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Research article

Reinvestigation of cochlear pathology in circling mice

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HIGHLIGHTS

- The IHCs and OHCs were relatively intact by P38.
- Spiral ganglion cell densities were not different with those of littermates at P22.
- Hair bundle defects were observable by SEM in OHCs at P18.

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ABSTRACT

The main causes of early hearing deficit in circling mice have been reported to be early degeneration of the organ of Corti and deterioration of spiral ganglion neurons. As an exact cochlear pathology is essential to explain our previous results regarding the auditory brainstem circuits of developing circling mice, we reinvestigated the cochlear pathology in developing circling mice (14, 22, and 38 days old).

It has been reported that the organ of Corti in circling mice completely degenerates as early as postnatal day (P) 21 and that circling mice are deaf by P18. Although we confirmed that circling mice were deaf at P15 and that hair bundles of outer hair cells were defective at P18, complete degeneration of the organ of Corti was not observed by P38 in circling mice. At P22, the type I cell-like spiral ganglion cell density in circling mice was reduced to 78% of that of control mice (ICR mice), but it was not significantly different from that of other control mice (heterozygous (*+/cir*) mice, littermates of circling mice) that could hear at P22.

Our data suggest that other factors, such as absence of neurotransmitter release from inner hair cells, should be considered to explain the early hearing deficit observed at P15 in circling mice.

1. Introduction

The most notable pathologic findings of the circling mouse, an animal model of human non-syndromic deafness (DFNB6 form) [2,8,9], are their completely degenerated organ of Corti, which can occur as early as P21, as well as markedly reduced cellularity of the spiral ganglion neurons, which were thought to be the major causes of total deafness observed at P18 [2].

Although cochlear pathology has been presented in previous papers, some issues remained unclear. A completely degenerated organ of Corti was reported at P21 in one paper [2], but in other reports, the organ of Corti in 100- and 150-day-old circling mice were described in which the basic structure was maintained, and remnants of the organ of Corti were observed [8,9]. Moreover, we have observed intact organs of Corti in P22 circling mice with H&E staining.

Before hearing onset, sound-independent activity originating from the cochleae plays an important role in the survival and maturation of auditory neurons [3,12], synapse development [11], and tonotopic precision of brain stem auditory circuits [5]. We reported some anomalies in the developing medial nucleus of the trapezoid body (MNTB) – lateral superior olive (LSO) synapses in circling mice aged under P14, which were explained in terms of early cochlear degeneration [4,7,15,16]. Because whether cochlear hair cells or spiral ganglion cells really do degenerate early is crucial in explaining the abnormal development of MNTB-LSO synapses, we decided to reinvestigate the cochlear pathology of developing circling mice.

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2. Material and methods

2.1. Animals and cochlear preparation

Female heterozygous (*+/cir*) mice were mated with male homozygous (*cir/cir*) mice (circling mice), and their offspring were used for this study. Circling mice strain has been maintained for more than 10 generations by breeding between female heterozygous (*+/cir*) mice and their male siblings (homozygous (*cir/cir*) mice) at the Animal Facility of Dankook University since 2007.

To investigate inner ear pathology of homozygous (*cir/cir*) mice, P13-14, P22-24, and P36-38 mice were used. More than three animals were included in each group. ICR mice and heterozygous (*+/cir*) mice of the same age were used as controls. Genotypes were assessed as detailed in our previous report [4].

After anesthesia with isoflurane, intracardiac perfusion was performed with 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) and the cochleae were removed in PBS. After overnight fixation in 4% PFA, cochleae were decalcified with 10% EDTA for 48 hr and embedded in paraffin. Mid-modiolar cochlear sections of 4 μm were prepared and stained with hematoxylin and eosin (H&E). We also did scanning electron microscope (SEM) study. Detailed procedure for SEM was described in our previous study [6].

2.2. Analysis

Sections were evaluated using an Olympus BX51 microscope (Olympus, Japan). Images were obtained with a digital camera (DP50, Olympus). Among sections at similar cutting levels, those with relatively well-maintained organs of Corti were selected for analysis. The organ of Corti and spiral ganglion neurons in the basal turn were evaluated.

For grading the organ of Corti, a rank order evaluation was performed according to the same criteria used in a previous study [2]. The assigned numbers indicate the following conditions: (1) complete degeneration of the organ of Corti to a single layer, (2) organ of Corti collapse with tunnel consolidation, (3) pillars were obviously altered but the tunnel of Corti remained recognizable, and (4) normal pillar cells and maintenance of the organ of Corti cytoarchitecture.

Spiral ganglion cell density was measured using cell counts and the area of Rosenthal's canal. Digital imaging and the ImageJ software (NIH, Bethesda, MD) were used to count cells and to measure the area of Rosenthal's canal. Cell density is expressed as cell numbers per square millimeter. A rank order evaluation and cell density comparison were performed with Origin 7.0 (Origin Lab). Data are expressed as means ± SEM. An independent *t*-test was used. *P*-values <0.05 were considered to indicate statistical significance.

2.3. Auditory brainstem response (ABR)

Auditory brainstem responses (ABRs) were recorded (System III, Tucker Davis Technologies, Alachua, FL, USA) with Broadband clicks. After anesthesia with zolazepam (Zoletil, Virbac, Carros Cedex, France) and xylazine (Rompun, Bayer, Leverkusen, Germany) mixture (50 μl/mouse), three electrodes (vertex and ventrolateral to the left and right ears) were inserted subcutaneously. The click auditory stimuli were delivered through ear tubes. ABR waveforms were recorded for 10 ms at a pulse width of 100 μs by using 3–100 Hz by RP filter settings. Hearing thresholds were determined from 10 to 90 dB SPL with 5 dB steps. One thousand and twenty signals were averaged to detect responses.

3. Results

By P38, histological findings in the organs of Corti located in the basal turn in homozygous (*cir/cir*) mice were not significantly different from those of the controls. Outer and inner hair cells, supporting cells, pillar cells, and the Tunnel of Corti were intact in all the cochleae observed (Fig. 1). Similar findings were observed in the apical turn or middle turn (data not shown). The relative conditions of the organs of Corti were assessed in the basal turns using the same rank order grading method (see Methods) used in a previous paper because the organs of Corti in the basal turns were reported to be more damaged than in the apical turns [2]. The assigned numbers in homozygous (*cir/cir*) mice were 4 ± 0 (n = 12, P14), 3.88 ± 0.1 (n = 13, P22), and 3.93 ± 0.1 (n = 15, P38; Fig. 3A), indicating that the organs of Corti in the basal turns were almost normal by P38. They were 4 ± 0 (n = 16, P14), 4 ± 0 (n = 17, P22), and 3.96 ± 0.1 (n = 14, P38) in heterozygous (*+/cir*) mice and 4 ± 0 (n = 15, P14), 4 ± 0 (n = 17, P22), and 3.94 ± 0.1 (n = 14, P38) in ICR mice.

As previous studies reported reduced cellularity of spiral ganglion neurons in homozygous (*cir/cir*) mice [2,8,9], we measured the cell density of the spiral ganglion cells at the basal turns. Two types of neuronal cell bodies are in the mammalian spiral ganglion: a majority group of neurons (Type I) with large cell bodies, comprising 90–95% of the population, and a minority group of neurons (Type II), comprising the remaining 5–10% [13]. We also observed at least two types of cells: (1) larger cells containing a rich cytoplasm stained purple with a surrounding halo (filled arrow in Fig. 2) and (2) relatively smaller cells, stained light violet, without a surrounding halo (hollow arrow in Fig. 2). Most of the larger cells seemed to be Type I cells, but we were unsure as to whether the smaller cells without a surrounding halo were type II cells because other cell types such as satellite cells [17] or Schwann cells [6] are reported. As we could not distinguish type II cells from others with morphology base, we just counted cells separately (larger cells only vs. smaller cells and larger cells together). Total cell densities (cell densities of larger cells and smaller cells) in homozygous (*cir/cir*) mice were 38.8 ± 2.7 mm^{-2} (n = 8, P14), 34.2 ± 1.7 mm^{-2} (n = 11, P22), and 28.6 ± 0.9 mm^{-2} (n = 14, P38; Fig. 3B(a)). They were 47.7 ± 0.8 mm^{-2} (n = 11, P14), 43.4 ± 1.1 mm^{-2} (n = 12, P22), and 43.0 ± 1.6 mm^{-2} (n = 7, P38) in ICR mice and 39.0 ± 1.1 mm^{-2} (n = 11, P14), 32.6 ± 1.5 mm^{-2} (n = 22, P22), and 32.5 ± 1.0 mm^{-2} (n = 12, P38) in heterozygous (*+/cir*) mice. Total cell densities of homozygous (*cir/cir*) mice and heterozygous (*+/cir*) mice were significantly lower than those of ICR mice at all ages tested. At P38, the total cell density of homozygous (*cir/cir*) mice was significantly lower than that of heterozygous (*+/cir*) mice.

Type I-like cell densities in homozygous (*cir/cir*) mice were 11.3 ± 0.7 mm^{-2} (n = 8, P14), 8.3 ± 0.5 mm^{-2} (n = 11, P22), and 5.8 ± 0.2 mm^{-2} (n = 14, P38; Fig. 3B(b)). They were 14.1 ± 0.5 mm^{-2} (n = 11, P14), 10.7 ± 1.0 mm^{-2} (n = 12, P22), and 8.6 ± 0.4 mm^{-2} (n = 7, P38) in ICR mice and 13.5 ± 1.1 mm^{-2} (n = 11, P14), 8.9 ± 0.3 mm^{-2} (n = 22, P22), and 8.3 ± 0.3 mm^{-2} (n = 12, P38) in heterozygous (*+/cir*) mice. At P14 and P38, Type I-like cell densities in homozygous (*cir/cir*) mice were significantly lower than those of ICR mice. At P38, type I like cell density of homozygous (*cir/cir*) mice was significantly lower than that of heterozygous (*+/cir*) mice.

As the previous study presented SEM findings at P10 and P18, we also performed a SEM study at the same ages. Grossly, three rows of outer hair cells and a single row of inner hair cells were well maintained at P10 (data not shown). However, we did observe shortened stereocilia at the margin of the stereocilliary bundles of some outer hair cells (Fig. 4, Homo P10 Middle) but we did not find any missing hair bundles in the three rows of outer hair cells in 4 homozygous (*cir/cir*) mice at P10. Similar shorter stereocilia were also observed at the margin of stereocilliary bundles of the outer hair cells of