Biochemical and Biophysical Research Communications 463 (2015) 143–147

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Pannexin 1 deficiency can induce hearing loss

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ARTICLE INFO

Article history:
Received 28 April 2015
Available online 20 May 2015

Keywords:
Pannexin 1
Deafness
Inner ear
Gap junction
Caspasae-3
Cell degeneration

ABSTRACT

Gap junctions play a critical role in hearing. Connexin gap junction gene mutations can induce a high incidence of hearing loss. Pannexin (Panx) gene also encodes gap junction proteins in vertebrates. Panx1 is a predominant pannexin isoform and has extensive expression in the cochlea. Here, we report that deletion of Panx1 in the cochlea could produce a progressive hearing loss. The auditory brainstem response (ABR) recording showed that hearing loss was moderate to severe and severe at high-frequencies. Distortion product otoacoustic emission (DPOAE), which reflects the activity of active cochlear mechanics that can amply acoustic stimulation to enhance hearing sensitivity and frequency selectivity, was also reduced. We further found that Panx1 deficiency could activate Caspase-3 cell apoptotic pathway in the cochlea to cause hair cells and other types of cells degeneration. These data indicate that like connexins Panx1 deficiency can also induce hearing loss. These data also suggest that pannexins play important rather than redundant roles in the cochlea and hearing.

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1. Introduction

As a gene family to encode gap junctional proteins in vertebrates, Pannexin (Panx1) was identified 10 years ago [1,2]. So far, three pannexin isoforms (Panx1, 2, and 3) have been cloned from the human and mouse genomes [2]. Despite the lack of similar sequences with connexins, pannexin proteins share large similarities at the structural and functional levels [3]. They have been found to play important and critical roles in many physiological and pathological processes, such as ATP release [4,5], Ca^{2+} homeostasis [6,7], release of synaptic neurotransmitters [8], mediation of cell apoptosis [9,10], and immunological response [11]. However, pannexin functions *in vivo* still remain largely undetermined.

It has been well-demonstrated that gap junctions play a critical role in hearing. Connexin (Cx) gene mutations can induce a high incidence of hearing loss [12,13]. Cx26 and Cx30 have extensive expression in the cochlea [14–16]. Deletion of Cx26 in the cochlea can induce hearing loss [17–22]. Like connexins, pannexins are also extensively expressed in the inner ear [23]. In particular, high expression of Panx1 was found in the cochlear spiral limbus (SLM), supporting cells in the organ of Corti (OC), and fibrocytes in the cochlear lateral wall [23]. In this study, we used Panx1 deficient mice to examine the function of Panx1 in the cochlea and hearing. We found that deletion of Panx1 in the cochlea can induce hearing loss. This study provides important information about the pannexin function in hearing.

2. Materials and methods

2.1. Creation of Panx1 knockout mice

$Panx1^{tm1a(KOMP)Wtsi}$ knockout first mice were purchased from KOMP (Knock Out Mouse Project) and crossed with Pax2-Cre transgenic mouse line (the Mutation Mouse Regional Center, Chapel Hill, NC) to generate Panx1 conditional knockout (KO) in the cochlea. The mouse genotyping was identified by PCR amplification with the following primers: Panx1-Mut1a: 5′-CAC TGC ATT CTA GTT GTG GTT TGT CC-3′, Panx1-Mut2 (gene specific primer): 5′-CTG GCT CTC ATA ATT CTT GCC CTG-3′, Panx1-WF (wildtype-F): 5′-CTG TAT CAC ACA ACC ACT TCA GAG AAG G-3′, and Panx1-WR (wildtype-R): 5′-GAG CTG ACC CCT TTC CAT TCA ATA G-3′, which generated a 579 bp wild-type (WT) band and a 421 bp mutation deletion band. The WT littermates served as controls in the experiment. The experimental procedures were approved by the University of Kentucky's Animal Care & Use Committee and conducted according to the standards of the NIH Guidelines for the Care and Use of Laboratory Animals.

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http://dx.doi.org/10.1016/j.bbrc.2015.05.049
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2.2. Auditory brainstem response and distortion product otoacoustic emission measurements

Auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) were measured by a Tucker–Davis' ABR & DPOAE workstation (Tucker–Davis Tech. Alachua, FL) [19–22]. ABR was measured by clicks and series tone bursts (4–40 kHz, 80–10 dB SPL, 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 110–70 dB SPL was added. DPOAE was measured by two-tone stimulation. A cubic distortion product of $2f_1-f_2$ was recorded with $f_0 = (f_1 \times f_2)^{1/2} = 4, 8, 16, 20$ kHz and $f_2/f_1 = 1.22$ [12,22]. The WT littermates were used as control.

2.3. Cochlear section preparation and immunofluorescent staining

As reported previously [23], the cochlea was fixed with 4% paraformaldehyde, decalcified, frozen, and cut by a cryostat. The tissue sections were directly mounted onto glass slides for staining and storage. The cochlear section 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. Then, the section was incubated with chicken anti-human Panx1 antibody (1:500; #4515, a gift from Dr. Gerhard Dahl at the University of Miami Medical School), monoclonal mouse anti-caspase-3 (1:100–200; #53295, AnaSpec Inc. CA), or polyclonal goat anti-prestin (1:100; sc-22496, Santa Cruz Biotech Inc. CA) in the blocking solution at 4 °C overnight, following reaction with corresponding Alexa Fluor 488- or 568 secondary antibodies (1:500, Molecular Probes) for 2 h at room temperature (23 °C). In some cases, the sections were further stained by 1% 4′, 6-diamidino-2-phenylindole (DAPI, D1306; Molecular Probes) for ~15–20 min following the 2nd antibody incubation to visualize cell nuclei. After washout, the sections were mounted and observed under a microscope.

2.4. Data processing and statistical analysis

Statistical analyses were performed by use of SPSS v18.0 (SPSS Inc. Chicago, IL). Data were expressed as mean ± s.e.m. other than indicated in text. Data were plotted by SigmaPlot software (SPSS Inc., Chicago, IL).

3. Results and discussion

3.1. Deletion of Panx1 in the cochlea

As shown in our previous study [23], Panx1 had strong labeling in the organ of Corti (OC), the spiral limbus (SLM), and the cochlear lateral wall (Fig. 1A). In Panx1 KO mice, Panx1 expression in the SLM was completely deleted (Fig. 1B). Most of Panx1 expression in the cochlea and the cochlear lateral wall were also deleted. Only small, scattered Panx1 labeling was visible. The labeling was also light (Fig. 1B). In addition, the cochlea appeared normal development (Fig. 1B).

3.2. Hearing loss in Panx1 KO mice

Fig. 2 shows that Panx1 KO mice had a progressive hearing loss. ABR recording shows that the ABR thresholds in Panx1 KO mice were progressively increased (Fig. 2B). At postnatal day 80 (P80), the thresholds were increased to above 80 dB SPL at 24 kHz. In comparison with WT littermates, the increase in the ABR threshold in Panx1 KO mice was greater than 40 dB SPL. The hearing loss also appeared severe at high frequency range (Fig. 2C). The ABR

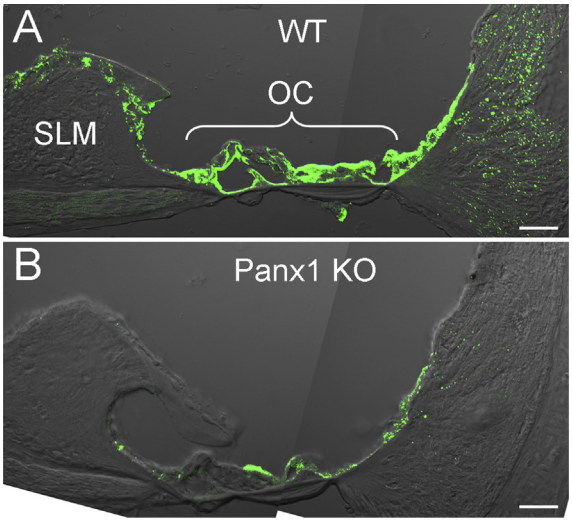

Fig. 1. Panx1 deletion in the cochlea. A: Immunofluorescent staining for Panx1 in the WT mouse cochlea. The intense labeling for Panx1 is visible in the cochlear lateral wall, the organ of Corti (OC), and the spiral limbus (SLM). The image was assembled by two pictures. B: Panx1 deletion in the Panx1 KO mouse cochlea. Most of Panx1 expression was deleted. Only light, scattered Panx1 labeling is visible at the cochlear supporting cells and the cochlear lateral wall. Scale bars: 25 μm.

thresholds in Panx1 KO mice at 8, 16, 24, 32, and 40 kHz were 56.9 ± 9.21, 51.3 ± 9.42, 80.3 ± 10.9, 91.3 ± 4.03, and 90.5 ± 4.58 dB SPL, respectively, at P80 (Fig. 2C). In comparison with WT littermate control, the increases in ABR threshold in Panx1 KO mice were significant ($P < 0.001$, one-way ANOVA with a Bonferroni correction) and larger at high-frequencies.

3.3. Reduction of DPOAE in Panx1 KO mice

DPOAE reflects *in vivo* activity of active cochlear mechanics, which is required for hearing and can amplify acoustic stimulation in the cochlea to increase hearing sensitivity and frequency selectivity [24,25]. In Panx1 KO mice, DPOAE was significantly decreased (Fig. 3). In comparison with WT littermates, the distortion product of $2f_1-f_2$ at $f_0 = 4, 8, 16$ and 20 kHz was reduced by 2.53 ± 1.14, 11.4 ± 1.20, 16.5 ± 3.73, and 17.7 ± 3.05 dB SPL, respectively, at P50 (Fig. 3B). The reduction was significant at 8, 16, and 20 kHz ($P < 0.001$, one-way ANOVA with a Bonferroni correction) and larger at higher frequencies. The reduction was also significant and larger at higher stimulus levels (Fig. 3C,D). The DPOAEs at the stimulus level of 40, 50, and 60 dB SPL were reduced by 10.3 ± 1.13, 14.8 ± 1.37, and 17.7 ± 3.05 dB ($P < 0.001$, one-way ANOVA with a Bonferroni correction), respectively.

3.4. Cell degeneration in Panx1 deficient mice

We also found that deletion of Panx1 could cause cell degeneration. Fig. 4 shows that there was positive reaction of the primary executioner of cellular apoptosis Caspase-3 in the organ of Corti and the cochlear lateral wall in Panx1 KO mice (Fig. 4C–E). However, there was no activity of Caspase-3 found in WT control mice at the same age (Fig. 4A,B). In the whole-mounting preparation, the positive responses of Caspase-3 activation were visible in hair cells and cochlear supporting cells in the cochlear sensory epithelium in Panx1 KO mice (Fig. 4F). Empty triangles in Fig. 4F indicate that outer hair cells had positive labeling for Caspase-3 with collapsed nuclei. Cell degeneration was also visible in the cochlear supporting cells in Panx1 KO mice as indicated by arrows in Fig. 4F.

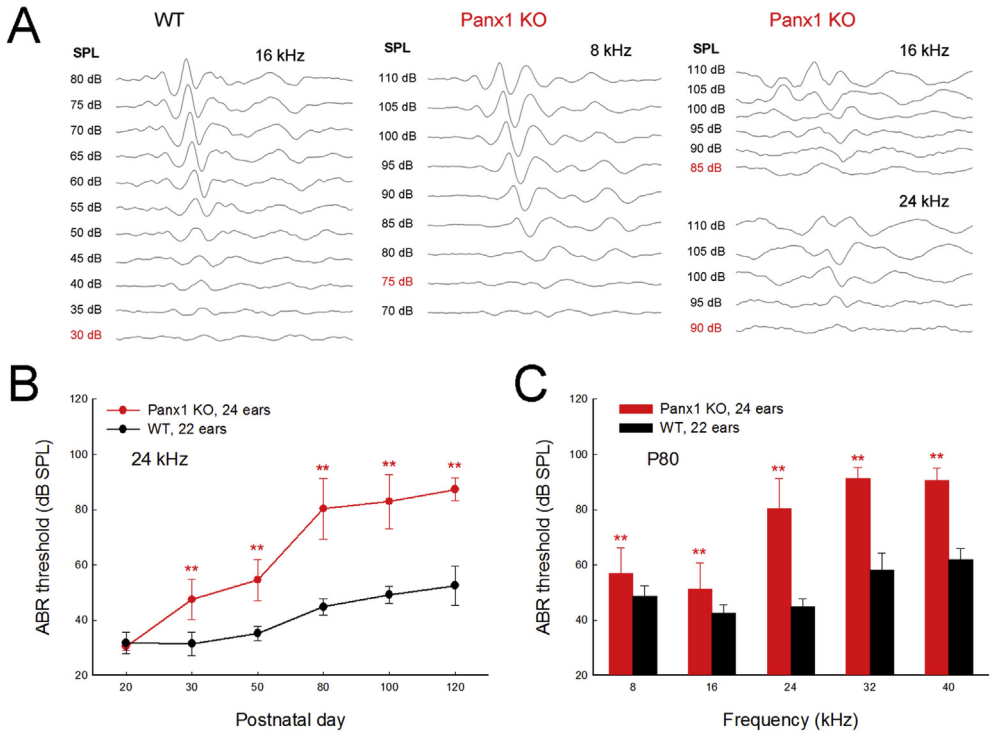

Fig. 2. Hearing loss in Panx1 KO mice. A: ABR waveforms recorded from Panx1 KO and WT littermate mice at P80. Red numbers represent the threshold. B: Progressive hearing loss in Panx1 KO mice. C: Hearing loss of Panx1 KO mice at P80. The hearing loss appears severe at high frequency range. Data are expressed as mean ± s.d. $^{**}P < 0.001$, one-way ANOVA with a Bonferroni correction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In this study, we found that deletion of Panx1 in the cochlea can induce hearing loss (Figs. 1 and 2). DPOAE were reduced (Fig. 3). DEOAE reflects activity of active cochlear amplification which is required for normal hearing. Reduction of active cochlear amplification can induce hearing loss [24,25]. Indeed, we recently found that reduction of active cochlear amplification by Cx26 deficiency can lead to progressive hearing loss; the hearing loss is also severe at high-frequency range [21,22]. This is consistent with our current finding that Panx1 deficiency induces progressive, moderate to severe hearing loss; hearing loss is also severe at high-frequencies (Fig. 2).

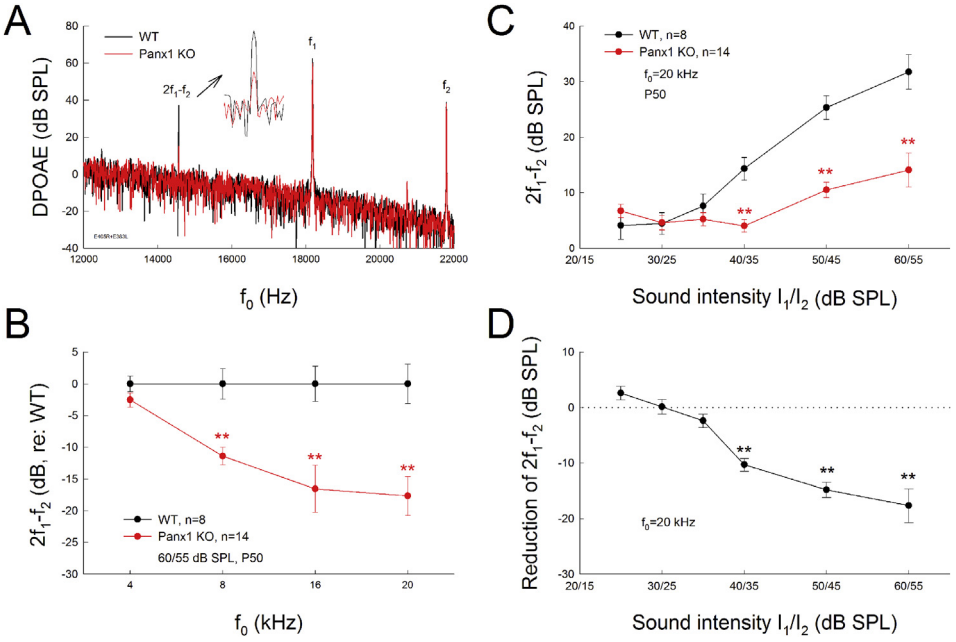

Fig. 3. DPOAE reduction in Panx1 KO mice. A: Spectrums of acoustic emission evoked in Panx1 KO and WT mice. Inset: A high-magnification plot of $2f_1-f_2$ peak. B: Reduction of DPOAE in Panx1 KO mice in frequency response. The responses of DPOAEs in Panx1 KO mice were normalized to those recorded from WT mice. Mice were P50 old. C–D: Reduction of $2f_1-f_2$ in Panx1 KO mice in I/O plot. DPOAE was measured at P50. $^{**}P < 0.001$ as determined by one-way ANOVA with a Bonferroni correction.

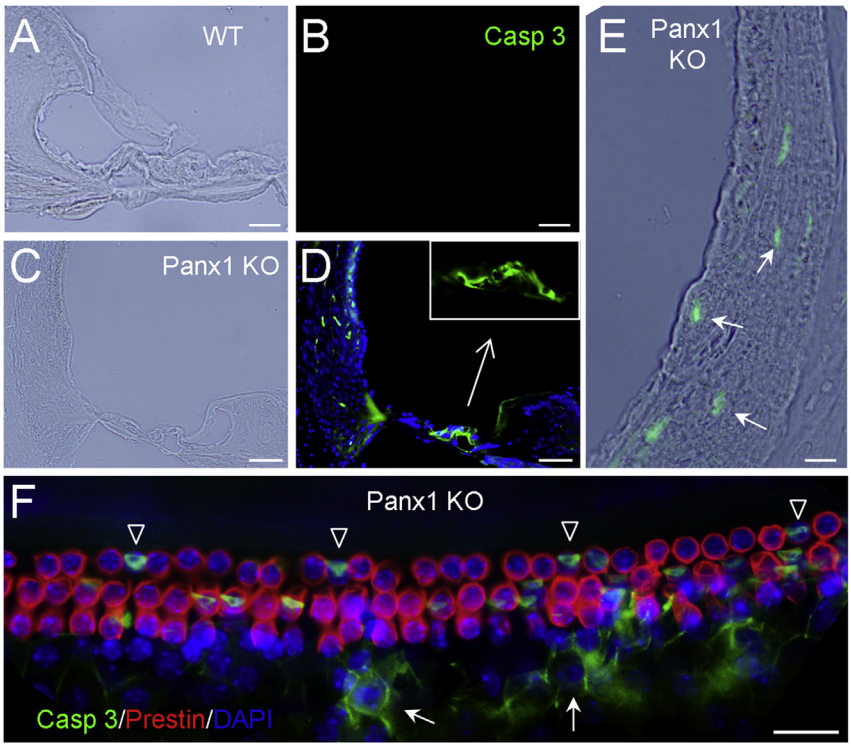

Fig. 4. Cell degeneration in the cochlea of Panx1 KO mice. Mice were 4-month old. A–B: Negative staining in immunofluorescent staining for Caspase-3 in the WT mouse cochlea. C–E: Positive-labeling for Caspase-3 in the cochlea in Panx1 KO mice. White arrows in panel E indicate positive-labeling of Caspase-3 in the cochlear lateral wall. F: Immunofluorescent staining for Caspase-3 in the cochlear sensory epithelium with whole-mounting preparation. Cell nuclei were labeled by DAPI staining (blue) and outer hair cells were visualized by prestin staining (red). Empty triangles indicate the degenerated outer hair cells with positive labeling for Caspase-3 and collapsed nuclei. White arrows indicate positive labeling for Caspase-3 in cochlear supporting cells. Scale bars: A–D: 50 μm, E–F: 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

It has been reported that the activation of Caspase-3 can activate Panx1 channels to mediate cell apoptosis [9,10,26]. In the experiments, we further found that Panx1 deficiency can activate Caspase-3 apoptotic pathway inducing cell degeneration in the cochlea (Fig. 4). However, as reported by previous publications [15,23], hair cells have neither connexin nor pannexin expression. How Panx1 deletion can cause hair cell degeneration in the cochlea currently remains unclear. Recently, we found that deletion of Panx1 could reduce ATP release and endocochlear potential (EP) generation in the cochlea [27]. ATP is an important energy source and also acts as an important cell signaling molecule, which can activate purinergic receptors to play broad roles in many physiological and pathological functions. It has been found that ATP is required for K^+-sinking in the cochlea [28]. Panx1 deletion reduced ATP release in the cochlea, which can consequently reduce K^+-sinking leading to K^+ ions accumulated in the extracellular space and eventually induce K^+-toxicity and cell degeneration (Fig. 4). In addition, Panx1 can form Ca^{2+}-leak channels on the endoplasmic reticulum (ER) membrane [6]. Panx1 deficiency may also result in mitochondrial Ca^{2+} overload leading to cell death [6]. Taken together, these data indicate that Panx1 plays a critical role in the cochlea and hearing. Panx1 deficiency can activate Caspase-3 cell apoptotic pathway to cause cell degeneration.

It has been well-known that connexin mutations can cause hearing loss [12,13]. However, pannexin mutation-induced hearing loss has not been identified yet. This requires further studies in future. Our findings strongly suggest that pannexin deficiency may also be able to induce hearing loss in humans. We found that Panx1 deficiency induced hearing loss is progressive, moderate to severe and severe at high-frequencies (Fig. 2). These findings provide important information about Panx1 deficiency induced hearing loss and also important clues for searching for Panx1 mutation induced hearing loss in humans.

Conflict of interest

There is no conflict of interest.

Acknowledgments

We are grateful to Dr. Gerhard Dahl at Miami University for kindly providing anti-Panx1 antibody. This work was supported by a grant (R01) from the National Institute on Deafness and Other Communication Disorders, DC 05989.

Transparency document

The Transparency Document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrc.2015.05.049.

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