Published in final edited form as:

Otolaryngol Head Neck Surg. 2014 April ; 150(4): 659–665. doi:10.1177/0194599813518876.

Heptanol Application to the Mouse Round Window: A Model for Studying Cochlear Lateral Wall Regeneration

Shawn M. Stevens, MD¹, Yazhi Xing, MD², Christopher T. Hensley², Juhong Zhu², Judy R. Dubno, PhD^{1,2}, and Hainan Lang, MD, PhD²

¹Department of Otolaryngology–Head and Neck Surgery, Medical University of South Carolina, Charleston, South Carolina, USA

²Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina, USA

Abstract

Objective—Identify cells supporting cochlear lateral wall regeneration.

Study Design—Prospective controlled trial.

Setting—Laboratory. Human presbycusis occurs, in part, secondary to age-related degeneration of cochlear lateral wall structures such as the stria vascularis and spiral ligament fibrocytes. This degeneration is likely linked to the diminished regenerative capacity of lateral wall cells with age. While lateral wall regeneration is known to occur after an acute insult, this process remains poorly understood and the cells capable of self-replication unidentified. We hypothesized that spiral ligament fibrocytes constitute these proliferative cells.

Subjects and Methods—To test the hypothesis, an acute ototoxic insult was created in 65 normal-hearing, young adult mice via cochlear exposure to heptanol. Sacrifice occurred at 1 to 60 days posttreatment. Auditory brainstem responses, 5-ethynyl-2'-deoxyuridine assay, and immunostaining were used to assess regeneration.

Results—Posttreatment hearing thresholds were elevated in nearly all treated mice. Selective fibrocyte apoptosis and stria injury were observed at the time of peak hearing loss around 1 to 7 days posttreatment. Cellular proliferation was detected in the region of type II fibrocytes during

© American Academy of Otolaryngology—Head and Neck Surgery Foundation

Corresponding Author: Hainan Lang, MD, PhD, Department of Pathology and Laboratory Medicine, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425, USA. langh@muscc.edu.

Reprints and permission: sagepub.com/journalsPermissions.nav

Author Contributions

Shawn M. Stevens, conception of design, acquisition of data, analysis of data, interpretation of data, drafting article, final approval; Yazhi Xing, acquisition of data, critical revision, final approval; Christopher T. Hensley, acquisition of data, final approval; Juhong Zhu, acquisition of data, final approval; Judy R. Dubno, analysis of data, critical revision, final approval; Hainan Lang, conception of design, analysis of data, critical revision, final approval.

This article was presented at the 2013 AAO-HNSF Annual Meeting & OTO EXPO; September 29–October 3, 2013; Vancouver, British Columbia, Canada.

Disclosures

Competing interests: None.

Sponsorships: None.

this time. Hearing thresholds plateaued at 7 days posttreatment followed by a significant recovery of both hearing and morphologic appearance. Permanent outer hair cell degeneration was observed.

Conclusions—Heptanol application to the round window of young adult mice is a rapid, selective, and reliable technique for investigating proliferation in the cochlear lateral wall. The data indirectly showed that spiral ligament fibrocytes may be the proliferative cells of the cochlear lateral wall. Further studies of this process are needed.

Keywords

heptanol; spiral ligament fibrocytes; cochlea; presbycusis; EdU

Age-related hearing loss (presbycusis) affects approximately two-thirds of adults older than 70 years.^{1,2} Histopathologic studies describe atrophy of cochlear lateral wall structures as the predominant mechanism underlying presbycusis.³ Studies have further shown age-related declines in endocochlear potential (EP), now thought to be related to the decline of spiral ligament fibrocytes (SLFs), cells bordering and supportive to the stria vascularis (SV) and EP maintenance.^{4–11}

Mechanisms underlying these declines remain poorly understood. Age-related loss of intrinsic regenerative capacity by lateral wall cells may be to blame. While aged cochlear cells have little regenerative ability, youthful cells have capacity for self-renewal following acute ototoxicity. Studies have shown that the lateral wall may harbor pluripotent cells able to support recovery of structure and function.^{12–15} Discerning the nature of these cells and their decline may greatly further our understanding of presbycusis.

Preliminary evidence has shown that SLFs may be closely associated with this regenerative capacity.¹⁵ However, the nature of this association is unclear. We hypothesize that SLFs are the regenerative cell population that confers regenerative capability to the cochlear lateral wall following an acute insult. The specific aims of this study were to (1) identify the proliferative cell type(s) within the cochlear lateral wall and (2) further demonstrate cochlear ability to regenerate structure/function within the lateral wall following acute insult. In support of these aims, normal-hearing, young adult mice were treated with 1-heptanol, an agent known for its targeted effects.¹⁶ Postexposure hearing and morphology were studied.

Methods

Animal Model

Research was conducted according to the Medical University of South Carolina Institutional Animal Care and Use Committee (IACUC) guidelines. Young adult (8–12 weeks old) CBA/CaJ mice of both genders were chosen as a well-established model of normal hearing. Original breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice received food and water ad libitum and were maintained on a 12-hour light/dark cycle. For all experiments, the right ear served as the experimental ear; the left served as an intra-animal control.

Anesthesia

Anesthesia via intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (20 mg/kg) was judged by toe-pinch reflex and supplemented as necessary prior to hearing assessment, surgery, and sacrifice. IP buprenorphine (0.1 mg/kg IP once 30 minutes before surgery) provided analgesia.

Auditory Brainstem Response Testing

Ears were inspected for signs of infection/obstruction. Tone pips were generated using Tucker Davis Technologies System III hardware (Gainesville, FL) and a SigGen software package. Needle electrodes were inserted at the vertex (+) and test/surgical side mastoid (–), with a ground in the control side leg. Tone pips were delivered in a sound/vibration-protected booth via a 10-mm-long (3- to 5-mm diameter) plastic tube with a tapered probe gently inserted into the external canal. Auditory brainstem responses (ABRs) were evoked at half-octave frequencies from 4 to 45 kHz using 5-millisecond tone pips with $\cos^2$ rise/fall times of 0.5 milliseconds delivered at 31/s. Sound levels were reduced in 10-dB steps from 90 to 10 dB SPL. Thresholds were defined as the lowest level (in dB SPL) at which the response peaks were clearly present and repeatable as read from stacked waveforms. Study inclusion required ABR threshold shifts at most frequencies of ≥ 20 dB. Ultimately, the entire available complement of 65 mice was tested over the 2-month period of the study. Fifty met inclusion criteria and were selected for further testing. As the study was exploratory in nature, initial power analysis was not conducted. Thresholds were measured preoperatively and longitudinally until a selected date of sacrifice at postoperative day (POD) 1, 2, 3, 4, 7, 14, 30, or 60.

EdU Assay

As an indicator of cellular proliferation, 5-ethynyl-2'-deoxyuridine (EdU), a thymidine analogue known to be incorporated into replicating cellular DNA, was used. An IP EdU dose of 50 mg/kg was administered on perioperative days –1, 0, and +1 based on established protocols.¹⁷ After sacrifice, tissue sections were exposed to a copper catalyzed “click” reaction entailing [3 + 2] cycloaddition of a fluorescent azide to an EdU ethynyl group. This was detectable by fluorescent microscopy.

Surgical Protocol

Aseptic technique conformed to IACUC guidelines. The postauricular tissue was prepped and scrubbed with betadine. A heating pad maintained body temperature at 36°C to 38°C. A specially designed bite block/head holder was used. The tympanic bulla was dissected as previously described.^{18,19} Using a dental drill, the bulla was perforated in the dorsal/caudal quadrant until the stapedial artery and round window niche (RWN) were visualized. One drop (20–30 μ L) of 98% 1-heptanol (Sigma, Atlanta, GA) was applied directly to the RWN with a blunt tip, 26-gauge needle under direct microscopic guidance. This solution was wicked free and replaced twice at 10-minute intervals. After final exposure, a small fragment of 1-heptanol-soaked gelfoam was packed into the RWN according to protocols established by Havenith et al.²⁰ Closure was at the skin only with a simple running synthetic

monofilament. A few mice underwent surgical exposure of the bulla without heptanol application.

Inner Ear Tissue Preparation

Sacrifice was performed at predetermined, postexposure time points. Sagittal hemisection of the cranium was followed by blunt removal of brain tissue. The cochlear/vestibular complex was dissected free. Four percent paraformaldehyde fixation for 1.5 hours at room temperature was followed by decalcification in 0.1 M EDTA at 4°C for 48 to 72 hours. Cryoprotection in 30% sucrose/phosphate-buffered saline (PBS) ensued followed by frozen sectioning. Tissue from treatment and control ears were co-mounted. Tissue sections then underwent staining and/or EdU assay. For surface preparations of the basilar membrane, we used a previously described technique.²¹ Briefly, the basilar membrane was dissected from the fixed cochlea and stained with fluorescein isothiocyanate green (FITC)-labeled phalloidin (1 µg/mL in PBS-labeling filamentous actin) for 20 minutes and propidium iodine (PI; 1 µg/mL in PBS-labeling nuclei) for 10 minutes.

Immunofluorescence Staining

The staining protocol is reported elsewhere.¹⁹ Tissue sections were dried under a hood, followed by fixation in acetone/methanol at -20°C. A permeability bath in 0.2% Triton-X100 for 4 minutes ensued, followed by washing and incubation with anti-rabbit Kir-4.1(1:400, APC-035, Alomone, Jerusalem, Israel) overnight at 4°C. The following day, sections were washed and incubated with a biotinylated secondary antibody for 3 hours at 4°C. Final incubation was with FITC-conjugated Avidin D (1:150; Vector, Burlingame, CA) at room temperature. Nuclear counterstaining was with PI. Additional control staining included omission (-) or substitution with similar dilutions of nonimmune serum of the appropriate species.

Microscopy

The sections were examined either with a Zeiss Axio Observer or a Zeiss LSM5 Pascal confocal microscope (Carl Zeiss, Inc). Excitation with 488-nm light yielded a fluorescence emission maximum of ~520 nm. Micrographs of frozen sections were taken at 40× magnification on a Zeiss Axio Observer D1 with a digital AxioCam camera.

Calculation of Staining Luminance

Kir 4.1 staining intensity was assessed using the Zeiss Axio Observer software package (AxioVision 4.8, Carl Zeiss Inc). Lateral wall structures of the middle turn were viewed under 40× oil immersion. Exposure times of 10 to 20 milliseconds were used. Image capture was in grayscale for uniformity of measurements. Using the circular measurement tool, a 470 µm² area (radius 13 µm) was drawn over 3 separate points within the bounds of the SV. The intensity of phosphorescence (luminance) within each circle was recorded and averaged.

Statistical Analyses

Mean values $\pm$ standard deviation (SD) were calculated for ABR thresholds at all frequencies. Data were grouped according to postexposure time point. Statistical comparisons were made between groups using unpaired Student *t* tests. The relative luminance (RL) of Kir 4.1 in treatment ears versus control ears was calculated via [(mean luminance treatment ear/mean luminance control ear) $\times$ 100]. The RL values were grouped into POD1, 2, 3, 4, 7, and 14+ (data combined for days 14–60). A mean $\pm$ SD was calculated for each group. Statistical analysis between groups was performed with 2-tailed, unpaired Student *t* tests. *P* $\leq$.05 was considered significant.

Results

Sixty-five mice were treated with 1-heptanol. The overall success rate was 77% (50/65), with 15 mice excluded for insufficient threshold shifts. Significant increases in pip tone thresholds (Figure 1) were observed across all frequencies (*P* $<$.001) except 4 and 5.6 kHz (*P* = .78 and .08, respectively). When comparing pretreatment to POD 14 thresholds, significant increases were observed only at 8, 11.3, and 45.2 kHz (*P* = .016, .022, and .016, respectively). When comparing thresholds at POD1 and POD14, significant threshold recoveries were observed at 8, 11.3, 16, and 22.6 kHz (*P* = .02, .03, .03, and .006, respectively).

Staining with Kir 4.1 demonstrated highly reproducible differences between treatment and control ears. Overall staining intensity was markedly decreased within the SV and among the SLFs between PODs 1 and 3. During this period, large vacuolated zones devoid of Kir 4.1 fluorescence were also noted within the SV (Figure 2) along with a marked decrease in Kir 4.1 staining intensity in the area of type II and type IV SLFs (Figure 3). Evidence of disrupted nuclear integrity and chromosomal condensation/blebs typical of cellular apoptosis was also seen on PI counterstains (Figure 3).

Staining conducted at a later POD saw these changes diminish noticeably or resolve. Beyond POD4, there was no evidence of apoptotic changes. Vacuolated zones of Kir 4.1 staining resolved markedly beyond POD7 and were absent entirely at 14 days. When Kir 4.1 RL was quantified, treated ears demonstrated an initial trough followed by a significant shift (*P* = .001) back toward control luminance after POD7 (Figure 4).

The EdU⁺ cells were demonstrated in nearly all treated ears measured for this purpose. Proliferating cells were visualized in greatest density (mean, 10; range, 2–15 EdU⁺ cells/per section) at POD2–4, with none detected beyond POD7 (Figure 5). Only 0–1 EdU positive cells per section were detected in control ears. The zone occupied by types II and IV fibrocytes had the vast majority of EdU⁺ cells. A few EdU⁺ cells were detected in the intermediate/basal stromal layers of the SV. Few to no EdU⁺ cells were detected in control ears, and none were seen in the lateral wall. Some EdU⁺ cells were detected in the region of the spiral ganglion, but findings were identical in both treatment and control ears. No other cochlea structures demonstrated EdU positivity.

Assessment of heptanol's effects on inner and outer sensory hair cells via basilar membrane surface preps demonstrated a notable disruption in outer hair cell (OHC) density and staining intensity in treated ears at all days tested (data not shown). When comparing a control ear with a treatment ear at day 28 after heptanol exposure, loss of OHCs was seen in the basal turn of treated ears (Figure 6). Loss of inner cells was not detected.

Discussion

Study of cochlear regenerative capacity has proven difficult. The microscopic anatomy and delicate interscalar ion balances of this structure demand meticulous study design to avoid confounding of data by iatrogenic effect. Several models using various acoustic and chemical insults have been described to date. Common to all is an intent to induce rapid, precise, and recoverable insults. Furosemide, aminoglycosides, and ouabain have all been described for these purposes.^{15,19} None are ideal for investigation of cochlear lateral wall regeneration. Of these agents, only furosemide has a targeted lateral wall effect, but its relative impotency requires use of an infusion pump to achieve consistent results.¹⁵

The second pitfall investigators face is selection of an acceptable delivery mechanism. Confounding of data may stem from chemical and/or mechanical insults. Both reproducibility and survivability of the model must be regarded. Damage mediated via acoustic exposure, while noninvasive, often creates a lesion of limited reproducibility.¹² Parenteral and IP delivery are associated with systemic toxicity. Currently described surgical procedures, while reliable, often directly violate the intrascalar compartment via some form of cochleostomy.^{15,16,22–24}

Given these aforementioned difficulties in study design, few investigators have managed to describe a precisely targeted insult followed by both structural and functional recovery. Fewer still have successfully targeted cochlear lateral wall structures. Roberson and Rubel¹² were of the first to demonstrate cellular proliferation within the lateral wall with subsequent hearing recovery after noxious acoustic stimuli in gerbils. Yamashita et al¹³ treated mice with aminoglycosides noting OHC loss and signs of cellular proliferation within the spiral ligament. Further investigation of these findings by Lang et al using a continuous furosemide infusion demonstrated recoverable declines in hearing and EP as well as evidence of cellular turnover among SLFs.¹⁵

The above works and others informed the development of our model. Surgical exposure with round window diffusion has been previously described as an ideal means for inducing atraumatic ototoxic lesions but may be inconsistent.^{18,19,25–29} The choice of heptanol, a gap junction uncoupler, was based on prior studies.^{16,30,31} All of these found heptanol to decrease EP selectively and disrupt interscalar potassium gradients, suggesting a targeted effect on cochlear lateral wall cells. Goddard et al (unpublished data) further confirmed a targeted effect at the SV and SLFs and were the first to detect partial functional recovery in the cochlear lateral wall after heptanol exposure.

In review of our results, the model did inflict a rapid, reproducible, and precise insult. The addition of a gelfoam carrier was felt to enhance experimental success rates. Hearing loss

was not felt to be secondary to surgical exposure as mice undergoing surgery without heptanol exposure had no threshold shift (data not shown). While the exact mechanism of heptanol on lateral wall cells remains unknown, morphologic analyses indicated highly selective damage to the SV and associated SLFs and appreciable sparing of other cochlear structures. Non-lateral wall damage was limited only to the OHCs. This did not recover. We postulate, however, that this effect was related more to a temporal decline in EP/nutrient support associated with stria and SLF damage than to direct heptanol toxicity. The incomplete hearing recovery also was likely due, in part, to OHC losses as the SLFs and SV appeared to fully recover.

Further support of a targeted heptanol effect on SLFs stems from the observation of selective apoptosis among cells in zones known to be occupied by type II and IV SLFs.^{4,7} This may easily be explained by the exposure model, as round window perfusion would lead to high heptanol concentrations within the scala tympani along the basilar membrane in this region. A notable lack of apoptosis among other cells occupying the basilar membrane (neurons, dendritic cells, inner hair cells, support cells) also suggests a selective ototoxic effect.

The precise identity of the proliferating cells observed and their role in the recovery of morphology and hearing cannot be fully ascertained from these data. Counterstains known to differentiate fibrocytes, at least as applied in this study, were not specific enough to individually label the EdU⁺ cells. However, based on the anatomic and temporal patterns of Kir 4.1 staining, some inferences may be made. For instance, EdU⁺ cells were detected almost exclusively in the region of type II and IV SLF apoptosis (also the time of greatest hearing loss) around POD3-4. At dates following the observation of EdU⁺ cells, a full morphologic return to baseline was observed among the SV/SLFs in tandem with a partial hearing recovery. Given the paucity of other known cellular subtypes in the region of EdU positivity, few candidates exist aside from a population of clonal SLFs. The nature of the postexposure regeneration is also uncertain, as recovery may have been secondary to either SLFs replenishing their own ranks or possibly de-differentiation into pluripotent progenitor cells. Ongoing studies will be required to determine the exact nature of this process and definitively identify the proliferating cell type identified by this model.

Conclusion

To our knowledge, these results represent one of the few demonstrations of an acute, reliable, and recoverable ototoxic insult targeted to the cochlear lateral wall. Recovery of both form and function was observed. The data indirectly support the hypothesis that SLFs may represent a pluripotent, or at least proliferative, cell line responsible for the cochlear lateral wall self-repair. Further studies are required to determine precisely the time course of lateral wall functional recovery and its underlying molecular mechanisms.

Acknowledgments

Funding source: This study was supported, in part, by NIH R01 DC012058 (to H.L.) and NIH P50 DC000422 (to H.L. and J.R.D.).

References

1. Lin FR, Thorpe R, Gordon-Salant S, et al. Hearing loss prevalence and risk factors among older adults in the United States. *J Gerontol A Biol Sci Med Sci*. 2011; 66(5):582–590. [PubMed: 21357188]
2. Lin FR, Yaffe K, Xia J, et al. Health ABC Study Group. Hearing loss and cognitive decline in older adults. *JAMA Intern Med*. 2013; 25:173(4):293–299. [PubMed: 23337978]
3. Schuknecht HF, Gacek MR. Cochlear pathology in presbycusis. *Ann Otol Rhinol Laryngol*. 1993; 102:1–16. [PubMed: 8420477]
4. Spicer SS, Schulte BA. Differentiation of inner ear fibrocytes according to their ion transport related activity. *Hear Res*. 1991; 56(1–2):53–64. [PubMed: 1663106]
5. Spicer SS, Schulte BA. Ultrastructural differentiation of the first Hensen cell in the gerbil cochlea as a distinct cell type. *Anat Rec*. 1994; 240(2):149–156. [PubMed: 7992881]
6. Schulte BA, Steel KP. Expression of alpha and beta subunit isoforms of Na,K-ATPase in the mouse inner ear and changes with mutations at the Wv or Sld loci. *Hear Res*. 1994; 78(1):65–76. [PubMed: 7961179]
7. Spicer SS, Schulte BA. The fine structure of spiral ligament cells relates to ion return to the stria and varies with place-frequency. *Hear Res*. 1996; 100(1–2):80–100. [PubMed: 8922982]
8. Spicer SS, Schulte BA. Evidence for a medial K⁺ recycling pathway from inner hair cells. *Hear Res*. 1998; 118(1–2):1–12. [PubMed: 9606057]
9. Cohen-Salmon M, Ott T, Michel V, et al. Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. *Curr Biol*. 2002; 12(13):1106–1111. [PubMed: 12121617]
10. Teubner B, Michel V, Pesch J, et al. Connexin30 (Gjb6)-deficiency causes severe hearing impairment and lack of endocochlear potential. *Hum Mol Genet*. 2003; 12(1):13–21. [PubMed: 12490528]
11. Hibino H, Kurachi Y. Molecular and physiological bases of the K⁺ circulation in the mammalian inner ear. *Physiology (Bethesda)*. 2006; 21:336–345. [PubMed: 16990454]
12. Roberson DW, Rubel EW. Cell division in the gerbil cochlea after acoustic trauma. *Am J Otol*. 1994; 15(1):28–34. [PubMed: 8109626]
13. Yamashita H, Shimogori H, Sugahara K, et al. Cell proliferation in spiral ligament of mouse cochlea damaged by dihydrostreptomycin sulfate. *Acta Otolaryngol*. 1999; 119(3):322–325. [PubMed: 10380736]
14. Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. *Nat Med*. 2003; 9(10):1293–1299. [PubMed: 12949502]
15. Lang H, Schulte BA, Schmiedt RA. Effects of chronic furosemide treatment and age on cell division in the adult gerbil inner ear. *J Assoc Res Otolaryngol*. 2003; 4(2):164–175. [PubMed: 12943371]
16. Suzuki M, Kikuchi T, Ikeda K. Endocochlear potential and endolymphatic K⁺ changes induced by gap junction blockers. *Acta Otolaryngol*. 2004; 124(8):902–906. [PubMed: 15513524]
17. Zeng C, Pan F, Jones LA, et al. Evaluation of 5-ethynyl-2'-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. *Brain Res*. 2010; 10:1319–1332.
18. Lang H, Schulte BA, Schmiedt RA. Ouabain induces apoptotic cell death in type I spiral ganglion neurons, but not type II neurons. *J Assoc Res Otolaryngol*. 2005; 6(1):63–74. [PubMed: 15735933]
19. Lang H, Li M, Kilpatrick LA, et al. Sox2 up-regulation and glial cell proliferation following degeneration of spiral ganglion neurons in the adult mouse inner ear. *J Assoc Res Otolaryngol*. 2011; 12(2):151–171. [PubMed: 21061038]
20. Havenith S, Versnel H, Agterberg MJ, et al. Spiral ganglion cell survival after round window membrane application of brain-derived neurotrophic factor using gelfoam as carrier. *Hear Res*. 2011; 272(1–2):168–177. [PubMed: 20969940]

21. Lang H, Schulte BA, Zhou D, Smythe N, Spicer SS, Schmiedt RA. Nuclear factor kappaB deficiency is associated with auditory nerve degeneration and increased noise-induced hearing loss. *J Neurosci*. 2006; 26(13):3541–3550. [PubMed: 16571762]
22. Dodson HC. Loss and survival of spiral ganglion neurons in the guinea pig after intracochlear perfusion with aminoglycosides. *J Neurocytol*. 1997; 26:541–556. [PubMed: 9350806]
23. Lee KS, Kimura RS. Effect of ototoxic drug administration to the endolymphatic sac. *Ann Otol Rhinol Laryngol*. 1991; 100:355–360. [PubMed: 1673834]
24. Wanamaker HH, Gruenwald L, Damm KJ, et al. Dose-related and cochlear effects of transtympanic gentamicin. *Am J Otol*. 1998; 19:170–179. [PubMed: 9520053]
25. Kimura RS, Iverson NA, Southard RE. Selective lesions of the vestibular labyrinth. *Ann Otol Rhinol Laryngol*. 1988; 97:577–584. [PubMed: 3264488]
26. Heydt JL, Cunningham LL, Rubel EW, Coltrera MD. Round window gentamicin application: an inner ear hair cell damage protocol for the mouse. *Hear Res*. 2004; 192(1–2):65–74. [PubMed: 15157964]
27. Palmgren B, Jin Z, Ma H, et al. beta-Bungarotoxin application to the round window: an in vivo deafferentation model of the inner ear. *Hear Res*. 2010; 265(1–2):70–76. [PubMed: 20184947]
28. Schmiedt RA, Lang H, Okamura HO, et al. Effects of furosemide applied chronically to the round window: a model of metabolic presbycusis. *J Neurosci*. 2002; 22(21):9643–9650. [PubMed: 12417690]
29. Husmann KR, Morgan AS, Girod DA, et al. Round window administration of gentamicin: a new method for the study of ototoxicity of cochlear hair cells. *Hear Res*. 1998; 125(1–2):109–119. [PubMed: 9833965]
30. Suzuki T, Matsunami T, Hisa Y, et al. Roles of gap junctions in glucose transport from glucose transporter 1-positive to -negative cells in the lateral wall of the rat cochlea. *Histochem Cell Biol*. 2009; 131(1):89–102. [PubMed: 18787834]
31. Matsunami T, Suzuki T, Hisa Y, et al. Gap junctions mediate glucose transport between GLUT1-positive and -negative cells in the spiral limbus of the rat cochlea. *Cell Commun Adhes*. 2006; 13(1–2):93–102. [PubMed: 16613783]

Figure 1.

Mean auditory brainstem response thresholds (in dB SPL) plotted as a function of tone pip frequency. Measurements are grouped according to pretreatment and postoperative day (POD) 1, 7, and 14. Error bars represent SD.

Figure 2. Changes in Kir 4.1 staining within the stria vascularis (StV). (A) Normal Kir 4.1 staining (green) in control (left) ear. (B) Treatment ear (right) at postoperative day 1. Nuclei counterstained with propidium iodide (red). Scale = 15 μ m.

Figure 3.

Changes in spiral ligament fibrocytes (SLFs) of treated ear versus control. (A) Control Kir 4.1 staining (green) with normal-appearing type II and IV SLFs (II). SL, spiral ligament. (B) Treatment ear showing apoptosis in these cells (arrows).

Figure 4.

Mean relative luminance of Kir 4.1 staining plotted by postoperative day (POD). POD14-28 grouped as single data point. Solid circles = means; error bars = SD. Significant recovery between POD7 and later dates ($P = .001^*$).

Figure 5.

EdU positivity in the treated ear. More than 10 EdU positive cells (arrows) among type II and IV spiral ligament fibrocytes on postoperative day 1. No EdU positivity was detected in controls (image not shown). Nuclei counterstained with propidium iodide (red). Scale = 15 μ m.

Figure 6.

Outer hair cell (OHC) disruption in treated ear versus control ear. (A) Normal staining pattern of fluorescein isothiocyanate green-labeled phalloidin-actin (green) in control ear. Nuclei counterstained with propidium iodine (red). (B) Postoperative day 28 treatment ear with disruption of OHC/nuclear staining (arrows).