

Role of Endothelin Receptor B in Spiral Ganglion Neurons

(14, 15). Melanocytes in the inner ear are specifically located in the SV, and these defects lead to impaired EP levels resulting in hearing loss (16). Thus, disturbance of these constituent cells in inner ears has been shown to cause hearing losses (10).

Dopamine β -hydroxylase (DBH) is an enzyme that converts the neurotransmitter dopamine to noradrenaline. DBH has been used as a specific marker of noradrenergic/adrenergic neurons because noradrenaline converted by DBH is secreted as a neurotransmitter from noradrenergic/adrenergic neurons. *DBH* promoter has been used as a valuable tool to allow a target gene to be expressed in peripheral neurons derived from the neural crest *in vivo* (17). A previous study showed that aganglionic megacolon disease in *Ednrb* homozygously deleted mice (*Ednrb*^{-/-} mice) was recovered by the introduction of an *Ednrb* transgene driven by the human *DBH* promoter (*Ednrb*^{-/-}; *DBH-Ednrb* mice) (18). However, there is no information about hearing levels in *Ednrb*^{-/-}; *DBH-Ednrb* mice. Previous studies have shown that endogenous DBH is expressed in SGNs of inner ears (19), whereas neither endogenous DBH nor a transgene driven by the *DBH* promoter is expressed in melanocytes or their precursors (17, 20). Thus, these results of previous studies suggest that the *DBH* promoter enables *Ednrb* protein to be specifically expressed in SGNs.

Previous studies demonstrated that impairments of *Ednrb*/EDNRB cause hypopigmentation and megacolon disease due to defects of melanocytes and peripheral neurons such as enteric ganglion neurons, respectively (4–6, 8, 9). There has been only one report showing that *Ednrb*-mediated hearing loss involved a congenital defect of melanocytes in the SV (9). However, there was no information in that report about the role of *Ednrb* in SGNs, which serve as peripheral neurons in inner ears for the auditory system, although it was shown in the present study that *Ednrb* protein is expressed in SGNs. The results of the present study indicate a novel etiology for *Ednrb*-mediated hearing loss in mice that can involve postnatal degeneration of SGNs besides congenital defects of melanocytes in the SV.

EXPERIMENTAL PROCEDURES

Mice—*Ednrb*^{-/-} mice (5) and *Ednrb*^{-/-}; *DBH-Ednrb* mice (18) were reported previously. All experiments were authorized by the Institutional Animal Care and Use Committee in Chubu University (approval number 2110017) and the Institutional Recombinant DNA Experiment Committee in Chubu University (approval number 06-01) and followed the Japanese Government Regulations for Animal Experiments.

Measurement of Hearing—Tone burst-evoked auditory brainstem response (ABR) measurements (AD Instruments Pty. Ltd.) were performed as described previously (13, 21). Tone burst stimuli were measured 5 dB stepwise from 0 decibel sound pressure level (dB SPL) to 70 or 90 dB SPL. The threshold was obtained by identifying the lowest level of the I wave of ABR recognized. Data are presented as means \pm S.E.

Morphological Analysis with a Light Microscope—After perfusion fixation by Bouin's solution, cochleae from postnatal day 19 (P19) mice were immersed in the same solution for 1 week. H&E staining and immunohistochemical analyses with anti-*Ednrb* (1:2000; Chemicon) and anti-Kir4.1 (1:500; Santa Cruz Biotechnology) antibodies were performed with paraffin sec-

tions. The VECTASTAIN ABC kit (Vector) and Envision kit/HRP (diaminobenzidine; DAKO) were used in the immunohistochemical analyses with a hematoxylin counterstain. In the case of anti-Kir4.1, the Vector VIP substrate kit for peroxidase (Vector) was used with counterstained methyl green. LacZ staining of dopachrome tautomerase (*Dct*)-LacZ melanocytes was performed as described previously (13). In brief, after fixation with PBS containing 0.25% glutaraldehyde, the inner ears were stained with 0.04% X-Gal by intracochlear perfusion. The samples were postfixed with 4% paraformaldehyde and decalcified with EDTA, and then paraffin sections were prepared. Estimation of cell density of SGNs with H&E staining basically followed the previous method (13, 22–24). In brief, the area of Rosenthal's canal in three sections from each mouse was measured with the software program WinROOF (version 6.2; Mitani Corp., Fukui, Japan) as reported previously (13, 24). Cell density of SGNs from three mice for each mouse strain was calculated by dividing the cell number of SGNs in the measured Rosenthal's canal by the area of the section examined. A total of 100–150 SGNs in three sections from each mouse were examined. Percentage of positive signals histochemically detected by antibodies or LacZ staining was estimated with WinROOF (version 6.2) as reported previously (13, 24). Briefly, the number of positive SGNs was divided by the total number of SGNs. A total of 100–150 SGNs in five sections from each mouse were examined. In the case of SV, positive signals in the measured SV were divided by the area of the section measured. To compare the positive levels among mouse strains, the normalized positive signals in *Ednrb*^{-/-} mice and *Ednrb*^{-/-}; *DBH-Ednrb* mice were divided by the normalized positive signals in WT mice. Rosenthal's canal or SV from three or four mice for each mouse strain was measured for each estimation.

Morphological Analysis by Transmission Electron Microscopy (TEM)—Preparation of tissues for TEM basically followed the previous method (13, 22). In brief, after perfusion fixation with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.3 M HEPES (pH 7.4), dissected murine cochleae were immersed in the same fixative solution overnight at 4 °C. The cochleae were then fixed with 2% osmium tetroxide in 0.3 M HEPES (pH 7.4) at 4 °C for 3 h. After rinsing off the fixative solution, the cochleae were dehydrated with a graded series of ethanol and embedded in epoxy resin (Quetol 651). Ultrathin sections ($t = 70$ nm) were observed under an electron microscope at 80 kV (JEOL JEM1200EX, Tokyo, Japan). Additional procedures are described in the supplemental Methods.

Statistics—Significant difference (*, $p < 0.01$; **, $p < 0.05$) from the control was analyzed by the Mann-Whitney *U* test.

RESULTS

Congenital Deafness in *Ednrb*^{-/-} Mice and Tissue Distribution of *Ednrb* Protein in Inner Ears—We first measured hearing levels of *Ednrb*^{-/-} mice (5) and littermate WT mice on P19 (Fig. 1, A–C). ABR thresholds for 4–32-kHz sound in *Ednrb*^{-/-} mice (90–95 dB SPL) were much higher than those in littermate WT mice (20–55 dB SPL) (Fig. 1A). This result indicates that the *Ednrb*^{-/-} mice suffer from severe congenital hearing loss. Immunohistochemical analyses of inner ears showed expression of *Ednrb* protein in SGNs (arrow in Fig. 1D) and the

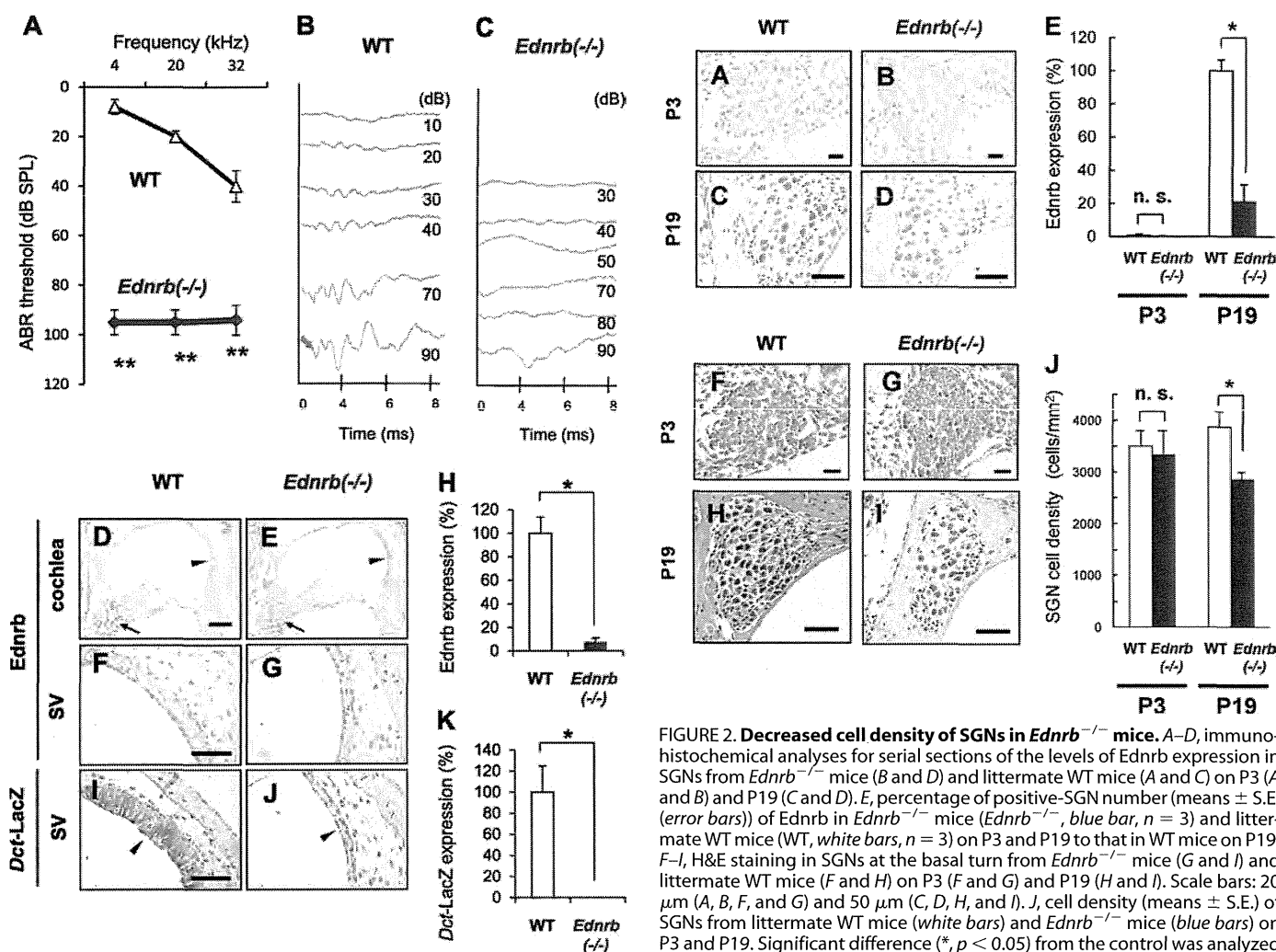


FIGURE 1. Congenital deafness in *Ednrb*^{-/-} mice and expression of *Ednrb* in inner ears. *A*, hearing levels (means \pm S.E. (error bars)) in WT mice ($n = 9$) and *Ednrb*^{-/-} mice ($n = 9$) on P19 measured by ABR. *B* and *C*, ABR waveforms of littermate WT mice (WT, *B*) and *Ednrb*^{-/-} mice (*Ednrb*^{-/-}, *C*) on P19 at 10–90 dB SPL of 12 kHz sound. *D–G*, immunohistochemical analysis of *Ednrb* expression in the cochlea (*D* and *E*) and the SV (*F* and *G*) from *Ednrb*^{-/-} mice (*E* and *G*) and littermate WT mice (*D* and *F*) on P19. Arrows and arrowheads in *D* and *E* indicate SGNs and the SV, respectively. *H*, percentage (means \pm S.E.) of *Ednrb* expression in the SV from *Ednrb*^{-/-} mice (*Ednrb*^{-/-}, blue bar, $n = 3$) and littermate WT mice (WT, white bar, $n = 3$) to that in the SV from WT mice. *I* and *J*, LacZ staining of melanocytes in the SV. We employed *Dct-LacZ* mice, in which the *Dct* promoter is known as a specific marker of melanocytes (intermediate cells) (31), to establish *Dct-LacZ;Ednrb*^{-/-} mice newly by crossing *Ednrb*^{-/-} mice and *Dct-LacZ* mice (32). *K*, percentage (means \pm S.E.) of LacZ-positive melanocytes in the SV from *Ednrb*^{-/-} mice (*Ednrb*^{-/-}, $n = 3$) and littermate WT mice (WT, white bar, $n = 3$) to that in the SV from WT mice. LacZ staining showed no positive cells in the SV from *Dct-LacZ;Ednrb*^{-/-} mice (arrowhead in *J* and *K*), whereas *Dct-LacZ* mice with intact *Ednrb* showed LacZ-positive melanocytes in the SV (blue signals indicated by arrowhead in *I* and *K*). Significant difference (*, $p < 0.05$; **, $p < 0.01$) from the control was analyzed by the Mann-Whitney *U* test. Scale bars: 100 μ m (*D* and *E*) and 50 μ m (*F–J*).

SV (Fig. 1*F*) in WT mice but not in *Ednrb*^{-/-} mice (Fig. 1, *E*, *G*, and *H*). Correspondingly, there were no melanocytes in the SV from *Ednrb*^{-/-} mice (Fig. 1, *I–K*).

Neurodegeneration of SGNs in *Ednrb*^{-/-} Mice—Immunohistochemical analysis of SGNs on P19 showed expression of *Ednrb* protein in WT mice (Fig. 2*C*) but not in *Ednrb*^{-/-} mice (Fig. 2*D*), whereas it was undetectable in SGNs from WT mice on P3 (Fig. 2, *A*, *B*, and *E*). *Ednrb* protein was not detectable in

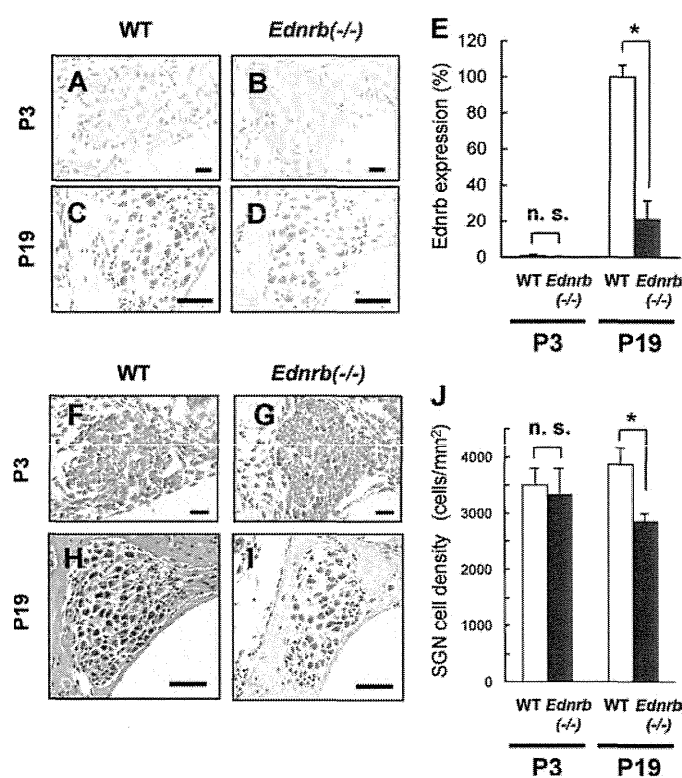


FIGURE 2. Decreased cell density of SGNs in *Ednrb*^{-/-} mice. *A–D*, immunohistochemical analyses for serial sections of the levels of *Ednrb* expression in SGNs from *Ednrb*^{-/-} mice (*B* and *D*) and littermate WT mice (*A* and *C*) on P3 (*A* and *B*) and P19 (*C* and *D*). *E*, percentage of positive SGN number (means \pm S.E. (error bars)) of *Ednrb* in *Ednrb*^{-/-} mice (*Ednrb*^{-/-}, blue bar, $n = 3$) and littermate WT mice (WT, white bars, $n = 3$) on P3 and P19 to that in WT mice on P19. *F–I*, H&E staining in SGNs at the basal turn from *Ednrb*^{-/-} mice (*G* and *I*) and littermate WT mice (*F* and *H*) on P3 (*F* and *G*) and P19 (*H* and *I*). Scale bars: 20 μ m (*A*, *B*, *F*, and *G*) and 50 μ m (*C*, *D*, *H*, and *I*). *J*, cell density (means \pm S.E.) of SGNs from littermate WT mice (white bars) and *Ednrb*^{-/-} mice (blue bars) on P3 and P19. Significant difference (*, $p < 0.05$) from the control was analyzed by the Mann-Whitney *U* test. *n.s.*, not significant.

hair cells from WT mice (supplemental Fig. S1). Moreover, cell density of SGNs in the basal turn from *Ednrb*^{-/-} mice was 20–30% lower than that in the basal turn from littermate WT mice on P19 (Fig. 2, *H–J*) but not on P3 (Fig. 2, *F*, *G*, and *J*). Hair cells in the inner ear showed comparable morphology in WT and *Ednrb*^{-/-} mice on P19 (supplemental Fig. S2). We further performed detailed morphological analyses of SGNs from *Ednrb*^{-/-} mice and littermate WT mice on P16 by TEM analysis (Fig. 3). Gap areas (arrows in Fig. 3*B*) between SGNs (SGN in Fig. 3*B*) and Schwann cells were observed in *Ednrb*^{-/-} mice but not in littermate WT mice on P16 (arrow in Fig. 3*A*). SGNs from *Ednrb*^{-/-} mice showed vacuolar degeneration of the Golgi apparatus (arrows in Fig. 3*D*) and mitochondria (arrowhead in Fig. 3*D*), whereas WT mice showed intact morphology of the Golgi apparatus (arrow in Fig. 3*C*) and mitochondria (arrowhead in Fig. 3*C*). Because gaps between SGNs and Schwann cells and vacuolar degeneration have been shown to be neurodegeneration markers (13, 25), our results suggest that decreased cell density of SGNs in *Ednrb*^{-/-} mice was caused by neurodegeneration.

Improved Hearing Levels of *Ednrb*^{-/-};DBH-*Ednrb* Mice—Our results showed not only a defect in melanocytes of the SV (Fig. 1) but also neurodegeneration of SGNs (Fig. 2) in *Ednrb*^{-/-}

Role of Endothelin Receptor B in Spiral Ganglion Neurons

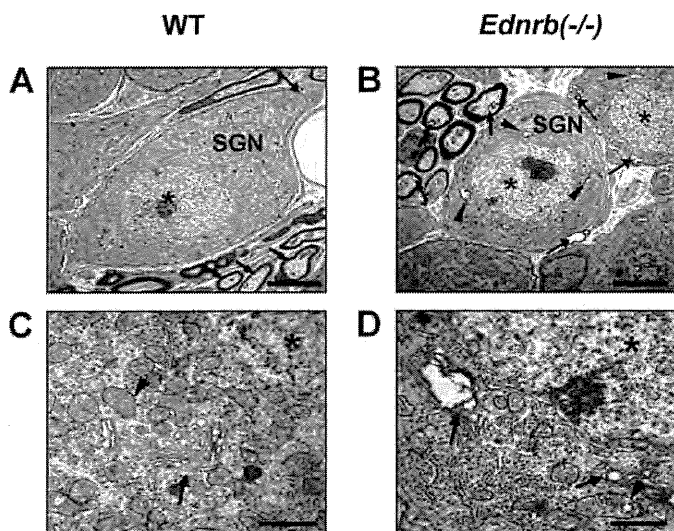


FIGURE 3. Neurodegeneration of SGNs in *EdnrB*^{-/-} mice. TEM of SGNs from *EdnrB*^{-/-} mice (B and D) and littermate WT mice (A and C) on P16. A and B, gap areas (arrows in B) between SGNs (SGN in B) and Schwann cells were observed in *EdnrB*^{-/-} mice but not in littermate WT mice (arrow in A). C and D, vacuolar degeneration of the Golgi apparatus (arrows in D) and mitochondria (arrowhead in D) in SGNs from *EdnrB*^{-/-} mice and intact morphology of the Golgi apparatus (arrow in C) and mitochondria (arrowhead in C) from WT mice. Asterisks indicate nuclei. Scale bars: 5 μ m (A and B) and 1 μ m (C and D).

mice. Previous studies showed that megacolon disease in *EdnrB*^{-/-} mice was recovered in *EdnrB*^{-/-};*DBH-EdnrB* mice, suggesting that *EdnrB* transgene driven by the human *DBH* promoter recovers development of enteric ganglion neurons (18, 26). To clarify the role of *EdnrB* expressed in SGNs in neurodegeneration of SGNs, we next crossed *EdnrB*^{-/-} mice with *DBH-EdnrB* transgenic mice (18, 26) to develop *EdnrB*^{-/-};*DBH-EdnrB* mice. Comparative analysis of ABR thresholds between *EdnrB*^{-/-} mice and littermate *EdnrB*^{-/-};*DBH-EdnrB* mice on P19 revealed marked improvement (more than 30 dB SPL at 4 kHz) of hearing levels in *EdnrB*^{-/-};*DBH-EdnrB* mice (Fig. 4A). Latencies of all four ABR waves in *EdnrB*^{-/-} mice were also recovered in *EdnrB*^{-/-};*DBH-EdnrB* mice (Fig. 4B). We next determined the rescue effect of *EdnrB* transgene driven by the *DBH* promoter on SGNs in *EdnrB*^{-/-} mice. Comparable expression levels of *EdnrB* in SGNs were observed in *EdnrB*^{-/-};*DBH-EdnrB* mice and littermate WT mice on P19 (Fig. 4, C–F). Cell density of SGNs from *EdnrB*^{-/-};*DBH-EdnrB* mice was also comparable with that of SGNs from littermate WT mice and was significantly higher than that of SGNs from *EdnrB*^{-/-} mice (Fig. 4, G–J). Gaps between SGNs and Schwann cells and vacuolar degeneration of the Golgi apparatus and mitochondria were nearly undetectable by TEM in *EdnrB*^{-/-};*DBH-EdnrB* mice as well as WT mice (Fig. 4, K–M). These results suggest that not only enteric ganglion neurons but also SGNs in *EdnrB*^{-/-};*DBH-EdnrB* mice were recovered by *EdnrB* transgene driven by the *DBH* promoter.

Defects of Melanocytes in the SV from *EdnrB*^{-/-};*DBH-EdnrB* Mice—We finally examined whether *EdnrB* transgene driven by the *DBH* promoter affects defects of melanocytes in the SV from *EdnrB*^{-/-} mice. Neither *EdnrB*-positive cells (Fig. 5, A–C, G) nor Kir4.1-positive cells (Fig. 5, D–F, H), a specific marker of melanocytes (27), were detectable in the SV from *EdnrB*^{-/-} mice (Fig. 5, B, E, G, and H) and *EdnrB*^{-/-};*DBH-EdnrB* mice

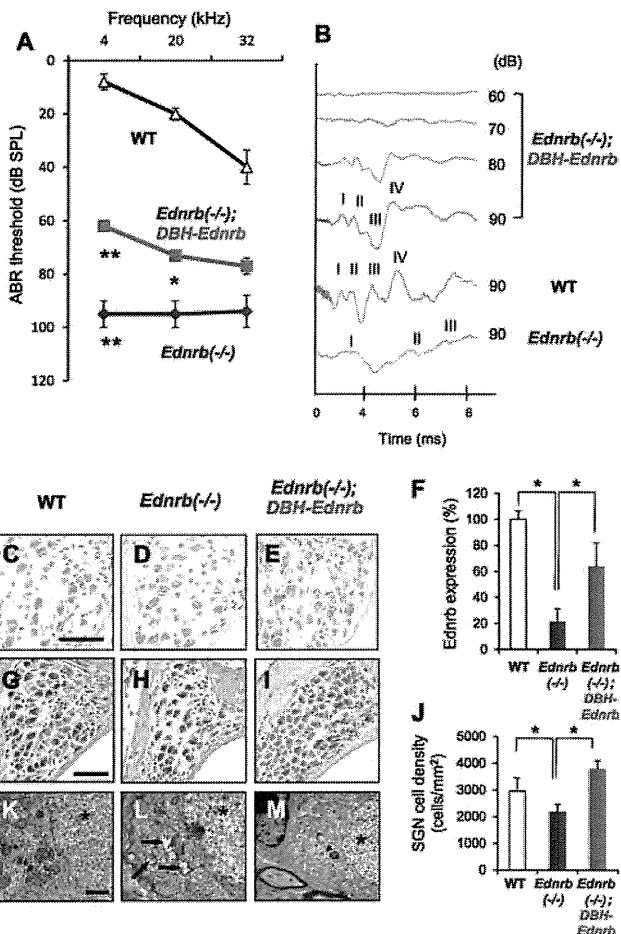


FIGURE 4. Improvements of hearing levels in *EdnrB*^{-/-} mice by *DBH-EdnrB* transgene. A, hearing levels (means \pm S.E. (error bars)) in WT ($n = 9$), *EdnrB*^{-/-} ($n = 9$), and *EdnrB*^{-/-};*DBH-EdnrB* mice ($n = 12$) on P19 measured by ABR. B, ABR waveforms of littermate WT, *EdnrB*^{-/-}, and *EdnrB*^{-/-};*DBH-EdnrB* mice on P19 at 12-kHz sound. ABR wave peaks correspond to cochlear nerve activity (wave I) and downstream neural activities (waves II–IV) (33, 34). C–E, immunohistochemical analysis of *EdnrB* expression in SGNs from WT (C), *EdnrB*^{-/-} (D), and *EdnrB*^{-/-};*DBH-EdnrB* mice (E) on P19. F, percentage of positive SGN number (means \pm S.E.) of *EdnrB* in *EdnrB*^{-/-} mice (*EdnrB*^{-/-}, blue bar, $n = 3$), *EdnrB*^{-/-};*DBH-EdnrB* mice (*EdnrB*^{-/-};*DBH-EdnrB*, red bar, $n = 3$) and littermate WT mice (WT, white bar, $n = 3$) to that in WT mice. G–I, H&E staining in SGNs at the basal turn from WT (G), *EdnrB*^{-/-} (H), and *EdnrB*^{-/-};*DBH-EdnrB* mice (I) on P19. J, cell density (means \pm S.E.) of SGNs from WT, *EdnrB*^{-/-}, and *EdnrB*^{-/-};*DBH-EdnrB* mice on P19. K–M, TEM of SGNs from WT (K), *EdnrB*^{-/-} (L), and *EdnrB*^{-/-};*DBH-EdnrB* mice (M) on P16. Vacuolar degeneration in SGNs from *EdnrB*^{-/-} mice (arrows in L) was not observed in *EdnrB*^{-/-};*DBH-EdnrB* mice (M). Asterisks indicate nuclei (K–M). Scale bars: 50 μ m (C–E, G–I), 1 μ m (K–M). Significant difference (*, $p < 0.05$; **, $p < 0.01$) from the control was analyzed by the Mann-Whitney *U* test.

(Fig. 5, C, F, G, and H). TEM analysis further revealed that *EdnrB*^{-/-} mice and *EdnrB*^{-/-};*DBH-EdnrB* mice similarly exhibited no melanocytes and many gap areas (red arrows in Fig. 5, J and K) among marginal cells and blood vessels, whereas WT mice showed melanocytes among marginal cells and blood vessels (Fig. 5I). In addition, EP of *EdnrB*^{-/-};*DBH-EdnrB* mice (79 ± 6 mV) was significantly ($p < 0.01$) lower than that of WT mice (109 ± 4 mV) (supplemental Fig. S4). These results suggest that defects of melanocytes in the SV from *EdnrB*^{-/-} mice were not recovered by *EdnrB* transgene driven by the *DBH* promoter. Correspondingly, there was no difference in coat color or defects of melanocytes between *EdnrB*^{-/-} mice and *EdnrB*^{-/-};*DBH-EdnrB* mice (supplemental Fig. S5). These results suggest

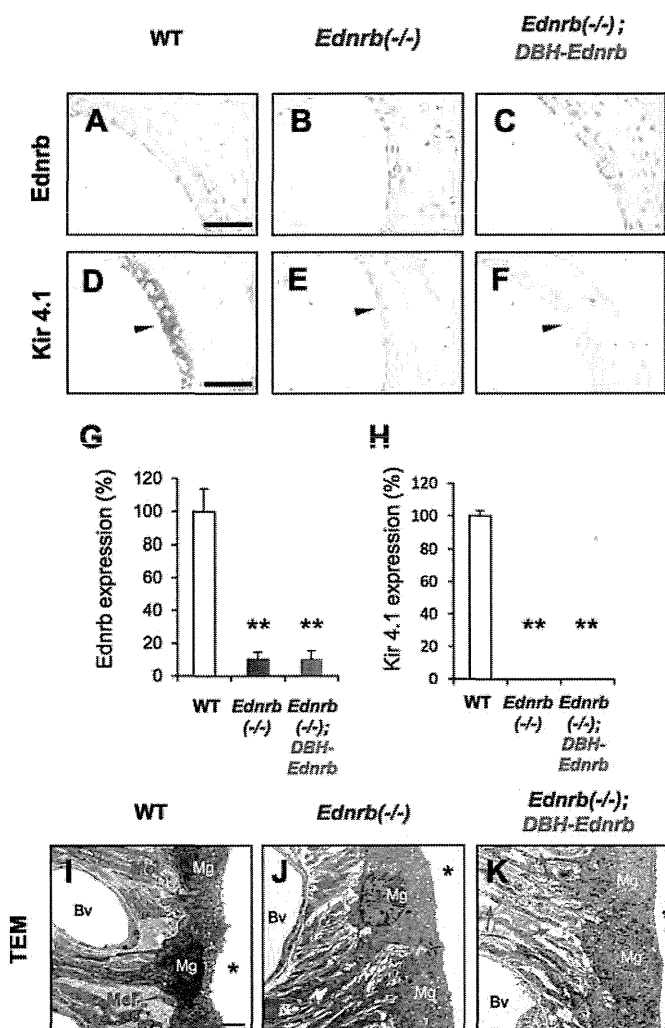


FIGURE 5. Melanocyte defect in the SV from *Ednrb*^{-/-} mice was not rescued in *Ednrb*^{-/-};DBH-*Ednrb* mice. A–C, immunohistochemical analysis of Ednrb expression in the SV from WT mice (A), *Ednrb*^{-/-} mice (B), and *Ednrb*^{-/-};DBH-*Ednrb* mice (C) on P19. D–F, immunohistochemical analysis of Kir4.1 expression, which is known as one of the melanocyte markers in the SV (27). Kir4.1-expressing cells were found in WT mice (purple signals indicated by arrowhead in D) but were not found in *Ednrb*^{-/-} mice and *Ednrb*^{-/-};DBH-*Ednrb* mice (E and F). The methods used for staining are described in detail under “Experimental Procedures.” G and H, percentage (means ± S.E. (error bars)) of Ednrb (G) and Kir4.1 (H) expression levels in the SV from *Ednrb*^{-/-} mice (*Ednrb*^{-/-}, blue bar, *n* = 3), *Ednrb*^{-/-};DBH-*Ednrb* mice (*Ednrb*^{-/-};DBH-*Ednrb*, red bar, *n* = 3) and littermate WT mice (WT, white bar, *n* = 3) to that in the SV from WT mice. Significant difference (*, *p* < 0.05; **, *p* < 0.01) from the control was analyzed by the Mann-Whitney *U* test. I–K, TEM of the SV from *Ednrb*^{-/-} mice (J), *Ednrb*^{-/-};DBH-*Ednrb* mice (K), and littermate WT mice (I) on P19. WT mice exhibited melanocytes (Mel in I) among marginal cells (Mg in I) and blood vessels (Bv in I), whereas *Ednrb*^{-/-} mice and *Ednrb*^{-/-};DBH-*Ednrb* mice exhibited no melanocytes and many gaps (indicated by red arrows in J and K) among marginal cells (Mg in J and K) and blood vessels (Bv in J and K). Asterisk indicates endolymphatic space (I–K). Scale bars: 50 μm (A–F), 2 μm (I–K).

that *Ednrb* transgene driven by the *DBH* promoter did not affect development of melanocytes in the skin and SV.

DISCUSSION

This study demonstrated that *Ednrb*^{-/-} mice had severe congenital deafness (ABR threshold > 90 dB SPL) with not only a defect of melanocytes in the SV (Figs. 1 and 5) but also neurodegeneration of SGNs (Figs. 2 and 3). These results indicate a novel etiology for *Ednrb*-mediated hearing loss in *Ednrb*^{-/-}

mice that involves degeneration of SGNs, which serve as peripheral neurons in inner ears, besides defects of melanocytes in the SV.

This study showed neurodegeneration of SGNs resulting in decreased numbers of SGNs in *Ednrb*^{-/-} mice on P19 (Figs. 2 and 3), whereas cell density and morphology of SGNs were comparable in *Ednrb*^{-/-} mice and WT mice on P3 (Fig. 2). These results suggest that SGNs in *Ednrb*^{-/-} mice developed normally at least until P3, when the level of *Ednrb* expression in SGNs from WT mice was undetectable (Fig. 2A). However, SGNs from *Ednrb*^{-/-} mice no longer survived on P19 (Fig. 2, I and J), when the level of *Ednrb* expression in SGNs from WT mice was clearly detectable (Fig. 2C). We therefore conclude that a defect of *Ednrb* expression affects survival of SGNs during hearing development after birth in mice.

Degeneration of SGNs but not the defect of melanocytes in the SV from *Ednrb*^{-/-} mice was recovered in *Ednrb*^{-/-};DBH-*Ednrb* mice (Figs. 4 and 5). The defect of *Ednrb* protein in SGNs, but not in melanocytes in the SV (Fig. 5) or in inner and outer hair cells (supplemental Fig. S1), from *Ednrb*^{-/-} mice was correspondingly rescued by *Ednrb* transgene driven by the *DBH* promoter. In addition, the suprathreshold ABR, which has been shown to reflect auditory nerve activity (28), showed similar growth rates in *Ednrb*^{-/-};DBH-*Ednrb* mice and littermate WT mice (supplemental Fig. S6), suggesting that development of SGNs was similar in *Ednrb*^{-/-};DBH-*Ednrb* mice and WT mice. On the other hand, *Ednrb*^{-/-};DBH-*Ednrb* mice showed a significantly lower level of EP than that in WT mice (supplemental Fig. S4), although a previous study has shown that there is a link between EP levels and auditory nerve activities (29). TEM analysis also showed no melanocytes with many gap areas in the SV from *Ednrb*^{-/-};DBH-*Ednrb* mice as well as *Ednrb*^{-/-} mice (Fig. 5, I–K), suggesting impairments of the SV in *Ednrb*^{-/-};DBH-*Ednrb* mice. Thus, these results suggest that degeneration of SGNs in *Ednrb*^{-/-} mice was specifically recovered in inner ears of *Ednrb*^{-/-};DBH-*Ednrb* mice. We further showed that hearing levels in *Ednrb*^{-/-};DBH-*Ednrb* mice were partially (20–30 dB SPL) recovered compared with those in *Ednrb*^{-/-} mice (Fig. 4). Thus, these results suggest that *Ednrb* expressed in SGNs is partially required for postnatal development of hearing.

Ednrb has been reported to mediate embryonic development of melanocytes (30) and the enteric nervous system (18, 26) derived from the neural crest. Our results indicate a novel possibility that *Ednrb* is essential for postnatal development of SGNs, although the development process of SGNs (e.g. differentiation or migration of precursors) during prenatal and postnatal hearing development has not been completely elucidated (12). Our previous study also showed that impairment of *c-Ret* causes severe congenital hearing loss with degeneration of SGNs and with intact morphology of hair cells and the SV (13). Because both *EDNRB* and *c-RET* cause megacolon disease with congenital intestinal aganglionosis in mice and humans, further study is needed to determine whether megacolon-related molecules such as *SOX10* and *PAX3* are involved in congenital hearing loss caused by degeneration of SGNs.

Role of Endothelin Receptor B in Spiral Ganglion Neurons

The degeneration of SGNs from *Ednrb*^{-/-} mice did not involve the hallmark of apoptotic signals (supplemental Fig. S3). The results of a previous study also showed that neurodegeneration of enteric neurons did not involve apoptotic signals during the developmental stage in mice with deletion of *Ednrb* (30). On the other hand, our results showed that hair bundles of inner and outer hair cells in *Ednrb*^{-/-} mice, which have already developed congenital hearing loss, were comparable with those in littermate WT mice (supplemental Fig. S2). Immunohistochemical analysis correspondingly showed that expression of *Ednrb* protein was nearly undetectable in hair cells from WT mice (supplemental Fig. S1). These results suggest that the congenital hearing loss in *Ednrb*^{-/-} mice involves postnatal degeneration of SGNs as well as defects of melanocytes in the SV rather than disturbance of hair cells.

Several mouse models for *Ednrb*-mediated WS-IV have been reported (summarized in supplemental Fig. S7). *sl* mice, in which exon 1 and intron 1 are spontaneously deleted, and WS-IV mice, in which exons 2 and 3 are spontaneously deleted, have been shown to develop megacolon disease and hearing loss. On the other hand, the hearing level of *Ednrb*^{-/-} mice with deletion of exon 3, which we analyzed in this study, has not been reported. In humans, although impairments of *EDNRB* caused by nonsense mutations of exon 3 have been reported also to result in the development of WS with hearing loss, the etiology has not been clarified. Thus, this study for the first time provides an insight into the pathogenesis of congenital hearing loss caused by impairment of *Ednrb*^{-/-} by deletion of exon 3 in mice.

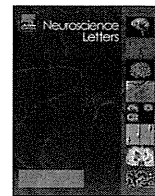
Our results suggest that 60–70 dB SPL of hearing levels could be maintained even if there are no melanocytes in the SV in inner ears of *Ednrb*^{-/-}; *DBH-Ednrb* mice. Because a previous study has shown that a transgene driven by the *Dct* promoter is expressed in melanocytes (31), further study is needed to determine the concurrent rescue effect of *Ednrb* transgene driven by the *Dct* promoter and the *DBH* promoter on congenital deafness in *Ednrb*^{-/-} mice.

In summary, this study demonstrates a novel role of *Ednrb* expression in SGNs distinct from that in melanocytes in the SV contributing partially to postnatal hearing development via survival of SGNs. A therapeutic strategy for congenital hearing loss in WS-IV patients has not been established. Enhancement of *EDNRB* expression in SGNs could be a novel potential therapeutic strategy for congenital hearing loss in WS-IV patients.

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REFERENCES

1. Pardono, E., van Bever, Y., van den Ende, J., Havrenne, P. C., Iughetti, P., Maestrelli, S. R., Costa, F. O., Richieri-Costa, A., Frota-Pessoa, O., and Otto, P. A. (2003) *Am. J. Med. Genet. A* **117A**, 223–235
2. Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Préhu, M. O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G., Amiel, J., Lyonnet, S., Ceccherini, I., Romeo, G., Smith, J. C., Read, A. P., Wegner, M., and Goossens, M. (1998) *Nat. Genet.* **18**, 171–173
3. Edery, P., Attié, T., Amiel, J., Pelet, A., Eng, C., Hofstra, R. M., Martelli, H., Bidaud, C., Munnich, A., and Lyonnet, S. (1996) *Nat. Genet.* **12**, 442–444
4. Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWit, D., Yanagisawa, M., and Chakravart, A. (1994) *Cell* **79**, 1257–1266
5. Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Cheung, J. C., Giaid, A., and Yanagisawa, M. (1994) *Cell* **79**, 1267–1276
6. Bagnato, A., Spinella, F., and Rosanò, L. (2005) *Endocr. Relat. Cancer* **12**, 761–772
7. Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., and Masaki, T. (1990) *Nature* **348**, 732–735
8. Garipey, C. E., Cass, D. T., and Yanagisawa, M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 867–872
9. Matsushima, Y., Shinkai, Y., Kobayashi, Y., Sakamoto, M., Kunieda, T., and Tachibana, M. (2002) *Mamm. Genome* **13**, 30–35
10. Gürtler, N., and Lalwani, A. K. (2002) *Otolaryngol. Clin. North Am.* **35**, 891–908
11. Brown, S. D., Hardisty-Hughes, R. E., and Mburu, P. (2008) *Nat. Rev. Genet.* **9**, 277–290
12. Rubel, E. W., and Fritzsche, B. (2002) *Annu. Rev. Neurosci.* **25**, 51–101
13. Ohgami, N., Ida-Eto, M., Shimotake, T., Sakashita, N., Sone, M., Nakashima, T., Tabuchi, K., Hoshino, T., Shimada, A., Tsuzuki, T., Yamamoto, M., Sobue, G., Jijiwa, M., Asai, N., Hara, A., Takahashi, M., and Kato, M. (2010) *Proc. Natl. Acad. Sci. U.S.A.* **107**, 13051–13056
14. Salt, A. N., Melichar, I., and Thalmann, R. (1987) *Laryngoscope* **97**, 984–991
15. Steel, K. P., and Barkway, C. (1989) *Development* **107**, 453–463
16. Nin, F., Hibino, H., Doi, K., Suzuki, T., Hisa, Y., and Kurachi, Y. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 1751–1756
17. Kapur, R. P., Yost, C., and Palmiter, R. D. (1992) *Development* **116**, 167–175
18. Laghmani, K., Preisig, P. A., Moe, O. W., Yanagisawa, M., and Alpern, R. J. (2001) *J. Clin. Invest.* **107**, 1563–1569
19. Trigueiros-Cunha, N., Renard, N., Humbert, G., Tavares, M. A., and Eybalin, M. (2003) *Eur. J. Neurosci.* **18**, 2653–2662
20. Kapur, R. P., Hoyle, G. W., Mercer, E. H., Brinster, R. L., and Palmiter, R. D. (1991) *Neuron* **7**, 717–727
21. Zheng, Q. Y., Johnson, K. R., and Erway, L. C. (1999) *Hear. Res.* **130**, 94–107
22. Lang, H., Schulte, B. A., Zhou, D., Smythe, N., Spicer, S. S., and Schmiedt, R. A. (2006) *J. Neurosci.* **26**, 3541–3550
23. Shimada, A., Ohta, A., Akiguchi, I., and Takeda, T. (1992) *J. Neuropathol. Exp. Neurol.* **51**, 440–450
24. Ohgami, N., Ida-Eto, M., Sakashita, N., Sone, M., Nakashima, T., Tabuchi, K., Hoshino, T., Shimada, A., Tsuzuki, T., Yamamoto, M., Sobue, G., Jijiwa, M., Asai, N., Hara, A., Takahashi, M., and Kato, M. (2011) *Neurobiol. Aging*, in press
25. Hubbs, A. F., Benkovic, S. A., Miller, D. B., O'Callaghan, J. P., Battelli, L., Schwegler-Berry, D., and Ma, Q. (2007) *Am. J. Pathol.* **170**, 2068–2076
26. Garipey, C. E., Williams, S. C., Richardson, J. A., Hammer, R. E., and Yanagisawa, M. (1998) *J. Clin. Invest.* **102**, 1092–1101
27. Knipper, M., Claussen, C., Rüttiger, L., Zimmermann, U., Lüllmann-Rauch, R., Eskelinen, E. L., Schröder, J., Schwake, M., and Saftig, P. (2006) *J. Physiol.* **576**, 73–86
28. Graham, C. E., and Vetter, D. E. (2011) *J. Neurosci.* **31**, 1267–1278
29. Sewell, W. F. (1984) *J. Physiol.* **347**, 685–696
30. Stanchina, L., Baral, V., Robert, F., Pingault, V., Lemort, N., Pachnis, V., Goossens, M., and Bondurand, N. (2006) *Dev. Biol.* **295**, 232–249
31. Steel, K. P., Davidson, D. R., and Jackson, I. J. (1992) *Development* **115**, 1111–1119
32. Mackenzie, M. A., Jordan, S. A., Budd, P. S., and Jackson, I. J. (1997) *Dev. Biol.* **192**, 99–107
33. Møller, A. R., and Jannetta, P. J. (1985) in *The Auditory Brainstem Response* (Jacobson J. T., ed) pp. 13–31, Taylor & Francis, London
34. Stockard, J. J., and Rossiter, V. S. (1977) *Neurology* **27**, 316–325



Effect of salicylate on potassium currents in inner hair cells isolated from guinea-pig cochlea

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ABSTRACT

Although salicylate is one of the most widely used nonsteroidal anti-inflammatory drugs, it causes moderate hearing loss and tinnitus at high-dose levels. In the present study, salicylate effects on the K currents in inner hair cells were examined. Salicylate reversibly reduced the outward K currents (I_{Kf}), but did not affect the inward current (I_{Kn}). Salicylate blocked the outward K currents in a concentration-dependent manner according to Hill equation with a half-blocking concentration of 1.66 mM, and the Hill coefficient of 1.86.

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Salicylate, one of the most widely used nonsteroidal anti-inflammatory drugs, has been effectively used to treat various inflammation diseases and connective tissue disorders. However, salicylate causes moderate hearing loss and tinnitus at high-dose levels [2,17]. Salicylate ototoxicity is reversible, which differs from that induced by other ototoxic drugs such as aminoglycosides and loop diuretics. The tinnitus subsides and hearing is restored within 24–72 h of stopping salicylate treatment. The exact mechanisms of salicylate's ototoxicity are unknown, but one of the possible mechanisms is an impairment of sound amplification by outer hair cells (OHCs) through its direct action on OHC motility [7,20]. A decrease in otoacoustic emissions after exposure to salicylate provides further evidence of the involvement of OHCs in salicylate ototoxicity [16].

Somatic motility of OHCs is driven by the membrane potentials, and the resting potential of OHCs is dependent on the potassium current I_{Kn} ; a low-voltage activated K^+ conductance, which is obviously influenced by salicylate [23]. Salicylates significantly reduce the I_{Kn} amplitude and lead to depolarization of OHCs. Inner hair cells (IHCs) also possess I_{Kn} [10,18] and other potassium currents such as I_{Kf} , which allow the cells to perform high frequency transduction by shortening the membrane time constant [11] and are crucial for maintaining the cell physiological functions. The morphological changes induced by salicylate were observed not only

in OHCs [9] but also in isolated IHCs [3] with transmission electron microscopy.

In order to evaluate the salicylate influence on potassium currents in IHCs, we isolated the IHCs from guinea-pig cochlea and recorded the potassium currents in the present study. Outward and inward currents were identified and the effects of salicylate were compared between these two potassium currents.

Adult albino guinea-pigs (200–350 g) with normal Preyer reflex were killed by rapid cervical dislocation, both bullae were removed and the cochlea exposed. The cochlea, fused to the bulla, was placed in a Ca^{2+} -free external solution (mM: 142 NaCl, 4 KCl, 3 $MgCl_2$, 2 NaH_2PO_4 , 8 Na_2HPO_4 , adjusted to pH 7.4 with NaOH). The otic capsule was opened, allowing removal of the organ of Corti attached to the modiolus. The organ of Corti was treated with trypsin (0.5 mg/ml, T-4665, Sigma) for 12 min, and gentle mechanical trituration was carried out. Trypsin was rinsed from the specimen by perfusing with a standard external solution (mM: 142 NaCl, 4 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 NaH_2PO_4 , 8 Na_2HPO_4 , adjusted to pH 7.4 with NaOH) for at least 10 min before starting any experiments. The most important landmarks for identifying IHCs are a tight neck and the angle between the cuticular plate and the axis of the cell [5].

Membrane currents were measured by conventional whole-cell voltage-clamp recordings using an EPC-10 (HEKA, Lambrecht, Germany). Data acquisition was controlled by the software PatchMaster (HEKA, Lambrecht, Germany). Recording electrodes were pulled with a two-stage vertical puller (PP830 Narishige, Tokyo, Japan) using 1.5 mm outside diameter borosilicate glass (GC-1.5,

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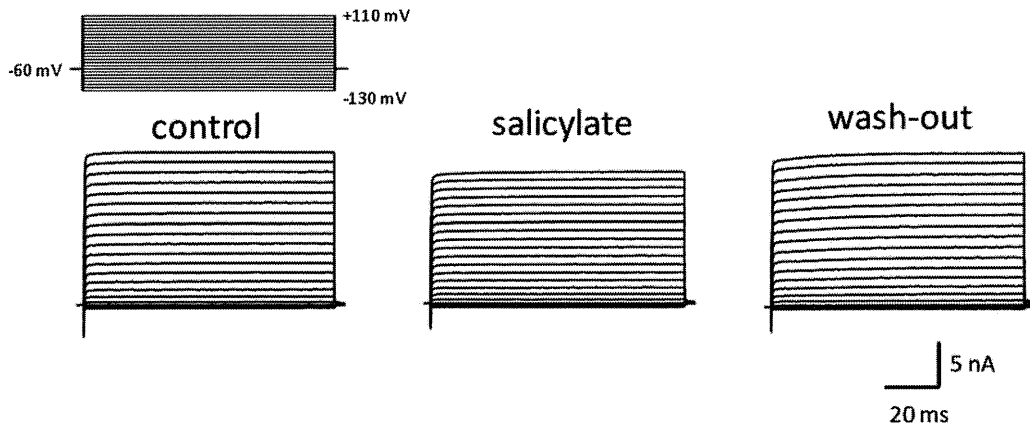


Fig. 1. Effects of 10 mM salicylate on K currents in IHCs. Original traces of K current recorded in normal saline (left), in 10 mM salicylate saline (middle), and in normal saline after salicylate had been washed out (right). The upper panel shows the voltage step protocol.

Narishige, Tokyo) filled with an internal solution (mM: 144 KCl; 2 MgCl₂; 1 NaH₂PO₄; 8 Na₂HPO₄; 2 ATP; 3 D-glucose; 0.5 EGTA; adjusted to pH 7.4 with KOH). Pipettes showed a resistance of 4–8 MΩ in the bath and were coated with ski wax (Tour-DIA, DIAWax, Otaru, Japan) to minimize capacitance. The cell's capacitance was 12.9 ± 6.2 pF (mean ± SD) and the series resistance was 14.8 ± 5.1 MΩ ($n = 21$). Sodium salicylate (S2679, Sigma–Aldrich, St. Louis, USA) was applied under pressure (Pressure micro-injector: PMI-200, Dagan, Minneapolis) using pipettes with a tip diameter of 2–4 μm positioned around 50 μm from the IHCs. Cells were continuously perfused with external saline and all experiments were performed at room temperature (20–25 °C).

The experimental design was reviewed and approved (Accession No. A21-085-0) by the Animal Care and Use Committee, Kyushu University. All procedures were conducted in accordance with the Guidelines for Animal Care and Use Committee, Kyushu University.

The membrane currents in response to various test potentials (from –130 mV to +110 mV) from a holding potential of –60 mV were recorded in IHCs and 10 mM salicylate was applied extracellularly under pressure using puff-pipettes (Fig. 1). Typical current recorded in the control solutions demonstrated outwardly rectifying currents ($I_{K,f}$ and $I_{K,s}$) in response to depolarizing voltage pulses,

with only a slight inward current ($I_{K,n}$) when hyperpolarized (Fig. 1, left). In the solution of 10 mM salicylate, the amplitude of outward K currents decreased by 15% (Fig. 1, middle), and the reduction induced by salicylate was reversed by washing (Fig. 1, right). In the salicylate solutions, fast activation was maintained, displaying the fast current rising phase. Six of 11 cells showed recovery by washing with control solutions.

Despite the apparent decrease in outward currents (Fig. 2A), small inward currents did not show any reduction (Fig. 2B). The relative conductances (ratio of the peak current amplitude in salicylate saline to that measured in salicylate-free control saline) were calculated at membrane potentials of +110 mV and –130 mV, and compared between the outward currents and inward currents (Fig. 3). The relative conductance of inward current and outward current was 0.78 ± 0.21 ($n = 13$) and 0.96 ± 0.14 ($n = 10$), respectively.

Salicylate blocked outward K currents in a concentration-dependent manner (Fig. 4). The currents after salicylate treatment relative to the control level were 0.946 ± 0.007 (1 mM, $n = 2$), 0.884 ± 0.046 (2 mM, $n = 4$), 0.862 ± 0.079 (5 mM, $n = 2$), 0.779 ± 0.059 (10 mM, $n = 13$), 0.826 ± 0.097 (20 mM, $n = 4$), 0.774 ± 0.036 (50 mM, $n = 4$), and 0.811 ± 0.016 (100 mM, $n = 2$). The concentration–response curve was fitted with the Hill

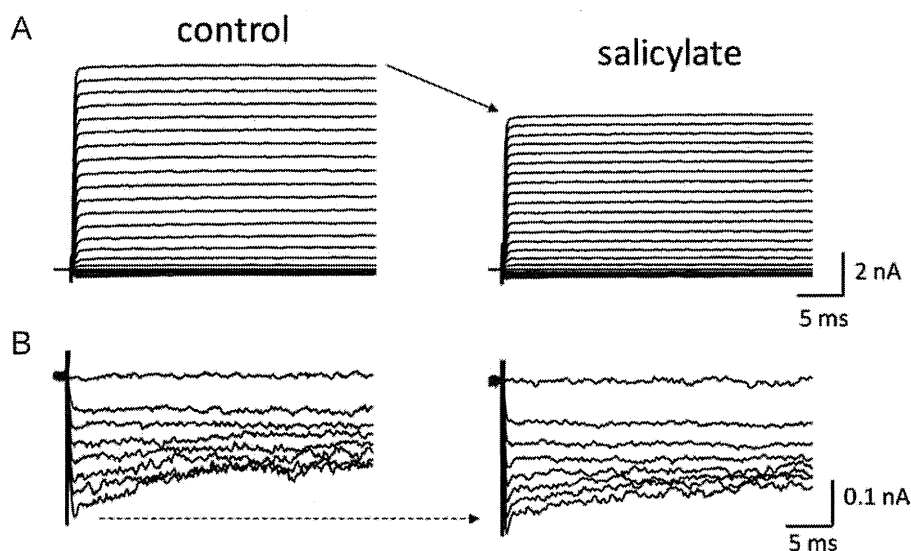


Fig. 2. Effects of 10 mM salicylate on outward and inward currents. In the outward (A), currents were reduced (arrow), however, in the inward (B), current amplitude was not changed (dotted arrow).

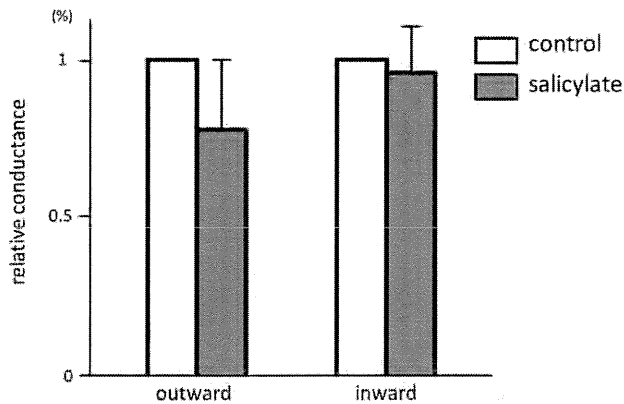


Fig. 3. Comparison of the amplitude change by 10 mM salicylate between the outward K current and inward K current.

equation, $V = 1 - V_{\max} \times [1/(1 + (IC_{50}/C)^h)]$, where V_{\max} is the maximal response, C is the concentration of salicylate, h is the Hill coefficient, and IC_{50} is the half-blocking concentration. The curve was best fitted when IC_{50} was 1.66 mM, h was 1.86, and V_{\max} was 0.196.

The effects of salicylate on the K channels in cochlear IHCs were studied. In rats and guinea pigs, serum salicylate concentrations can reach around 1–3 mM after treatment with high doses of salicylate, and the salicylate concentrations in perilymph was around a half of those in serum [6]. These values are comparable to our observation that IC_{50} was 1.66 mM (Fig. 4). Salicylate inhibited channel activity especially in the outward K currents, but did not affect the inward current ($I_{K,n}$). $I_{K,n}$ determine the resting potential, so salicylate might not change the resting potentials in IHCs. However, salicylate might change the discharge pattern onto spiral ganglion neurons because outward potassium currents are known to participate in repolarization and discharge behaviors of action potentials in neurons [4,19,21].

In OHCs, outward-going potassium currents underwent transient increase and then declined after salicylate administration [13]. With regard to the inward-going $I_{K,n}$, a lower concentration of salicylate (IC_{50} was 10^{-5} M) caused reduction and subsequent depolarization [23]. Nonstationary fluctuation analysis shows that

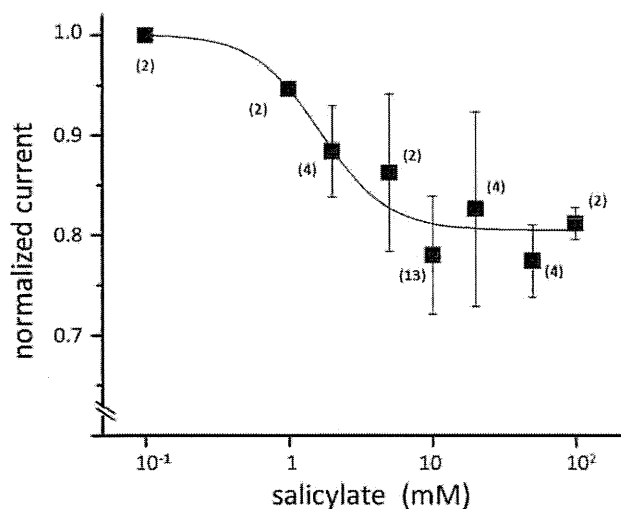


Fig. 4. Concentration–response relationship of salicylate. The conductance of K current in salicylate saline relative to the control recorded in normal saline was calculated at the membrane potential of +110 mV and plotted against the concentration of salicylate. Each plot is the average of 2–13 sets of data. Bars indicate the standard deviation. The fitted curve was drawn according to the Hill equation.

salicylate has a direct blocking action on $I_{K,n}$ (KCNQ4 channel) by significantly reducing the estimated single-channel current amplitude and numbers. In Deiter's cells, 5 mM salicylate reduced the outward K currents by approximately 15% and then recovered 3–4 min after return to control solutions [22]. In rat inferior colliculus neurons, salicylate blocked both the transient outward potassium current ($I_{K(A)}$) and the delayed rectifier potassium current ($I_{K(DR)}$) in a concentration-dependent manner with IC_{50} values of 2.27 and 0.80 mM, respectively [14]. In dissociated rat pyramidal neurons in the auditory cortex, salicylate reduced $I_{K(DR)}$ with a IC_{50} of 2.13 mM and shifted the steady-state activation and inactivation curves negatively [15].

Reduction of the single-channel current amplitude and numbers by salicylate [23] suggested that salicylate has a direct blocking action on potassium channel, which is the major mechanism of the drug. The reversible effects of salicylate in the present study (Fig. 1) were highly consistent with the direct mechanism of the drug. However, salicylate-elicited intracellular acidosis is another possible mechanism to be considered. The salicylate anion contains a benzyl ring and exhibits both hydrophilic and lipophilic properties, therefore salicylic acid could permeate the cell membrane and dissociate to acidify the cell cytoplasm [22]. Acidification can release free Ca^{2+} from its bound form and accordingly increase free intracellular Ca^{2+} . In OHCs, Ca^{2+} imaging study showed that salicylate increased intracellular Ca^{2+} in both the supranuclear and basal cytoplasmic regions [23]. The outward K currents in IHCs mostly consist of $I_{K,f}$, which resemble Ca^{2+} -activated K^+ currents (K_{Ca} , BK) due to their kinetics and pharmacology [1,12]. Therefore the current reduction observed in the present study is readily explained by an interaction between protons and calcium at a regulatory site on the K_{Ca} channel. $I_{K,f}$ is dominant in basal IHCs [8], so high-frequency hearing loss and high-pitch tinnitus might be explained by the different effect of salicylate between the apical and basal IHCs.

Outward K currents in IHCs ($I_{K,f}$) possess fast activating properties and 10 mM salicylate did not influence these fast kinetics (Fig. 1). In inferior colliculus neurons, salicylate attenuated $I_{K(A)}$ but did not affect activation and inactivation gating. However, 1 mM salicylate significantly affected the kinetic properties of $I_{K(DR)}$, including shifting the steady-state activation and inactivation curves in the negative direction [14]. In auditory cortex neurons, the application of 1 mM salicylate significantly produced a negative shift in the steady-state activation curve and inactivation process [15]. Unlike the central auditory pathway, salicylate did not seem to influence K channel kinetics in peripheral organs especially in IHCs.

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References

- [1] J.J. Art, R. Fettiplace, Variation of membrane properties in hair cells isolated from the turtle cochlea, *J. Physiol.* 385 (1987) 207–242.
- [2] Y. Cazals, Auditory sensori-neural alterations induced by salicylate, *Prog. Neurobiol.* 62 (2000) 583–631.
- [3] E.E. Douek, H.C. Dodson, L.H. Bannister, The effects of sodium salicylate on the cochlea of guinea pigs, *J. Laryngol. Otol.* 97 (1983) 793–799.
- [4] E. Ficker, U. Heinemann, Slow and fast transient potassium currents in cultured rat hippocampal cells, *J. Physiol.* 445 (1992) 431–455.
- [5] D.Z. He, J. Zheng, R. Edge, P. Dallos, Isolation of cochlear inner hair cells, *Hear. Res.* 145 (2000) 156–160.
- [6] P.J. Jastreboff, R. Hansen, P.G. Sasaki, C.T. Sasaki, Differential uptake of salicylate in serum, cerebrospinal fluid, and perilymph, *Arch. Otolaryngol. Head Neck Surg.* 112 (1986) 1050–1053.
- [7] S. Kakehata, J. Santos-Sacchi, Effects of salicylate and lanthanides on outer hair cell motility and associated gating charge, *J. Neurosci.* 16 (1996) 4881–4889.

- [8] T. Kimitsuki, K. Kawano, K. Matsuda, A. Haruta, T. Nakajima, S. Komune, Potassium current properties in apical and basal inner hair cells from guinea-pig cochlea, *Hear. Res.* 180 (2003) 85–90.
- [9] T. Kimitsuki, Y. Kakazu, N. Matsumoto, T. Noda, N. Komune, S. Komune, Salicylate-induced morphological changes of isolated inner hair cells and outer hair cells from guinea-pig cochlea, *Auris Nasus Larynx* 36 (2009) 152–156.
- [10] T. Kimitsuki, N. Komune, T. Noda, K. Takaiwa, M. Ohashi, S. Komune, Property of $I(K)_n$ in inner hair cells isolated from guinea-pig cochlea, *Hear. Res.* 261 (2010) 57–62.
- [11] C.J. Kros, A.C. Crawford, Potassium currents in inner hair cells isolated from the guinea-pig cochlea, *J. Physiol.* 421 (1990) 263–291.
- [12] R.S. Lewis, A.J. Hudspeth, Voltage- and ion-dependent conductances in solitary vertebrate hair cells, *Nature* 304 (1983) 538–541.
- [13] Y. Liang, N.C. Zhong, Effects of sodium salicylate and extracellular K^+ on outward potassium current and resting potential of outer hair cell isolated from guinea pig cochlea, *Di Yi Jun Yi Da Xue Xue Bao* 22 (2002) 137–140.
- [14] Y. Liu, X. Li, Effects of salicylate on transient outward and delayed rectifier potassium channels in rat inferior colliculus neurons, *Neurosci. Lett.* 369 (2004) 115–120.
- [15] Y. Liu, H. Zhang, X. Li, Y. Wang, H. Lu, X. Qi, C. Ma, J. Liu, Inhibition of voltage-gated channel currents in rat auditory cortex neurons by salicylate, *Neuropharmacology* 53 (2007) 870–880.
- [16] G.R. Long, A. Tubis, Modification of spontaneous and evoked otoacoustic emissions and associated psychoacoustic microstructure by aspirin consumption, *J. Acoust. Soc. Am.* 84 (1988) 1343–1353.
- [17] E.N. Myers, J.M. Bernstein, Salicylate ototoxicity: a clinical and experimental study, *Arch. Otolaryngol.* 82 (1965) 483–493.
- [18] D. Oliver, M. Knipper, C. Derst, B. Fakler, Resting potential and submembrane calcium concentration of inner hair cells in the isolated mouse cochlea are set by KCNQ-type potassium channels, *J. Neurosci.* 23 (2003) 2141–2149.
- [19] A.G. Sanz, S. Hospital, A. Badia, M.V. Clos, Presynaptic effect of 7-OH-DPAT on evoked $[3H]$ -acetylcholine release in rat striatal synaptosomes, *Brain Res.* 874 (2000) 116–122.
- [20] W.E. Shehata, W.E. Brownell, R. Dieler, Effects of salicylate on shape, electromotility and membrane characteristics of isolated outer hair cells from guinea pig cochlea, *Acta Otolaryngol.* 111 (1991) 707–718.
- [21] C. Summers, M.A. Fleegal, M. Zhu, Angiotensin AT1 receptor signalling pathways in neurons, *Clin. Exp. Pharmacol. Physiol.* 29 (2002) 483–490.
- [22] M.J. Tunstall, J.E. Gale, J.F. Ashmore, Action of salicylate on membrane capacitance of outer hair cells from the guinea-pig cochlea, *J. Physiol.* 485 (1995) 739–752.
- [23] T. Wu, P. Lv, H.J. Kim, E.N. Yamoah, A.L. Nuttall, Effect of salicylate on KCNQ4 of the guinea pig outer hair cell, *J. Neurophysiol.* 103 (2010) 1969–1977.

Daily Short-Term Intratympanic Dexamethasone Treatment Alone as an Initial or Salvage Treatment for Idiopathic Sudden Sensorineural Hearing Loss

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Key Words

Idiopathic sudden sensorineural hearing loss · Intratympanic dexamethasone treatment · Initial treatment · Salvage treatment · Laser-assisted myringotomy

Abstract

Objective: Intratympanic (IT) steroid therapy has been proposed as an alternative treatment option for patients with idiopathic sudden sensorineural hearing loss (ISSNHL). However, the number and frequency of IT treatments and drug delivery methods remain to be determined. The purpose of this study was to evaluate the efficacy of daily short-term IT dexamethasone (DEX) treatment alone in ISSNHL patients using laser-assisted myringotomy (LAM) for the drug delivery route as an initial and/or salvage treatment. **Study Design:** Retrospective study. **Setting:** University hospital. **Patients:** Seventy-six ISSNHL patients receiving IT DEX. Patients with low-tone hearing loss, unilateral or bilateral fluctuating hearing loss or contralateral hearing loss were excluded. **Intervention:** DEX (4 mg/ml) was injected through a perforation made by LAM. IT DEX administration was performed on 8 sequential days. **Main Outcome Measures:** Pre-

and postprocedure hearing levels. The average hearing level was determined by 5 frequencies (250, 500, 1000, 2000 and 4000 Hz). **Results:** Nineteen out of 76 patients fit the criteria for initial treatment in the study (group I), while 24 patients, who had failed systemic therapy, received salvage treatment (group S). The mean age of the patients in groups I and S was 56.2 years with a range from 31 to 73 years of age and 46.0 years with a range from 11 to 76 years of age, respectively. The mean number of days from onset of symptoms to IT therapy in groups I and S was 4.8 days with a range of 1–23 days and 15.3 days with a range of 6–28 days, respectively. In group I, 18 of the 19 patients (95%) showed improvement of more than 10 dB in the pure-tone audiogram, with a mean improvement of 40 dB. Twelve patients (63%) recovered completely and 16 patients (84%) demonstrated successful results with an improvement of more than 30 dB. In group S, 14 of the 24 patients (58%) showed improvement of more than 10 dB with a mean improvement of 16 dB. Two (8%) of the 7 patients (29%) with successful results recovered completely. **Conclusions:** Daily short-term IT DEX administration using LAM for ISSNHL patients without concurrent therapy showed a high response rate and high cure rate and proved to be an alternative therapeutic option to high-dose systemic steroids as a first- and/or second-line treatment.

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Introduction

Idiopathic sudden sensorineural hearing loss (ISSNHL) is the most frequent acute sensorineural hearing loss and is one of the few types of sensorineural hearing loss which can be cured. One of the main problems in the treatment of ISSNHL is that the therapeutic options are limited [Conlin and Parnes, 2007]. Systemic glucocorticoids have been the mainstay for treatment of ISSNHL [Wilson et al., 1980], but as the etiology of ISSNHL is thought to be multifactorial and the mechanism of action of glucocorticoids in the inner ear remains unclarified, the optimal dose is currently unknown. Up to now, higher concentrations of glucocorticoids in the inner ear have been considered desirable because they were thought to exert a stronger effect on hearing recovery. However, systemic application of glucocorticoids, oral or intravenous (IV), had limitations in providing higher concentrations in the perilymph [Parnes et al., 1999; Chandrasekhar et al., 2000; Niedermeyer et al., 2003] because of the blood-labyrinthine barrier. In addition, high-dose systemic administration of glucocorticoids causes higher occurrences of undesirable side effects, in particular, the potential risk of avascular necrosis of the femoral head, and should be avoided in patients with diabetes mellitus, hypertension, gastric ulcer, tuberculosis and so on. Given this background, intratympanic (IT) injection of glucocorticoids for ISSNHL has attracted attention. It could induce higher concentrations of the agent in the target organ and produce less side effects in other parts of the body. Several lines of evidence in animal models and human studies revealed that IT administration results in significantly higher perilymph concentrations of steroids than IV or oral administration [Parnes et al., 1999; Chandrasekhar et al., 2000; Chandrasekhar, 2001; Niedermeyer et al., 2003].

Since Parnes et al. [1999] reported efficacy of IT steroid (ITS) in animal models and a human study, clinical reports of ITS therapy for ISSNHL have been increasing (see review by Hu and Parnes [2009]). Most of the studies have focused on the ITS therapy as a salvage treatment option for patients with ISSNHL [Parnes et al., 1999; Chandrasekhar et al., 2000; Gianoli and Li, 2001; Guan-Min et al., 2004; Dallan et al., 2006; Haynes et al., 2007; Kilic et al., 2007; Plaza and Herraiz, 2007; Van Wijck et al., 2007; Plontke et al., 2009; Dallan et al., 2010]. On the other hand, there are several studies which investigated the efficacy of the ITS treatment alone as an initial treatment [Kakehata et al., 2006; Battaglia et al., 2008; Han et al., 2009; Hong et al., 2009; Kara et al., 2010], although

some reported efficacy of the concurrent use of ITS with high-dose steroids as an initial treatment [Battista, 2005; Lautermann et al., 2005; Ahn et al., 2008; Battaglia et al., 2008].

An ideal treatment of ISSNHL should have a high cure rate as well as a high response rate. A high cure rate is important because patients are not satisfied with results, even an improvement of 30 dB, if their hearing ability does not return to its previous level.

We previously reported efficacy of IT dexamethasone (DEX) treatment on 8 sequential days in ISSNHL patients with diabetes mellitus using laser-assisted myringotomy (LAM) for the drug delivery route as an initial treatment [Kakehata et al., 2006]. The administration of DEX through the small perforation made by LAM [Kakehata et al., 2004] is an easy, secure and confirmable delivery with minimal or no pain. Although a blinded, controlled and randomized study is preferred, as the number and frequency of IT treatments and drug delivery methods remain to be determined, we undertook a retrospective study to ascertain the effective protocol for the treatment of ISSNHL.

The purpose of this study was to evaluate the efficacy of daily short-term IT DEX treatment using LAM without concurrent therapy in ISSNHL patients as an initial treatment as well as a salvage treatment and whether it could be a therapeutic option to high-dose systemic steroids.

Materials and Methods

Seventy-six ISSNHL patients were treated with IT DEX between April 2002 and December 2009. Patients were included in this study if they had a sensorineural hearing loss of 30 dB or more with over 3 contiguous audiometric frequencies that occurred in fewer than 3 days. Patients with low-tone hearing loss, unilateral or bilateral fluctuating hearing loss or contralateral hearing loss and Ménière's disease were excluded. Patients with no identifiable cause of sudden hearing loss were considered to have ISSNHL. Additionally, patients with inadequate follow-up after the treatment (less than 4 weeks) or late IT DEX therapy (beginning more than 4 weeks after onset) were excluded. In the initial treatment group (group I), patients who had received any preceding therapy were excluded. On the other hand, patients who had received systemic steroids for more than 5 subsequent days before the IT DEX therapy were included in the salvage group (group S).

Criteria for Outcome

Pure-tone audiograms were obtained before, during and after the procedure and at periodical checkups. Average hearing was determined by 5 frequencies (250, 500, 1000, 2000 and 4000 Hz). A final audiogram was obtained at least 4 weeks after the final injection. Criteria for audiologic improvement were based on the classification prepared by the Acute Severe Hearing Loss Study

Table 1. Patient profiles

	Group I	Group S
Patients	19	24
Sex (male/female)	12/7	10/14
Age, years		
Mean \pm SD	56.2 \pm 9.7	46.0 \pm 18.7
Range	31–73	11–76
Vertigo	4 (21%)	12 (50%)
Diabetes mellitus	14 (74%)	3 (13%)
Initial hearing level, dB		
Mean \pm SD	77.7 \pm 18.2	74.6 \pm 15.3
Range	49–102	42–102
Duration between onset and IT DEX, days		
Mean \pm SEM	4.8 \pm 5.0	15.3 \pm 6.4
Range	1–23	6–28

Group [Kanzaki et al., 2003] of Japan, which classifies the outcomes as complete recovery, marked recovery, slight recovery, or nonrecovery. Complete recovery is defined as recovery of hearing to within 10 dB of the contralateral pure-tone average, marked recovery as an improvement of the average hearing of 30 dB or more, and slight recovery as an improvement of the average hearing of between 10 and 30 dB. Complete recovery or marked recovery is considered successful treatment.

Treatment Procedure

Patients only received IT DEX treatment and did not undergo additional treatment. Delivery methods were described elsewhere [Kakehata et al., 2006]. Briefly, DEX was injected through a perforation made by LAM, or through a tympanostomy tube (Paparella type II) for the first 6 cases. In the outpatient clinic, a perforation with a diameter of 1.4–2 mm was made in the tympanic membrane with a CO₂ laser unit (OtoLAM; LUMENIS, Yokneam, Israel) using a single pulse of 10–13 W after tympanic membrane anesthesia with iontophoresis [Kakehata et al., 2004]. The location of the perforation was between the oval window and the round window (RW).

The patient lies flat with the affected ear upward and the head is tilted 45° away with the chin upward so that the RW membrane is bathed. 0.5 ml of DEX (4 mg/ml) is injected through the perforation made by LAM using a 1-ml tuberculin syringe with a 26-gauge needle. DEX 4 mg/ml was used because it is the only DEX available in our country. Under direct visualization with an otomicroscope or a magnifying otoscope, one can confirm that the tip of the needle is inserted through the perforation and the mesotympanum is filled with DEX, replacing the air. 0.5 ml is usually more than enough to fill the mesotympanum and a small amount of DEX spills to the outside of the tympanic membrane as a reservoir. The patient is instructed to remain in this position for 30 min without swallowing. DEX administration is performed on 8 sequential days because our previous study showed that daily IT DEX treatment for 8 sequential days was at least as effective as IV DEX treatment (tapering over 8 days) for ISSNHL patients with diabetes mellitus [Kakehata et al., 2006].

Results

Profile of Patients

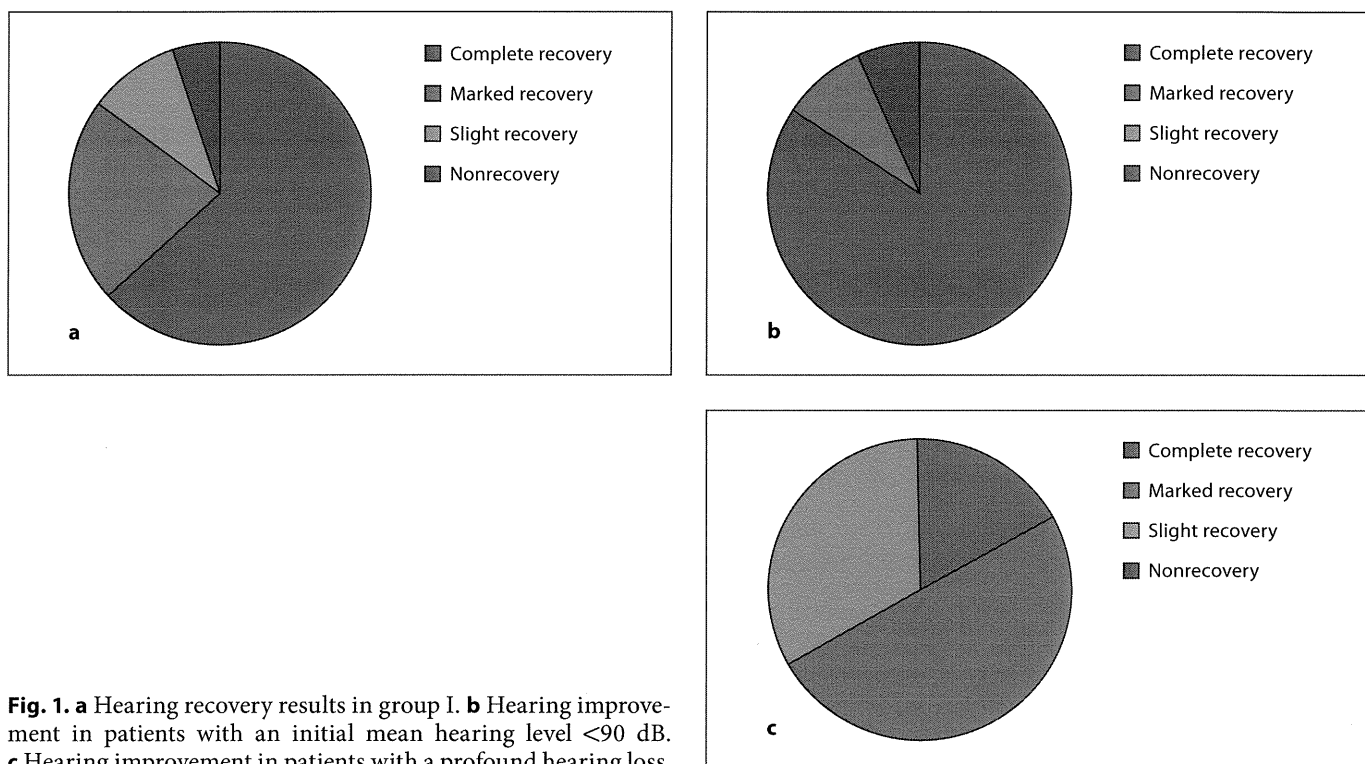
Table 1 summarizes the profiles of the patients in groups I and S. Nineteen out of 76 patients fit the criteria outlined in Materials and Methods for group I, while 24 patients, who had failed systemic therapy, received the IT DEX treatment as a salvage treatment (group S). Twelve men and 7 women were included in group I and 10 men and 14 women in group S. The mean age of groups I and S was 56.2 years with a range from 31 to 73 and 46.0 years with a range from 11 to 76, respectively. The mean number of days from onset of symptoms to IT DEX treatment in group I was 4.8 days with a range of 1–23 days and in group S it was 15.3 days with a range of 6–28 days.

The average hearing level before the IT DEX treatment in group I was 77.7 \pm 18.2 dB (mean \pm standard deviation) with a range from 49 to 102 dB. There were 4 (21%) patients who had vertigo and 14 (74%) patients with diabetes mellitus. On the other hand, in group S, the average hearing level before the IT DEX treatment was 74.6 \pm 15.3 dB with a range from 42 to 102 dB. There were 12 (50%) patients with vertigo and 3 (13%) patients with diabetes mellitus. There was no significant difference in the hearing level before the IT DEX treatment between the 2 groups (Welch's t test). The difference in duration between onset and the IT DEX treatment was significant ($p < 0.001$).

In group S, the mean duration between onset of the symptoms and the initial systemic steroids treatment was 1.9 days with a range from 0 to 20 days. All patients but 1 started the initial treatment within 6 days. Twelve (50%) patients received the treatment on the day of onset. The patients in group S received various kinds of systemic steroids at least 5 consecutive days before being referred to our hospital as well as other therapies such as agents that decrease blood viscosity (dextran), prostaglandin E₁ (PGE₁), ATP and vitamin B₁₂. In 18 out of 24 patients, DEX was used intravenously or orally, usually starting from an amount of 8 mg/day followed by tapered doses for 8 days, with a total amount of 40 mg. Prednisolone or betamethasone was also used in other patients. Six patients received PGE₁ therapy after the failure of the initial steroid therapy. One patient who failed the initial systemic IV DEX therapy starting from an amount of 32 mg received PGE₁ therapy and oxygen at hyperbaric pressure as a salvage treatment.

Clinical Outcomes

Figure 1a depicts the overall results in group I. Eighteen of 19 patients (95%) showed improvement in the



pure-tone audiogram of more than 10 dB. After the treatment, the mean pure-tone average reached 38.8 dB from 77.7 dB with a mean improvement of 39.7 ± 18.4 dB. Sixteen (84%) patients demonstrated successful treatment and 12 (63%) patients recovered completely, reaching a hearing level identical to that of the unaffected ear. However, a 62-year-old man, who underwent treatment 23 days after the onset with an initial hearing level of 61 dB without vertigo, showed no improvement. As it is known that patients with a profound hearing loss (initial pure-tone average worse than 90 dB) have a poor prognosis, those patients were evaluated separately. There were 13 patients who did not have a profound hearing loss. This group had 11 (85%) with complete recovery, 1 (8%) with marked recovery and 1 (8%) with no hearing recovery (fig. 1b). The mean hearing improvement was 40.5 ± 19.2 dB. In the profound hearing loss group, all patients responded to the treatment with a mean hearing improvement of 37.8 ± 18.2 dB; 1 (17%) with complete recovery, 3 (50%) with marked and 2 (33%) with slight recovery (fig. 1c). Hearing improvement between the 2 groups was not statistically different.

The results for all patients in group S are presented in figure 2a. Fourteen of 24 (58%) patients showed improve-

ment. The mean pure-tone average reached 57.8 from 74.6 dB with a mean improvement of 16.8 ± 21.6 dB. Seven (29%) patients demonstrated successful treatment, including 2 (8%) with complete recovery. Ten patients did not show improvement. There were 20 patients who did not have a profound hearing loss, whose mean hearing improvement was 16.9 ± 23.6 dB. This group had 11 (55%) with improvement and 7 (35%) with successful treatment and 2 (10%) with complete recovery (fig. 2b). In the profound hearing loss group, all patients responded to the treatment with a mean improvement of 16.5 ± 7.2 dB.

In the responders of group S, the mean hearing improvement was 30.1 ± 18.3 dB. Several factors which are known to affect the prognosis of patients were compared between the responders and nonresponders. The mean duration between onset of symptoms and IT DEX treatment was 14.5 ± 5.9 days and 16.5 ± 7.1 days in the responders and nonresponders, respectively, which is not statistically different. On the other hand, the average hearing level before the IT DEX treatment was 82.0 ± 12.4 dB and 64.2 ± 13.0 dB in the responders and nonresponders, respectively, which is statistically different ($p < 0.01$).

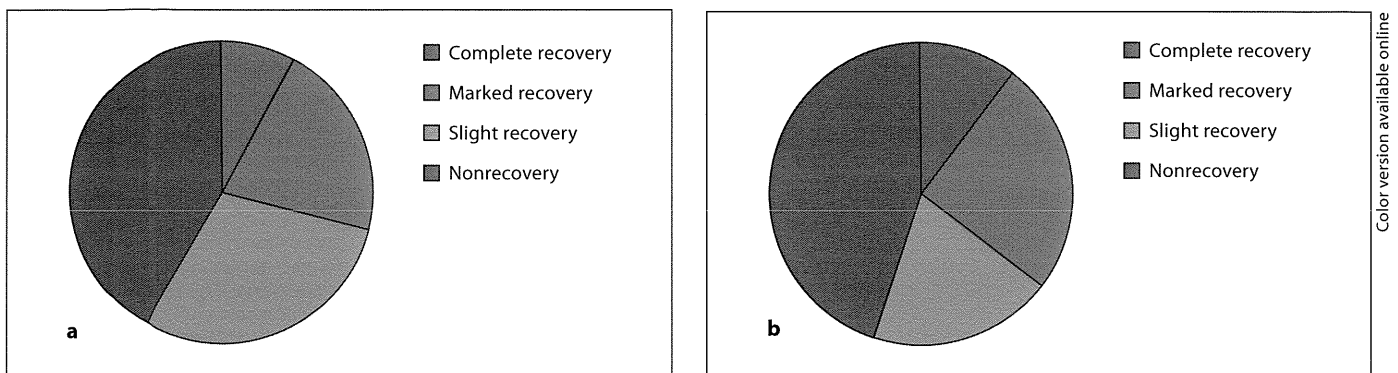


Fig. 2. a Hearing recovery results in group S. **b** Hearing improvement in patients with an initial mean hearing level <90 dB.

Side Effects

There was transient vertigo of about a minute associated with the injections. Some patients had tolerable pain after the injection for a minute. No patients had otitis media. The perforations were closed spontaneously or with a chitin sheet patch in most patients. For 12 patients, autologous serum ear drops and/or basic fibroblast growth factor with a chitin sheet patch therapy in the outpatient clinic [Kakehata et al., 2008] were performed and the perforations were closed successfully except in 1 patient. Small perforations remained in 2 patients. One patient underwent myringoplasty.

Discussion

Recently, there has been increased interest in IT administration of steroids for the treatment of ISSNHL, whether as an initial or salvage treatment [Parnes et al., 1999; Gianoli and Li, 2001; Guan-Min et al., 2004; Battista, 2005; Lautermann et al., 2005; Dallan et al., 2006; Kakehata et al., 2006; Haynes et al., 2007; Kilic et al., 2007; Plaza and Herraiz, 2007; Van Wijck et al., 2007; Ahn et al., 2008; Battaglia et al., 2008; Han et al., 2009; Hong et al., 2009; Plontke et al., 2009; Dallan et al., 2010; Kara et al., 2010], since ITS seems to be a potent alternative treatment option to systemic steroid therapy. As a first-line treatment, several clinical studies reported efficacy of combination therapies of ITS with systemic steroid therapy [Battista, 2005; Lautermann et al., 2005; Ahn et al., 2008; Battaglia et al., 2008]. However, there has been no ITS treatment protocol that seems to be superior [Hu and Parnes, 2009]. Thus, before discussing the efficacy of combination therapy, it seems necessary to elucidate a preferable ITS treatment

protocol without concurrent or previous treatments. Here, we performed daily short-term IT DEX treatment on fresh ISSNHL patients without concurrent treatments in order to investigate the efficacy of ITS alone.

An ideal treatment of ISSNHL should have a high cure rate as well as a high rate of response. To improve the cure rate is especially important because patients are not satisfied, even if their hearing level is improved by 30 dB, if it does not improve to near the hearing level of the unaffected ear. In this study, the rate of response and the cure rate of daily short-term IT DEX administration alone as an initial treatment reached 95 and 63%, respectively. This cure rate is higher than that of IT DEX treatment without concurrent systemic steroids in the recently published studies. Battaglia et al. [2008] reported a cure rate of 29% (5/17) by IT DEX therapy of 3 weekly injections. In their study, a high-dose oral prednisone treatment (tapering from 60 mg for 14 days; total 660 mg) and a combination therapy (IT DEX plus high-dose oral steroids) were also attempted. The cure rate of the oral steroid therapy and the combination therapy was 17% (3/18) and 63% (10/16), respectively. Battaglia et al. [2008] advocated the efficacy of the combination therapy over the systemic high-dose steroid therapy. Our cure rate was higher than that of the IT DEX therapy of 3 weekly injections and comparable to that of the combination therapy. This suggests that daily administration through LAM for 8 days is more effective than 3 weekly injections. In addition, in patients who did not have a profound hearing loss, our cure rate of 85% is higher than that of the combination therapy (63%). In the profound hearing loss group, the mean hearing improvement reached 37.8 dB, which was not statistically significantly different compared to the 40.5 dB in the other group. However, the cure rate was

low (17%) compared to that of 66% (deduced from figure 3 in Battaglia et al. [2008]) in the combination therapy, which might suggest a limitation of the IT DEX therapy in this form for patients with a profound hearing loss. This may also suggest that there are other pathological conditions in the case of profound hearing loss which cannot be reached by ITS treatment.

On the other hand, Ahn et al. [2008] reported an additional effect of IT DEX (3 injections on days 1, 3 and 5) through a 25-gauge spinal needle on oral methylprednisolone therapy (tapering from 48 mg for 14 days) in a study with a larger number of subjects. The cure rate by the combination therapy of IT DEX plus oral methylprednisolone was 25% (15/60), which did not show significant improvement compared to the oral methylprednisolone alone (27%, 16/60).

Battaglia et al. [2008] and Ahn et al. [2008] used a treatment protocol of 3 transtympanic injections of DEX on infrequent days. In most studies, steroids were administered by transtympanic injection through a fine needle under local anesthesia at 1–5 injections over 1–3 weeks [Hu and Parnes, 2009]. Recently, Kara et al. [2010] reported a high cure rate of 48% (14/29) by 5 transtympanic injections of IT DEX on 5 consecutive days as an initial treatment. Taking into account our high cure rate by 8 injections on 8 consecutive days, daily application of ITS may be more effective than infrequent application.

Recent studies on cochlear pharmacokinetics with local ear drug delivery revealed that after the end of the 30-min application, the concentration in the base of the scala tympani rapidly declines due to clearance from the cochlea and diffusion into other compartments and apical regions [Plontke et al., 2007]. To conquer this drawback, several delivery methods have been devised for the sustained application of the drug to the RW membrane, such as the Silverstein MicroWick [Silverstein et al., 1997] or the MicroCatheter [Kopke et al., 2001; Plontke et al., 2009]. However, the latter is no longer available because the FDA removed it from the market. With the Silverstein MicroWick, the patient can apply the steroid several times a day. In our previous study, we speculated that our high response rate and the degree of improvement might be due mainly to the delivery methods used and/or the frequency of the applications. The delivery method with a wide opening to the mesotympanum assures the certain filling of the mesotympanum with the treatment agent and allows the air to escape from the mesotympanum, permitting the treatment agent to contact the RW membrane. The high cure rate as an initial treatment in this study might support this speculation.

As a salvage treatment, improvement of more than 10 dB was achieved in 58% and the cure rate was 8% with successful treatment in 29%. The mean improvement was 16.8 dB. In the responder group, the mean improvement reached 30.1 dB. There are a number of studies of ITS as a salvage treatment. Reported response rates were between 27.5 and 73.6% [Parnes et al., 1999; Chandrasekhar et al., 2000; Gianoli and Li, 2001; Guan-Min et al., 2004; Dallan et al., 2006; Haynes et al., 2007; Kilic et al., 2007; Plaza and Herraiz, 2007; Van Wijck et al., 2007; Plontke et al., 2009; Dallan et al., 2010].

Initial severity of hearing loss is one of the known prognostic factors. Although there were no significant differences between the initial and salvage groups regarding the hearing level before the IT DEX treatment (77.7 ± 18.2 vs. 74.6 ± 15.3 dB) ($p < 0.01$), the hearing level after the treatment (38.8 ± 22.3 vs. 57.8 ± 21.8 dB) and gain of improvement (39.7 ± 18.4 vs. 16.8 ± 21.6 dB) were significantly higher in group I ($p < 0.01$). One of the reasons for these better results is the shorter duration between the onset and IT DEX treatment (4.8 ± 5.0 vs. 15.3 ± 6.4 days; $p < 0.01$). However, between the patients with successful treatment and those with no response in group S, the difference in the mean duration between onset of symptoms and IT DEX treatment (12.3 ± 3.5 vs. 16.5 ± 7.1 days) was not statistically significant, although the patients starting IT DEX treatment later than 19 days after onset did not have successful outcomes, suggesting the therapeutic window of this treatment. It is also likely that group S may include those with poor response to steroid therapy, which was not overcome by the high dose of steroid induced by the ITS therapy.

There are at least 3 requirements for successful IT treatment. Firstly, a secure and confirmable delivery method is necessary. For the agent to perfuse via the RW membrane, it is important to replace the air around the RW membrane [Nomura, 1984; Silverstein et al., 1997] with the solution containing the agent. Secondly, sequential or continuous administration of the drug might be desirable because it is expected to maintain the concentration of the drug in the target cells at a high level [Plontke et al., 2007]. Finally, it should be an easy and painless delivery method. The daily short-term IT DEX treatment using LAM seems to meet these 3 requirements.

In conclusion, daily short-term IT DEX treatment using LAM for ISSNHL patients without concurrent therapy is effective as an initial treatment as well as a salvage one and proved to be an alternative therapeutic option to high-dose systemic steroids. However, a prospective study is necessary to validate the conclusion.

References

- Ahn JH, Yoo MH, Yoon TH, Chung JW: Can intratympanic dexamethasone added to systemic steroids improve hearing outcome in patients with sudden deafness? *Laryngoscope* 2008;118:279–282.
- Battaglia A, Burchette R, Cueva R: Combination therapy (intratympanic dexamethasone + high-dose prednisone taper) for the treatment of idiopathic sudden sensorineural hearing loss. *Otol Neurotol* 2008;29:453–460.
- Battista RA: Intratympanic dexamethasone for profound idiopathic sudden sensorineural hearing loss. *Otolaryngol Head Neck Surg* 2005;132:902–905.
- Chandrasekhar SS: Intratympanic dexamethasone for sudden sensorineural hearing loss: clinical and laboratory evaluation. *Otol Neurotol* 2001;22:18–23.
- Chandrasekhar SS, Rubinstein R, Kwartler J, Gatz M, Connelly PE, Huang E, Baredes S: Dexamethasone pharmacokinetics in the inner ear: comparison of route of administration and use of facilitating agents. *Otolaryngol Head Neck Surg* 2000;122:521–528.
- Conlin AE, Parnes LS: Treatment of sudden sensorineural hearing loss. I. A systemic review. *Arch Otolaryngol* 2007;133:573–581.
- Dallan I, Bruschini P, Nacci A, Bruschini P, Traino C, Rognini F, Fattori B: Transtympanic steroids as a salvage therapy in sudden hearing loss: preliminary results. *ORL J Otorhinolaryngol Relat Spec* 2006;68:247–252.
- Dallan I, De Vito A, Fattori B, Casani AP, Panicucci E, Berrettini S, Marchetti M, Nacci A: Intratympanic methylprednisolone in refractory sudden hearing loss: a 27-patient case series with univariate and multivariate analysis. *Otol Neurotol* 2010;31:25–30.
- Gianoli GJ, Li J: Transtympanic steroids for treatment of sudden hearing loss. *Otolaryngol Head Neck Surg* 2001;125:142–146.
- Han CS, Park JR, Boo SH, Jo JM, Park KW, Lee WY, Ahn JG, Kang MK, Park BG, Lee H: Clinical efficacy of initial intratympanic steroid treatment on sudden sensorineural hearing loss with diabetes. *Otolaryngol Head Neck Surg* 2009;141:572–578.
- Haynes DS, O'Malley M, Cohen S, Watford K, Labadie RF: Intratympanic dexamethasone for sudden sensorineural hearing loss after failure of systemic therapy. *Laryngoscope* 2007;117:3–15.
- Ho HG, Lin HC, Shu MT, Yang CC, Tsai HT: Effectiveness of intratympanic dexamethasone injection in sudden-deafness patients as salvage treatment. *Laryngoscope* 2004;114:1184–1189.
- Hong SM, Park CH, Lee JH: Hearing outcomes of daily intratympanic dexamethasone alone as a primary treatment modality for ISSHL. *Otolaryngol Head Neck Surg* 2009;141:579–583.
- Hu A, Parnes L: Intratympanic steroids for inner ear disorders: a review. *Audiol Neurootol* 2009;14:373–382.
- Takehata S, Futai K, Kuroda R, Shinkawa H: Office-based endoscopic procedure for diagnosis in conductive hearing loss cases utilizing OtoScan laser-assisted myringotomy. *Laryngoscope* 2004;114:1285–1289.
- Takehata S, Hirose Y, Kitani R, Futai K, Maruya S, Ishii K, Shinkawa H: Autologous serum eardrops therapy with a chitin membrane for closing tympanic membrane perforations. *Otol Neurotol* 2008;29:791–795.
- Takehata S, Sasaki A, Oji K, Futai K, Ota S, Makinae K, Shinkawa H: Comparison of intratympanic and intravenous dexamethasone treatment on sudden sensorineural hearing loss with diabetes. *Otol Neurotol* 2006;27:604–608.
- Kanzaki J, Inoue Y, Ogawa K, Fukuda S, Fukushima K, Gyo K, Yanagihara N, Hoshino T, Ishitoya J, Toriyama M, Kitamura K, Murai K, Nakashima T, Niwa H, Nomura Y, Kobayashi H, Oda M, Okamoto M, Shitara T, Sakagami M, Tono T, Usami S: Effect of single-drug treatment on idiopathic sudden sensorineural hearing loss. *Auris Nasus Larynx* 2003;30:123–127.
- Kara E, Cetik F, Tarkan O, Sürmelioglu O: Modified intratympanic treatment for idiopathic sudden sensorineural hearing loss. *Eur Arch Otorhinolaryngol* 2010;267:701–707.
- Kilic R, Safak MA, Oquz H, Kargin S, Demirci M, Samim E, Ozluoglu LN: Intratympanic methylprednisolone for sudden sensorineural hearing loss. *Otol Neurotol* 2007;28:312–316.
- Kopke RD, Hoffer ME, Wester D, O'Leary MJ, Jackson RL: Targeted topical steroid therapy in sudden sensorineural hearing loss. *Otol Neurotol* 2001;22:475–479.
- Lautermann J, Sudhoff H, Junker R: Transtympanic corticoid therapy for acute profound loss. *Eur Arch Otorhinolaryngol* 2005;262:587–591.
- Niedermeyer HP, Zahneisen G, Lupp P, Busch R, Arnold W: Cortisol levels in the human perilymph after intravenous administration of prednisolone. *Audiol Neurootol* 2003;8:316–321.
- Nomura Y: Otological significance of the round window. *Adv Otorhinolaryngol* 1984;33:66–72.
- Parnes LS, Sun AH, Freeman DJ: Corticosteroid pharmacokinetics in the inner ear fluids: an animal study followed-up by clinical application. *Laryngoscope* 1999;109:1–17.
- Plaza G, Herraiz C: Intratympanic steroids for treatment of sudden hearing loss after failure of intravenous therapy. *Otolaryngol Head Neck Surg* 2007;137:74–78.
- Plontke SK, Löwenheim H, Mertens J, Engel C, Meisner C, Weidner A, Zimmermann R, Preyer S, Koitschev A, Zenner HP: Randomized, double-blind, placebo-controlled trial on the safety and efficacy of continuous intratympanic dexamethasone delivered via a round window catheter for severe to profound sudden idiopathic sensorineural hearing loss after failure of systemic therapy. *Laryngoscope* 2009;119:359–369.
- Plontke SK, Siedow N, Wegener R, Zenner HP, Salt AN: Cochlear pharmacokinetics with local inner ear drug delivery using a three-dimensional finite-element computer model. *Audiol Neurootol* 2007;12:37–48.
- Silverstein H, Rowan PT, Olds MJ, Rosenberg SI: Inner ear perfusion and the role of round window patency. *Am J Otol* 1997;18:586–589.
- Van Wijck F, Staecker H, Lefebvre PP: Topical steroid therapy using the Silverstein Microwick in sudden sensorineural hearing loss after failure of conventional treatment. *Acta Otolaryngol* 2007;127:1012–1017.
- Wilson WR, Byl FM, Laird N: The efficacy of steroids in the treatment of idiopathic sudden hearing loss: a double-blind clinical study. *Arch Otolaryngol* 1980;106:772–776.

