neither gap junctional coupling nor connexin expression (Kikuchi et al., 1995; Zhao and Santos-Sacchi, 1999; Zhao, 2000; Zhao and Yu, 2006; Yu and Zhao 2009). As we previously mentioned (Yu and Zhao, 2009; Zhu et al., 2013), deletion of Cx26 may alter cytoskeleton formation and tension in OHC supporting cells to change OHC loading influencing OHC electromotility. This may also change the phase difference between OHC electromotility and active force generation (Gummer et al., 1996; Fridberger et al., 1998; Zha et al., 2012) and hair bundle-based cochlear amplification (Chan and Hudspeth, 2005; Kennedy et al., 2005).

In the experiment, we found that deletion of Cx26 in the cochlea after birth shifted OHC NLC to the right (Fig. 8) and reduced DPOAE (Fig. 5). This is consistent with a previous report in which right-shift of OHC electromotility could reduce active cochlear amplification (Dallos et al., 2008). However, we previously reported that targeted-deletion of Cx26 in Deiters cells and outer pillar cells shifted OHC electromotility to the left (Zhu et al., 2013). There are several differences between these two mouse models. First, they have different genetic backgrounds. In both studies, we used WT littermates as control groups. The  $V_{pk}$  values in both WT control groups are different. In the current study,  $V_{pk}$  in WT mice was  $-100.5 \pm 3.11$  mV (Fig. 8b) whereas the  $V_{pk}$  in WT mice in our previous study was  $-73.8 \pm 2.14$  mV (Zhu et al., 2013). Second, EP was reduced in the current Cx26 cKO mice (Fig. 7) whereas EP was normal with targeted-deletion of Cx26 in DCs and OPCs (Zhu et al., 2013). Finally, time of deletion of Cx26 is different. In the current study, we deleted Cx26 at P10, when the cochlear postnatal development is almost completed. In our previous study, we deleted Cx26 at P0, when the postnatal development is just starting. It has been reported that both left and right shift of OHC electromotility can reduce OHC electromotility amplification and active force generation (Fridberger et al., 1998; Rajagopalan et al., 2007). Indeed, both mouse models had DPOAE reduction (Fig. 5 and also see Fig. 3 in Zhu et al., 2013).

We also found that EP was significantly reduced in Cx26 cKO mice (Fig. 7). However, the EP reduction was not associated with progressive hearing loss. The EP in Cx26 cKO mice at P35 and P80 was  $52.3 \pm 4.77$  and  $48.6 \pm 5.27$  mV, respectively. There was no significant change in EP between P35 and P80 (Fig. 7a). However, the ABR threshold significantly increased from  $34.8 \pm 0.69$  dB SPL at P35 to  $66.1 \pm 6.40$  dB SPL at P80, showing apparent hearing loss (Fig. 7b). Thus, the EP reduction is not a determining factor in Cx26 deficiency induced late-onset hearing loss. In addition, the Cx26 cKO mice had normal cochlear development and had no substantial hair cell loss (Figs. 3, 6), indicating that late-onset hearing loss is not a result of cell degeneration and developmental disorders.

Cx26 mutations can induce a high incidence of nonsyndromic hearing loss (Zhao et al. 2006; Castillo and Castillo, 2011). However, the underlying deafness mechanism(s) is still unclear. Several deafness mechanisms, such as  $K^+$ -recycling and  $Ca^{++}$ -wave propagation hypotheses, have been proposed (Kikuchi et al., 1995; Zhao et al. 2006; Beltramello et al., 2005). It has been hypothesized that Cx26 mutations may impair  $K^+$ -recycling from hair cell's extracellular space to endolymph leading to  $K^+$  accumulation, thereby damaging hair cells and causing hearing loss (Kikuchi et al., 1995; Zhao et al. 2006). It also has been hypothesized that Cx26 deficiency may impair  $Ca^{++}$ -wave propagation inducing hearing loss (Beltramello et al., 2005), because Cx26 deafness mutant p.R75W can impair the spread of  $Ca^{++}$ -waves between cells (Beltramello et al., 2005; Yum et al., 2010; Zhang et al., 2011; Kamiya et al., 2014). However, so far, these hypotheses still lack direct evidence as to whether Cx26 deficiency impairs  $K^+$ -recycling in the cochlea, and how such  $Ca^{++}$ -wave propagation is related to hearing. In this study, we found that after deletion of Cx26 in the cochlea (Fig. 3), mice still retained normal hearing at young ages (Figs. 1, 2, 9). These data imply that Cx26 deficiency may not impair  $K^+$ -recycling and  $Ca^{++}$ -wave propagation in the cochlea (which could result from possible compensations), or that impaired  $K^+$ -recycling



and  $\text{Ca}^{++}$ -wave propagation do not lead to hearing loss (if Cx26 deficiency can in fact impair  $\text{K}^{+}$ -recycling and  $\text{Ca}^{++}$ -wave propagation in the cochlea). In any case, both  $\text{K}^{+}$ -recycling and  $\text{Ca}^{++}$ -wave impairments cannot be the principle deafness mechanisms underlying Cx26 deficiency induced hearing loss.

Cx26 mutations can induce congenital deafness and late-onset hearing loss (Castillo and Castillo, 2011; Chan and Chang, 2014). We recently found that congenital deafness induced by Cx26 deficiency is associated with cochlear developmental disorders rather than hair cell loss and EP reduction (Liang et al., 2012; Chen et al., 2014). In this study, we further found that that Cx26 deficiency induced late-onset hearing loss may result from the reduction of active cochlear amplification rather than possible impairment in  $\text{K}^{+}$ -recycling and  $\text{Ca}^{++}$ -wave propagation (Figs. 1, 2, 5, 8). Thus, these studies provide a full image for deafness mechanisms underlying Cx26 mutation induced hearing loss and also provide important information for developing new protective and therapeutic strategies for this common deafness, which clinically accounts for more than 50% of nonsyndromic hearing loss.

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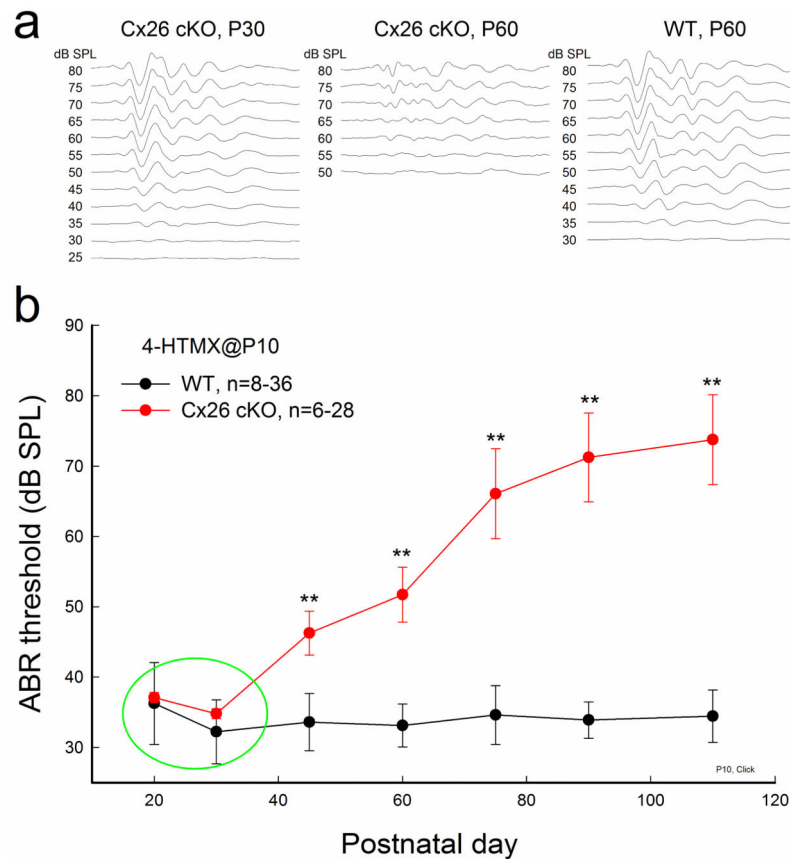
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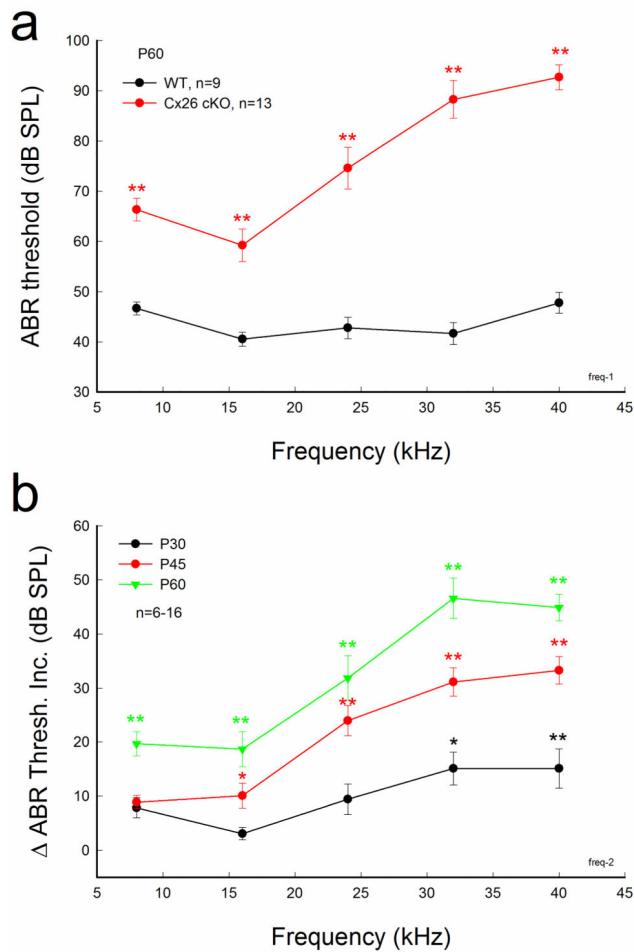
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### Highlights

- Cx26 deletion after birth can lead to late-onset, progressive hearing loss
- The deletion leads to hearing loss by the reduction of active cochlear amplification
- The deletion shifts outer hair cell NLC to reduce active amplification
- This study provides a new deafness mechanism due to Cx26 deficiency

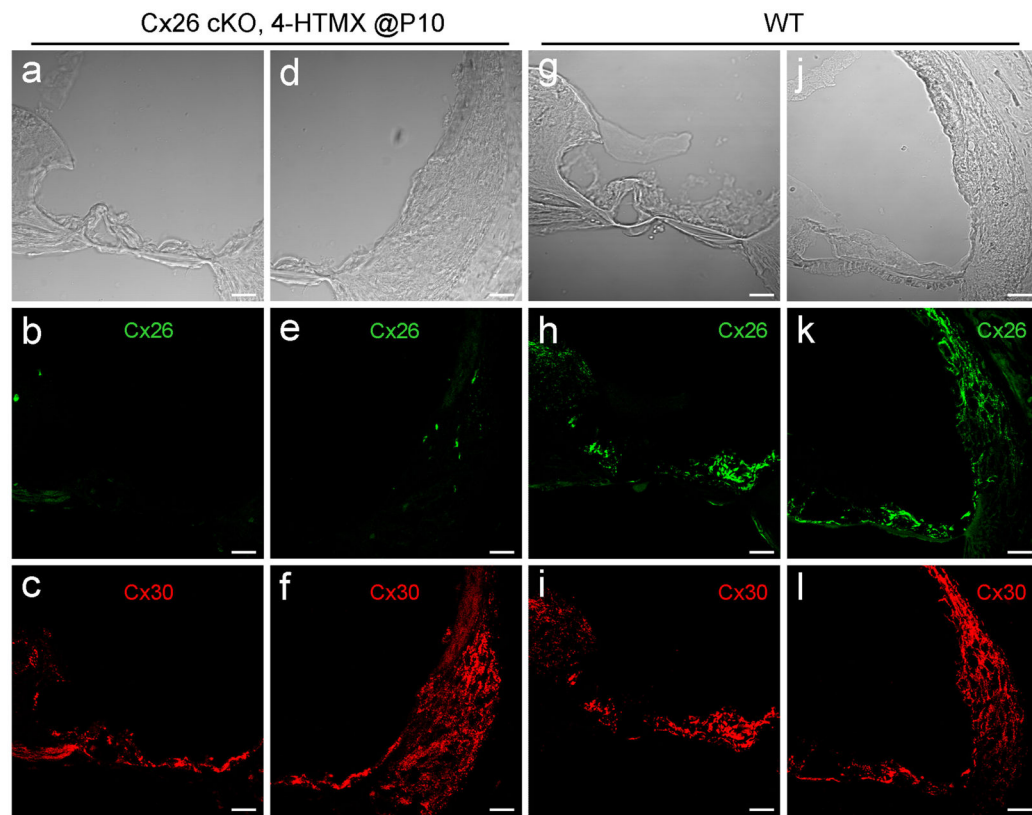


**Fig. 1.** Late-onset, progressive hearing loss in Cx26 cKO mice. The mice were injected with 4-HTMX at P10. ABR was evoked by click stimulations. WT littermates with 4-HTMX injection served as controls. **a:** ABR waveforms recorded from Cx26 cKO and WT mice. The Cx26 cKO mice retain normal hearing sensitivity at P30 (left panel) but have hearing loss at P60 (middle panel), demonstrating a late-onset hearing loss. Right panel: ABR in WT mice at the same age. **b:** Hearing loss in Cx26 cKO mice is late-onset and progressive. A green circle indicates that ABR thresholds at P20 and P30 in Cx26 cKO mice are not significantly different from WT mice. Data are expressed as mean  $\pm$  S.D; \*\*:  $P < 0.001$  as determined by one-way ANOVA with a Bonferroni correction.

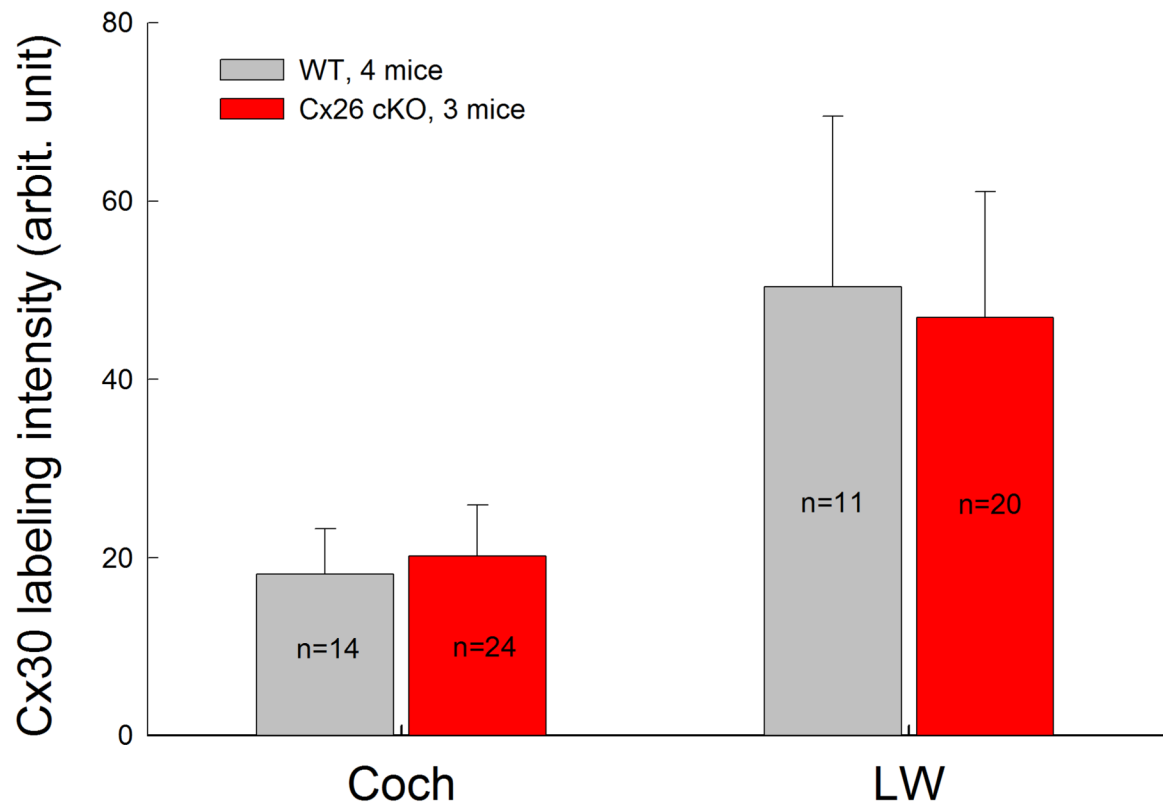


**Fig. 2.** Severe hearing loss in high-frequency range in Cx26 cKO mice. The mice were injected with 4-HTMX at P10. **a:** Frequency tone burst-evoked ABR thresholds at P60. The ABR thresholds were further elevated at high frequencies. **b:** Progressive increase in ABR threshold in Cx26 cKO mice. The increments were obtained from comparison with ABR thresholds of WT littermate mice at the same time point. The increments are apparent at high-frequencies. Data are expressed as mean  $\pm$  S.D; \*\*:  $P < 0.001$  as determined by one-way ANOVA with a Bonferroni correction.



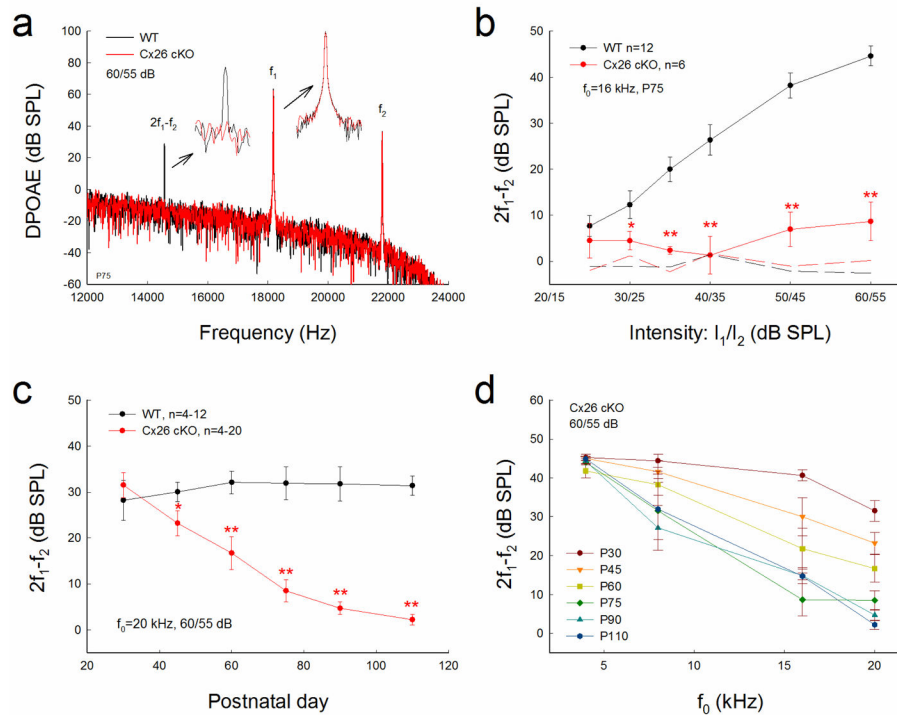


**Fig. 3.** Deletion of Cx26 in the cochlea in Cx26 cKO mice. **a–f:** Immunofluorescent staining of the cochlea for Cx26 (green) and Cx30 (red) in Cx26 cKO mice. The mouse was P30 old and 4-HTMX was injected at P10 for Cx26 deletion. **g–l:** Immunofluorescent staining of the cochlear cross-section for Cx26 and Cx30 in WT mice. Scale bars: 25  $\mu$ m.

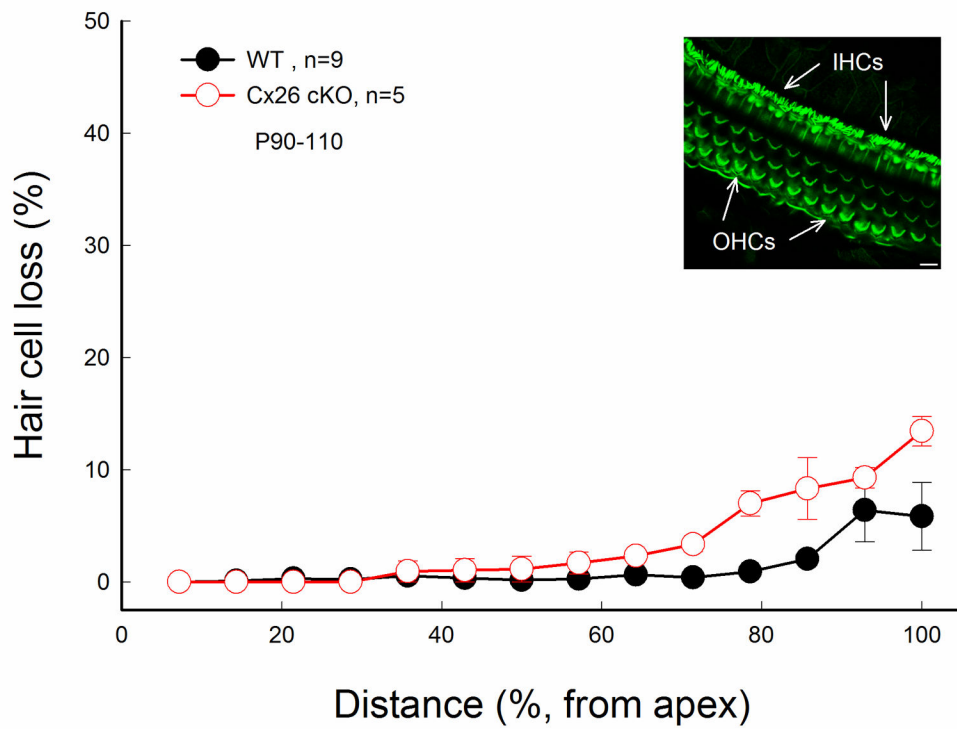


**Fig. 4.**

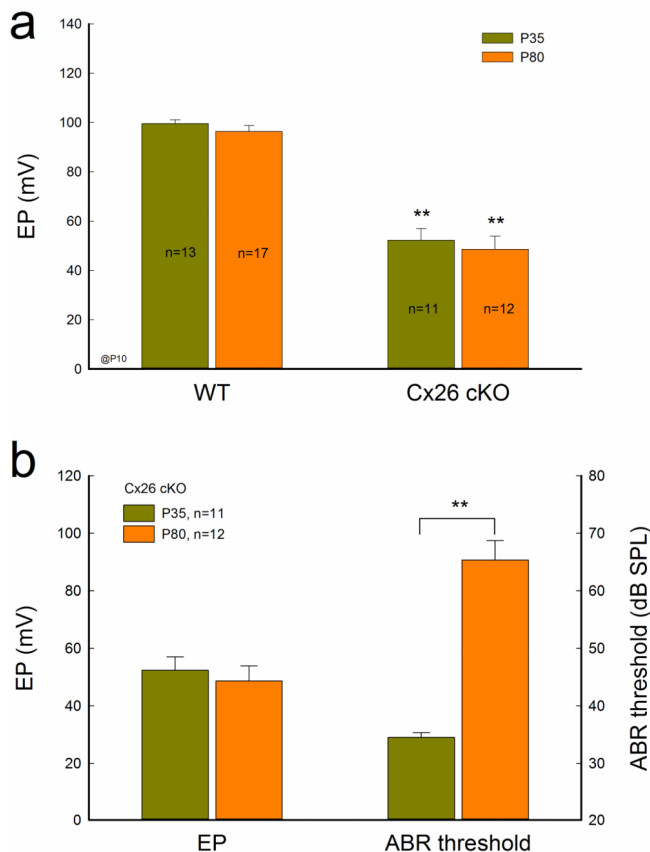
Quantitative analysis of Cx30 expression in the cochlea and lateral wall (LW) in the Cx26 cKO mice. The section was serially scanned along the Z-axis, and then the intensity of Cx30 labeling was measured. n represents measurement times. The data were collected from 4 WT mice and 3 Cx26 cKO mice, and then averaged. Error bars represent SD. There is no significant difference in Cx30 expression between WT mice and Cx26 cKO mice ( $P=0.14$ , Cochlea;  $P=0.31$ , LW, t-test).

**Fig. 5.**

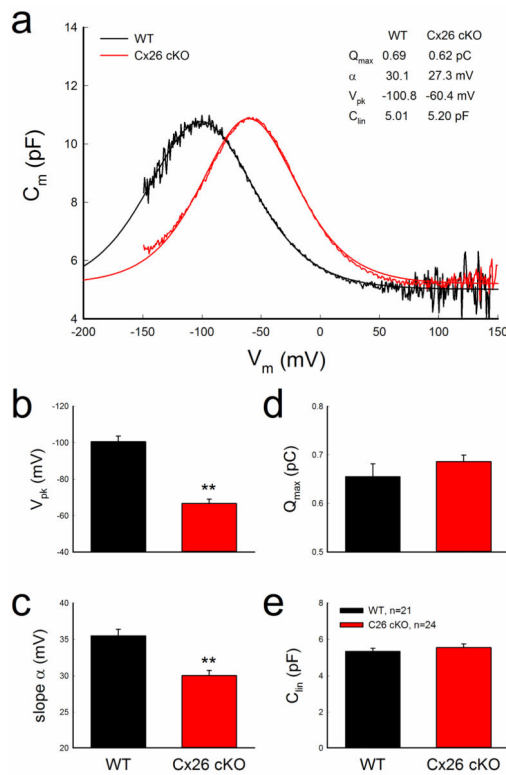
Progressive reduction of DPOAE in Cx26 cKO mice. The mice were injected with 4-HTMX at P10. WT littermates served as controls. **a**: Spectrum of acoustic emission recorded from Cx26 cKO mice and WT mice. Insets: Large scale plotting of  $2f_1-f_2$  and  $f_1$  peaks. The peak of DPOAE ( $2f_1-f_2$ ) in Cx26 cKO mice was reduced but  $f_1$  and  $f_2$  peaks remained the same as those in WT mice.  $f_0=20$  kHz. **b**: Reduction of DPOAE in Cx26 cKO mice in I/O plot. Dashed lines represent the noise levels of recording. **c**: Progressive reduction of DPOAE in Cx26 cKO mice. **d**: Large reduction of DPOAE at high-frequencies in Cx26 cKO mice. \*:  $P<0.05$ , \*\*:  $P<0.001$  as determined by one-way ANOVA with a Bonferroni correction.



**Fig. 6.** No substantial hair cell loss in Cx26 cKO mice. Mice were injected with 4-HTMX at P10 and were P90–110 old. WT littermates with 4-HTMX injection served as control. Insets: Stereocilia of inner hair cells (IHCs) and outer hair cells (OHCs) in Cx26 cKO mice were stained with phalloidin-Alexa 488 in whole-mount preparation and show normal shape. Scale bar: 10 μm.

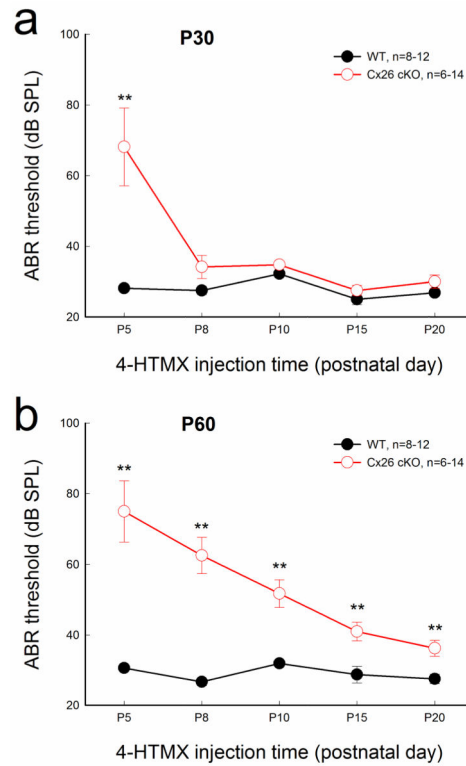


**Fig. 7.** EP reduced but not associated progressive hearing loss in Cx26 cKO mice. Mice were injected with 4-HTMX at P10 for deletion of Cx26. **a:** The EP has significant reduction in Cx26 KO mice. **b:** The EP reduction is not associated with progressive hearing loss in Cx26 cKO mice. The ABR thresholds were measured by click stimulations. EP has no significant changes in Cx26 cKO mice between P35 and P80. However, the ABR threshold at P80 has significant increase in comparison with that at P35. \*\*: P<0.001 (t-test).



**Fig. 8.** Shift of OHC electromotility associated NLC in Cx26 cKO mice. Mice were injected with 4-HTMX at P10 and were recorded at P45–90. **a**: NLC recorded from Cx26 cKO and WT mice. Smooth lines represent fitting by the first derivative of Boltzmann equation. **b–e**: Parameters of NLC fitting. WT littermates served as a control group. \*\*:  $P < 0.001$  (t-test).





**Fig. 9.** Effect of Cx26 deletion at different postnatal times on hearing. Mice were injected with 4-HTMX to delete Cx26 at different postnatal times. ABR was evoked by click stimulations. **a:** The ABR thresholds were measured at P30. Mice with deletion of Cx26 after P5 show normal ABR thresholds. **b:** The ABR thresholds were measured at P60. Data are expressed as mean  $\pm$  S.D; \*\*:  $P < 0.001$  as determined by one-way ANOVA with a Bonferroni correction.