Published in final edited form as:

Hear Res. 2009 July ; 253(1-2): 32–41. doi:10.1016/j.heares.2009.03.001.

Response of mechanosensory hair cells of the zebrafish lateral line to aminoglycosides reveals distinct cell death pathways

Kelly N. Owens^{a,b,c}, Allison B. Coffin^{b,c}, Lisa S. Hong^{c,d}, Keri O'Connell Bennett^{c,d}, Edwin W. Rubel^{b,c}, and David W. Raible^{a,c}

^aDepartment of Biological Structure University of Washington, Box 357420 Seattle, WA 98195-7420 USA

^bDepartment of Otolaryngology-HNS University of Washington, Box 356515 Seattle, WA 98195-6515 USA

^cV. M. Bloedel Hearing Research Center University of Washington, Box 357923 Seattle, WA 98195-7923 USA

^dDepartment of Speech and Hearing Science University of Washington, Box 354875 Seattle, WA 98105-6246 USA

Abstract

We report a series of experiments investigating the kinetics of hair cell loss in lateral line neuromasts of zebrafish larvae following exposure to aminoglycoside antibiotics. Comparisons of the rate of hair cell loss and the differential effects of acute versus chronic exposure to gentamicin and neomycin revealed markedly different results. Neomycin induced rapid and dramatic concentration-dependent hair cell loss that is essentially complete within 90 minutes, regardless of concentration or exposure time. Gentamicin induced loss of half of the hair cells within 90 minutes and substantial additional loss, which was prolonged and cumulative over exposure times up to at least 24 hr. Small molecules and genetic mutations that inhibit neomycin-induced hair cell loss were ineffective against prolonged gentamicin exposure supporting the hypothesis that these two drugs are revealing at least two cellular pathways. The mechanosensory channel blocker amiloride blocked both neomycin and gentamicin-induced hair cell death acutely and chronically indicating that these aminoglycosides share a common entry route. Further tests with additional aminoglycosides revealed a spectrum of differential responses to acute and chronic exposure. The distinctions between the times of action of these aminoglycosides indicate that these drugs induce multiple cell death pathways.

Keywords

ototoxicity; aminoglycoside; hair cell; mechanosensory; lateral line; zebrafish; hair cell

© 2009 Elsevier B.V. All rights reserved.

Corresponding author: Kelly N. Owens Department of Biological Structure, Box 357420 University of Washington Seattle, WA 98195-7420 USA Phone: 1-206-616-7467 Fax: 1-206-221-5685 kowens@u.washington.edu.

Allison B. Coffin coffina@u.washington.edu

Lisa S. Hong lisaness@gmail.com

Keri O'Connell Bennett Keri.Oconnell2@va.gov

Edwin W. Rubel rubel@u.washington.edu

David W. Raible draible@u.washington.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Mechanosensory hair cells are the key sensory cells of the auditory, vestibular and lateral line (in fish and amphibians) systems responsible for hearing, balance and various aspects of swimming behavior. Loss of mechanosensory hair cells is a leading cause of hearing loss and balance deficits in humans. Aging, trauma, intense or prolonged noise exposure, and certain therapeutic drugs are thought to be the major causes of hair cell death, and thus result in hearing and balance disorders (Saunders, et al., 1991; Nakashima, et al., 2000; Rauch, et al., 2001; Nelson & Hinojosa 2006; Rybak, et al., 2007). Of these, we focus on aminoglycosides, a class of antibiotics long recognized to induce hair cell death (reviewed in Forge & Schacht 2000).

In bacteria, aminoglycosides bind rRNA subunits and interfere with translation by affecting ribosome translocation, peptide release, mRNA coding and ribosome recycling (Magnet & Blanchard 2005; Borovinskaya, et al., 2007, 2008). Divergence of rRNA sites in the eukaryotic ribosome may explain the relative insensitivity of most eukaryotic cells to aminoglycosides. However, while the ribosomal residues targeted by aminoglycosides in bacteria have diverged in the eukaryotic cytoplasmic ribosome, the analogous residues are present in the mitochondrial ribosome (Lynch, et al., 2001), leading to the proposal that the mitochondrial ribosome may be an aminoglycoside target (Hutchin & Cortopassi 1994). Furthermore, mutations of mitochondrial 12S rRNA and tRNAs have been associated with aminoglycoside-associated hearing loss in humans (Fishel-Ghodsian 2003; Yan, et al., 2005; Guan, et al., 2006). Production of reactive oxygen species (ROS) occurs with aminoglycoside exposure and may thereby induce hair cell damage (Hirose, et al., 1997; Schacht 1999; Rybak & Whitworth 2005) and activation of caspase-dependent and caspase independent cell death pathways (e.g., Cunningham, et al., 2002; Cheng, et al., 2003; Mangiardi, et al., 2004; Jiang, et al., 2006). Gentamicin can interact with iron to form complexes that may contribute to hair cell toxicity (Schacht 1993; Sha & Schacht 2000). Membrane targets for aminoglycosides have also been proposed (Au, et al., 1987; Shakil, et al., 2008). A better understanding of the mechanisms involved in hair cell death may lead to methods to prevent such loss.

Initial observation of ototoxicity and nephrotoxicity in humans following streptomycin treatment prompted development of alternative aminoglycosides for clinical use (Hawkins & Lurie 1952; Rizzi & Hirose 2007; Forge & Schacht, 2000). While some aminoglycosides show milder ototoxicity, all induce hair cell death albeit to differing degrees and with tissue-dependent distinctions between cochlear and vestibular hair cells (e.g., Smith, et al., 1977; Wersall 1980; Wanamaker, et al., 1999). Current use of aminoglycosides in the U.S. is primarily limited to particular patient populations (e.g. neonates and patients with cystic fibrosis, tuberculosis or Meniere's disease) but remains widespread globally as an inexpensive antibiotic (Forge & Schacht 2000).

Experimentally, numerous studies also use aminoglycoside-induced hair cell death to study regeneration and the effects of hair cell loss on spiral ganglion cell viability and CNS development and integrity (e.g., Leake, et al., 2008; Parks, et al., 2004; Tucci & Rubel 1990; Matsui & Cotanche 2004; Bermingham-McDonogh, et al., 2001; Hernandez, et al., 2007; Ma, et al., 2008; Izumikawa, et al., 2008). The majority of work in a given experimental system has used a particular aminoglycoside (predominantly gentamicin in chicken, kanamycin in guinea pig, amikacin or tobramycin in mouse). This confounds interpretation between systems of whether differences are either species- or drug-specific. There have been relatively few studies comparing the impact of various aminoglycosides within a single experimental system (Dulon, et al., 1986; Nakashima, et al., 2000; Selimoglu, et al., 2003).

Studies in mammalian systems have often been limited to a single concentration or treatment paradigm (e.g. Aran, et al., 1982; Day, et al., 2007). The zebrafish lateral line offers a useful model system to rapidly evaluate hair cell death induced by different aminoglycosides across a range of concentrations and times.

We have used the zebrafish lateral line as a model system for studying hair cell death, protection and regeneration because of the ease of visualizing hair cells *in vivo* and the utility of zebrafish as a genetic system (Harris, et al., 2003; Murakami, et al., 2003; Santos, et al., 2006; Ou, et al., 2007; Chiu, et al., 2008; Ma, et al., 2008; Owens, et al., 2007; 2008). The lateral line is a mechanosensory organ comprised of a series of clusters of hair cells and support cells called neuromasts located on the outer surface of the head and body that functions to detect perturbation in the surrounding water current (Coombs & Montgomery 1999; Dambly-Chaudiere, et al., 2003; Montgomery, et al., 2000). Our prior work on the earliest response to neomycin in the zebrafish lateral line indicates that the mitochondrion is an early target (Owens, et al., 2007). Swelling of mitochondria and depolarization of mitochondrial membrane potential was observed within 15-30 min of exposure to neomycin in the zebrafish lateral line. In other work we have identified several genetic and small molecule modulators that protect against neomycin-induced hair cell death (Owens, et al., 2008). Here we address how other aminoglycosides act in the zebrafish lateral line and find evidence for at least two temporally distinct mechanisms of hair cell loss.

MATERIALS AND METHODS

Animals

Larval zebrafish (*Danio rerio*) were produced through paired mating of *AB wildtype fish unless otherwise noted. Animals were tested at 5-6 days post-fertilization (dpf) and held in an incubator at 28.5°C during treatments. A 50 ml conical tube with one end cut off and a mesh-covered bottom was used as a transfer device (Harris, et al., 2003). Larvae were immersed in 6-8 ml of embryo medium (EM: 1 mM MgSO₄, 0.15 mM KH₂PO₄, 1 mM CaCl₂, 0.5 mM KCl, 15 mM NaCl, 0.05 mM Na₂HPO₄, and 0.7 mM NaHCO₃ in dH₂O) in a 6 well culture plate. Larvae mutant for the *sentinel* (*snl*) gene (Owens, et al., 2008) and their wildtype siblings (both *snl*/+ and +/+) were produced by crossing *snl*/+ parents heterozygous for the *w38* allele. Sinusoidal body shape was used to prospectively identify mutants.

Drug exposure

Stock solutions of aminoglycosides were diluted in EM to final concentrations of 0.1 µM to 400 µM (neomycin, gentamicin, streptomycin, Sigma; tobramycin, Fluka; amikacin, Bedford Laboratories; kanamycin, Abraxis Pharmaceutical Products). For acute exposure, larvae were mock-treated (embryo medium only) or drug treated for 30 min, rinsed in fresh EM four times, then held in the final wash for 1, 2.5, 5.5, 23.5 hours (total treatment times of 1.5, 3, 6, 24 hours). For chronic treatments, larvae were treated with drug or embryo media (mock treated) for 1.5, 3, 6, or 24 hours continuously, rinsed rapidly four times in EM, anesthetized with buffered tricaine (Westerfield 2000) and examined live or euthanized for subsequent fixation. For analysis of kanamycin, streptomycin, amikacin, and tobramycin, zebrafish larvae were treated either acutely for 30 min with one hour recovery or chronically for 6 hours continuously. For experiments with the otoprotective agent PROTO1 (Owens, et al., 2008), larvae were pretreated for 1 hr in embryo medium supplemented with 10 µM PROTO1, then neomycin or gentamicin was added and fish were treated acutely (30 min, followed by 1 hr recovery in fresh EM without PROTO1) or chronically (6 hrs continuous treatment with aminoglycoside and PROTO1). Hair cell survival was assessed with DASPEI labeling. For experiments with amiloride and acute aminoglycoside exposure, zebrafish larvae were pretreated for 15 minutes with 1 mM amiloride (Sigma) in embryo medium,

transferred to 200 μ M neomycin or gentamicin plus 1 mM amiloride for 30 min, washed four times in embryo medium and held in the final rinse for one hour prior to evaluation of hair cells with DASPEI. For chronic exposure in the presence of amiloride, larvae were treated with 100-500 μ M amiloride and either 100 μ M neomycin or 50 μ M gentamicin for six hours prior to evaluation of hair cells with DASPEI.

DASPEI labeling of hair cells

For rapid *in vivo* assessment, hair cells of larvae were labeled with the vital dye DASPEI (0.005% in EM, 2-[4-(Dimethylamino)-styryl]-1-ethylpyridinium iodide, Sigma St. Louis MO). As described previously (Harris, et al., 2003), DASPEI was added to the embryo medium for 15 min prior to anesthetization. DASPEI labeling was evaluated on a Leica epifluorescent microscope equipped with a DASPEI filter (Chroma Technologies, Brattleboro, VT) for ten neuromasts (SO1, SO2, IO1-4, M2, MI1, MI2 and O2; Raible & Kruse 2000). Each neuromast was assigned a score of 0 (no/little staining), 1 (reduced staining) or 2 (wildtype-like staining) for a composite score of 0 - 20. For each treatment group, 5 - 12 fish (50-120 neuromasts) were assessed. DASPEI scores were averaged for each group and normalized as a percentage of mock-treated controls.

FM1-43 labeling of hair cells

For counts, hair cells were pre-labeled with the mechanotransduction marker FM1-43 FX (3 μ M in EM; Molecular Probes, Eugene, OR) for 45 - 60 seconds. The larvae were then quickly rinsed four times with EM and held in the final rinse for 20 minutes prior to drug treatment. Following drug treatment, animals were euthanized and fixed in cold 4% paraformaldehyde for 1 hour, fresh cold fixative overnight, and then washed 3 times with 0.1M PBS (pH 7.2). For examination, larvae were mounted in Fluoromount-G on double bridge coverslips (created by gluing two 22 mm $\times$ 22 mm squares at each end of a 24 mm $\times$ 60 mm coverglass) to prevent the neuromast from being crushed and to allow viewing of both sides of the animal. Hair cells of the SO2 and IO2 neuromasts were counted on each side of the animal (i.e. 4 neuromasts/fish) using an epifluorescent Zeiss AxioPlan microscope with a 40x objective. 10-30 fish were evaluated per treatment per recovery time. Hair cell survival was denoted by FM1-43 FX positive cytoplasm surrounding the nucleus and intact cell morphology. Data was analyzed by 2-way or 1-way ANOVA, with Bonferroni corrected post-hoc comparisons using Prism v5.0 (GraphPad Software, San Diego, CA) and analysis is shown in Supplemental Figure 1.

Animal care and experimental procedures were reviewed and approved by the University of Washington Institutional Animal Care Committee.

RESULTS

Response of hair cells to gentamicin differs from neomycin

To evaluate the response of hair cells to different aminoglycosides, we treated 5 dpf zebrafish larvae with neomycin or gentamicin at varying concentrations for 30 min followed by 1 hr recovery in normal embryo medium. We then labeled hair cells with the vital dye DASPEI. Groups of animals were evaluated for each condition and their average score was compared to that of mock-treated control animals. The response to neomycin was rapid and concentration-dependent (Fig 1A, solid line). After exposure to 200-400 μ M neomycin, there was little DASPEI labeling remaining and few hair cells survived. These data replicate earlier results (e.g., Harris, et al., 2003; Owens, et al., 2008). In contrast, the response to gentamicin was attenuated (Fig 1A, dashed line). With 200-400 μ M gentamicin exposure, nearly 60% of the hair cell staining was retained. This acute response of hair cells to gentamicin differed substantially from that of neomycin (2-way ANOVA revealed

significant main effects of both drug concentration and drug type as well as a significant interaction term; $p < 0.0001$). Substantial additional damage was observed after continuous treatment with gentamicin for 6 hours (Fig 1B). This result was also supported by 2-way ANOVA with significant main effects of drug, concentration and an interaction ($p < 0.0001$). While neomycin elicited little additional damage with prolonged treatment, prolonged gentamicin treatment resulted in significantly increased hair cell loss. Concentrations of gentamicin as low as 50 μM resulted in almost 80% damage as evaluated by DASPEI staining.

This delayed response of hair cells to gentamicin in comparison to neomycin might reflect either a difference in the speed of action or cumulative effects. To test these hypotheses, we evaluated the results with two additional experimental protocols. In the first protocol (hereafter called “chronic” exposure), zebrafish larvae were exposed to aminoglycoside continually for 0.5-24 hours to detect cumulative effects. In the second protocol (hereafter called “acute” exposure), zebrafish larvae were exposed to aminoglycoside for 30 minutes, rinsed with fresh embryo medium and held for varying length of time (1-24 hours from onset of treatment). In each protocol, animals were pre-labeled with a fixable mechanotransduction marker, FM1-43FX (Gale, et al., 2001; Marcotti, et al., 2005; Santos, et al., 2006; Owens, et al., 2008), treated with drug, euthanized and fixed and the number of hair cells was counted in four neuromasts (see Methods). By pre-labeling hair cells before treatment, we avoided counting regenerated hair cells, which begin to arise by 24 hours after damage (Ma, et al., 2008).

Chronic exposure to neomycin and gentamicin again reflected differences in response to the two antibiotics. The responses of hair cells to 50 μM or 200 μM neomycin were essentially unchanged with longer drug exposure. No significant additional hair cell death was observed between 3 hours and 24 hours (Fig 2A and C, solid lines; one-way ANOVA). By contrast, loss of hair cells increased with prolonged gentamicin exposure (Fig 2A and C, dashed lines). Gentamicin induced significant additional hair cell death from 1.5 to 24 hours exposure time (one-way ANOVA, $p < 0.0001$). Hair cell loss due to chronic gentamicin exposure was relatively concentration independent. That is, chronic exposure to modest, 50 μM (Fig. 2A) concentration of gentamicin elicited levels of cell death similar to concentrations 4-8 fold higher (200 μM , Fig. 2C; 400 μM , not shown; 2-way ANOVA, $p > 0.05$). Moreover, low and high concentrations affected hair cell loss on a similar time scale, predominantly between 3 and 6 hours.

To test whether the prolonged period of hair cell loss after chronic gentamicin exposure was due to a prolonged cell death process rather than to cumulative exposure, animals were exposed acutely to 50 μM or 200 μM aminoglycoside for 30 min, rinsed and then held in fresh embryo media for up to 24 hours. Loss of hair cells was unchanged with longer recovery time following exposure to neomycin (Fig 2B and 2D solid lines, respectively). That is, maximal hair cell loss was induced within 90 min of exposure to the drug and little additional loss occurred afterwards. These data are supported by 2-way ANOVA. For 50 μM neomycin, there was no evidence of increased loss at any time after 1.5 hr ($p > 0.05$) and for 200 μM neomycin there was no significant difference after 3 hr ($p > 0.5$). In contrast, additional loss of hair cells occurred with time following exposure to gentamicin (Fig 2B and 2D dashed lines, respectively, 2-way ANOVA $p < 0.0001$ with a main effect of time and concentration). These results demonstrate that additional damage occurs after drug has been washed out.

Although these experiments demonstrate that there is some gentamicin-induced damage that occurs from acute exposure, considerably more damage occurs after chronic exposure. This point is made most clearly when hair cell loss after both treatments is compared for 50 μM

gentamicin exposure. While hair cells continue to be lost 6 hours after acute exposure, a substantial number remain. In comparison, almost all hair cells are lost after a 6-hour chronic treatment (compare Fig 2B dashed line to Fig 2A dashed line).

These data lead us to suggest that there are at least two distinct processes resulting in hair cell death: an early, rapid process (<1.5 hours) and a later, slower process (between 1.5 hours and 24 hours). Neomycin is very effective in triggering the rapid process, and is less effective at triggering the second slow process. Gentamicin kills hair cells via both processes.

Protective mutants and drugs block initial rapid process of hair cell death but not the slower second process

We next tested whether these proposed rapid and slow processes of hair cell death could be distinguished with genetic and small molecule tools. We previously identified mutants in zebrafish and small drug-like molecules that protect lateral line hair cells against neomycin-induced hair cell death (Owens, et al., 2008). The mutant *sentinel* (*snl*) appears to block the action of neomycin, although it is not known whether this effect is direct or indirect. To determine whether this mutation also protected against gentamicin-induced hair cell death acutely, we exposed *snl* homozygotes or their wildtype siblings to gentamicin for 30 min with a 1 hr recovery and assessed the hair cell loss by DASPEI staining (Fig 3A). As with wildtype fish, wildtype siblings showed partial hair cell loss, while homozygous *snl* mutants exhibited complete protection against gentamicin under the acute exposure protocol. This data is supported by 2-way ANOVA with a main effect of genotype, gentamicin concentration and an interaction ($p < 0.0001$). With chronic exposure to gentamicin for 6 hr, *snl* mutants responded similarly to their wildtype siblings (Fig 3B), with concentration-dependent loss of DASPEI labeling that were indistinguishable from wildtype animals. Analysis by 2-way ANOVA indicates there is a main effect of genotype, drug concentration and a significant interaction between genotype and drug concentration ($p < 0.0001$). Bonferroni post-hoc comparisons indicate that the differences between mutants and siblings without drug or treated chronically with 200 μ M gentamicin are not significant ($p > 0.05$).

Pretreatment with the small drug-like molecule PROTO1 protects against acute neomycin damage (Owens, et al., 2008). We tested whether PROTO1 offered protection against gentamicin under acute or chronic exposure conditions (Fig 4). Under an acute exposure paradigm (30 min in drug and 1 hr recovery), animals treated with 10 μ M PROTO1 showed protection, with retention of hair cells as compared to those exposed only to gentamicin (Fig 4A, 2-way ANOVA with main effects of otoprotectant and aminoglycoside concentration and interaction, $p < 0.0001$). By contrast, PROTO1 conferred only modest, if any, protection from chronic exposure to gentamicin (Fig 4B). These data are supported by 2-way ANOVA with main effects of otoprotectant and aminoglycoside ($p < 0.0001$) and an interaction ($p < 0.01$). Bonferroni post-hoc comparison indicate only 200 μ M gentamicin ($p < 0.001$) is significantly different between controls and animals treated with PROTO1. Taken together, these results support the idea that there are multiple cellular processes that cause aminoglycoside-induced hair cell death, and neomycin elicits only early processes while gentamicin elicits early and later processes.

The mechanotransduction channel blocker amiloride inhibits both neomycin and gentamicin induced hair cell death

We considered whether differences in entry of aminoglycosides could account for the differences in time course observed between neomycin and gentamicin. Entry of aminoglycosides via the mechanotransduction channel is supported by work in bullfrog and mammalian systems (Steyger, et al., 2003; Dai & Steyger 2008). To test whether there is a

distinction in mechanotransduction-dependent entry of gentamicin versus neomycin, we used the mechanosensory channel blocker amiloride. We treated zebrafish larvae with amiloride 15 min prior to exposure to a combination of aminoglycoside and amiloride. Loss of hair cells was reduced in the presence of 1mM amiloride when animals were treated with either 200 μ M neomycin or gentamicin acutely (Fig 5A, 30 min exposure with washout and one hour recovery time). These data are supported by 2-way ANOVA with a main effect of amiloride presence and aminoglycoside ($p<0.0001$). Treatment with amiloride alone versus mock treated controls does not show a significant effect on hair cell survival (Bonferroni post-hoc analysis, $p>0.05$).

Amiloride inhibited hair cell loss in a concentration-dependent manner when zebrafish were treated chronically with neomycin or gentamicin (Fig 5B, 6 hour continuous exposure). These data are supported by 2-way ANOVA with a main effect of amiloride concentration, aminoglycoside and an interaction term ($p<0.0001$). These data suggest that blocking the mechanotransduction channel is sufficient to block slower process(es) of hair cell death induced by chronic gentamicin treatment.

Response to other aminoglycosides

We next tested whether other aminoglycosides induce hair cell death in the zebrafish lateral line. We evaluated the response of hair cells to the aminoglycosides amikacin, kanamycin, streptomycin, and tobramycin (Fig 6A-D respectively) by DASPEI labeling following acute and chronic exposure. For acute exposure, animals were exposed to drug for 30 min followed by a one hour recovery. For chronic exposure, animals were exposed to drug continually for 6 hours. Acute exposure to amikacin, kanamycin or streptomycin caused only modest reduction in hair cell labeling relative to mock-treated controls (Fig 6A-C, dashed lines). There was no statistical difference between the hair cell response to acute amikacin, kanamycin or streptomycin (2-way ANOVA, $p>0.05$). On the other hand, clear concentration-dependent loss of hair cell staining was observed with acute exposure to tobramycin (Fig 6D, dashed lines) although the loss of hair cell staining was still significantly less than that observed with neomycin (Fig. 1A). These data are supported by 2-way ANOVA with a main effect of drug, concentration and interaction ($p<0.0001$).

With chronic exposure to amikacin, kanamycin, streptomycin or tobramycin, hair cell staining was reduced to a greater extent than that observed with acute exposure (Fig 6A, C, D, solid lines versus dashed lines). Notably, although acute exposure to kanamycin led to only modest loss of hair cell staining, chronic exposure to kanamycin resulted in markedly greater hair cell death as compared to acute exposure (Fig 6B, dashed and solid lines) indicating that the hair cell death induced by this aminoglycoside may be affecting predominantly the second slower process. These data are supported by 2-way ANOVA with main effects of drug, concentration and interaction ($p<0.0001$). These results demonstrate that different aminoglycosides show markedly different ranges of effects to either acute or chronic exposure, with neomycin causing damage predominantly during the first, rapid process, gentamicin causing damage during both processes, and kanamycin effecting mainly a later, slower process.

DISCUSSION

The experiments reported here suggest that aminoglycoside-induced hair cell death in the zebrafish lateral line appears to occur by at least two processes: first, a rapid process (or processes) in which hair cells die within 30-90 minutes and, second, a slower process (or processes) that kills most of the remaining hair cells if exposure time is sufficient (3-6 hours). Rapid hair cell death is observed with all of the aminoglycosides tested, although the amount of death induced by a particular aminoglycoside differs. Neomycin shows little

appreciable increase in hair cell death beyond the first 90 min whereas the other aminoglycosides tested have significant hair cell loss by this second process. The distinctions between these processes and potential interpretations are discussed below. It should be underscored at this time we do not know which cellular process or processes lead to these forms of aminoglycoside-induced hair cell death. These processes will be a subject of continued investigation.

We suggest that our observations are best explained by a model where hair cell death occurs by two or more mechanisms; one mechanism results in an early, rapid phase that occurs over 30-90 minutes and a second mechanism that is first evident between 3-6 hours and continues for at least 24 hours, perhaps much longer. Several observations support this conclusion. First, we observe different temporal kinetics in response to different aminoglycosides. Second, mutations and hair cell protective drugs act differently on these processes. The hair cell protectant PROTO-1 and mutant *snl* block the first process but neither blocks the second process. These observations suggest that the first and second processes are separable. Our results suggest that variations in response between aminoglycosides reflect both differences in the extent that a particular aminoglycoside induces hair cell death but also the degree to which it triggers different cell death processes. While we have evidence for at least two processes impacting the route of hair cell death, we cannot rule out that there may be more than two pathways involved. Whether the slow hair cell death induced by different aminoglycosides (gentamicin, kanamycin, streptomycin) is due to a common later cell death pathway or multiple pathways is not known.

Our observation that amiloride blocks both neomycin- and gentamicin-induced hair cell loss both acutely and chronically suggests that there is a common, mechanotransduction-dependent route of entry for both neomycin and gentamicin in the zebrafish lateral line. Gale et al. (2001) and Marcotti et al. (2005) demonstrated that the mechanotransduction channel blocker FM1-43 reduces neomycin and dihydrostreptomycin hair cell toxicity in mouse, respectively. Previous observation that gentamicin tagged with the fluorophore Texas Red enters hair cells in less than 1 min is consistent with this entry route (Steyger, et al., 2003). Fluorescently-labeled gentamicin also enters hair cells of the zebrafish lateral line within 1 min. (Santos, et al, 2006; Owens, et al. 2007). While multiple entry routes of these drugs are possible, the observation that amiloride can inhibit the second wave of hair cell loss induced by chronic gentamicin treatment suggests that these aminoglycoside share a common entry route. Our washout experiments further support this hypothesis. Exposure of zebrafish hair cells to neomycin for 30 min followed by a washout period of 1 to 24 hours led to no significant additional hair cell death whereas parallel exposure to gentamicin led to a time-dependent increase in hair cell death. Thus, we suggest that the distinctions between a rapid process and a second, slower process or processes are dependent on differences in the mode of action of neomycin and gentamicin intracellularly. Our data do not directly address loading kinetics among the aminoglycosides. The ability of gentamicin to induce hair cell death in nearly half of the hair cells acutely, in the same amount of time as neomycin, suggests that there is sufficient drug present at short (90 min) time periods to induce hair cell death. Differences in intracellular sequestration of aminoglycosides or in the ability of the cell to pump these drugs out of the cell could contribute to the response of hair cells to each aminoglycoside. There is precedence for aminoglycosides targeting multiple cellular processes in bacteria and different aminoglycosides impact these processes to different extents (Magnet & Blanchard 2005; Borovinskaya, et al., 2007, 2008).

Hair cell death can be triggered rapidly with aminoglycosides

Rapid hair cell death after acute aminoglycoside exposure is consistent with our prior observations following neomycin treatment (Owens, et al., 2007). Here we show that gentamicin also induces rapid cell death in a subset of lateral line hair cells. Arguably, bath

application of aminoglycosides during *in vitro* experiments in other systems may be the most similar to the immersion exposure of the fish lateral line to aminoglycosides. Studies with acute or cultured explants revealed a hair cell response within 3-8 hours of aminoglycoside exposure in rat Organ of Corti (Wei, et al., 2005; Nagy, et al., 2004; Lahne & Gale 2008) and within 6 hours of gentamicin exposure in guinea pig or gerbil utricle (Forge & Li 2000). Ototoxic damage to mouse cochlear cultures has been reported with aminoglycosides following one hour treatment (Kotecha & Richardson 1994). With *in vivo* aminoglycoside exposure in mammalian systems (introduced systemically or intratympanically), the onset hair cell death is often reported as taking 24 hours or more. Intracochlear perfusion of gentamicin in guinea pigs induced damage to hair cells in a basal-to-apical gradient at 1 hour post-treatment and death of all hair cells by 3 days post treatment (Dodson 1997). Recently, Taylor, et al. (2008) observed loss of hair cells in the cochlea as soon as 18 hours post-treatment using kanamycin and the loop diuretic bumetanide. Suzuki, et al. (2008) observed dying hair cells in the cochlea of guinea pigs treated intratympanically with gentamicin by 12 hours post-treatment and subsequent shifts in hearing thresholds by 18 hours post-treatment. It is unclear whether the slower onset of hair cell loss in these systems reflects species-specific differences, organ-specific differences or differences in the aminoglycosides under investigation. Preliminary studies (Schmitt, et al., 2009), suggest that direct exposure of the guinea pig cochlea to neomycin *in vivo* results in a large threshold shift and loss of hair cells within 90 minutes.

We observe differences between aminoglycosides in the extent to which they induce acute hair cell loss. Of the aminoglycosides we tested, amikacin and kanamycin elicit only modest rapid hair cell death (~80% hair cell survival with 400 μ M), streptomycin, gentamicin, and tobramycin have more intermediate impact (~50-60% hair cell survival with 400 μ M), and neomycin elicits dramatic rapid hair cell death (~0-20% survival with 400 μ M). The rank order of ototoxicity we observe following acute treatment with different aminoglycosides (neomycin > gentamicin=tobramycin> streptomycin=amikacin=kanamycin) agrees with the rank order observed by Wang et al. (1994) with *in vitro* assays looking at phospholipid binding. Kotecha & Richardson (1994) reported a similar order of ototoxicity (neomycin > gentamicin > dihydrostreptomycin > amikacin > neamine > spectinomycin) using *in vitro* murine cochlear cultures. However, our evidence suggests that aminoglycosides cannot be simply ranked as more or less ototoxic, and that time of exposure must be taken into account. Notably, kanamycin induced little hair cell loss with acute treatment and induced substantial hair cell loss with chronic treatment.

We cannot explain fully why some hair cells die and others survive acute aminoglycoside treatment. Earlier work (Murakami, et al., 2003) demonstrated that hair cells become more sensitive to neomycin with developmental maturity (4 dpf versus older larvae). Santos, et al. (2006) demonstrated that hair cell immaturity contributes to neomycin susceptibility with ~ 0.5 hair cells/neuromast showing resistance to neomycin. However, this is insufficient to account for the amount of hair cell survival we observe with other aminoglycosides. A plausible alternative explanation is that there may be underlying difference between hair cells within a neuromast that are not readily apparent but which contribute to differences in aminoglycoside susceptibility. Heterogeneity among hair cells in the zebrafish inner ear has been reported (Chang, et al., 1992, Platt 1993; Bang, et al., 2001). Similarly, in adult zebrafish, there are differences in response to gentamicin between superficial and canal neuromasts (Song et al., 1995). We do not observe gross morphological distinctions between the hair cells of the lateral line neuromasts at this age, although subtle differences in cytoplasmic density between hair cells are observed (Owens, et al., 2007). We do not know whether this is correlated with a differential drug response and, if so, how it might relate to molecular distinctions related to differential susceptibilities.

One conundrum in the field has been why inhibiting cell death pathways often provides only partial hair cell protection (Cheng, et al., 2005). Our work supports the idea that more than one cell death pathway is triggered by aminoglycosides and that inhibition of only one pathway would therefore be insufficient to confer complete protection. Mitochondrial mutations and nuclear modifiers of mitochondrial function have been demonstrated to account for altered aminoglycoside susceptibility in some human families and in mice (Prezant, et al., 1993; Fishel-Ghodsian 2003; Guan, et al., 2006). However, only a portion of the variation in aminoglycoside susceptibility is explained by these mutations. The Ras/Rac/JUN kinase (JNK) pathway has also been implicated in hair cell death (Wang, et al., 2007). JNK activation occurs following aminoglycoside exposure (Pirvola, et al., 2000; Cheng, et al., 2005). Inhibitors of the JNK pathway (Pirvola, et al., 2000; Bodmer, et al., 2002; Wang, et al., 2003; Matsui, et al., 2004; Sugahara, et al., 2006) or inducers of the heat shock pathway (Cunningham & Brandon 2006; Taleb, et al., 2008) are partially protective against aminoglycoside-induced hair cell death. Inhibition of the anti-apoptotic factor, Bcl-2, and intrinsic caspase cascades (cas 9, cas 3) attenuates aminoglycoside-induced hair cell death in chick and rodent (Cheng, et al., 2003; Cunningham, et al., 2004), whereas inhibiting inhibitors of this path accentuates hair cell death (Tabuchi, et al., 2007). The MEK/ERK pathway has been implicated to affect hair cell death in a Ras-dependent (Battaglia, et al., 2003) and Ras-independent manner (Chung, et al., 2006). Furthermore, Lahne & Gale (2008) recently demonstrated that the ERK1/2 pathway is important in mammalian support cells for promoting cell death in damaged neighboring hair cells. It remains to be seen which of these (or other) pathways are involved with aminoglycoside-induced hair cell death in the zebrafish lateral line.

Hair cell death continues after aminoglycoside exposure ends

We found continued hair cell death after gentamicin washout, but little after neomycin washout. Onset or increase in hearing loss in human patients has been reported well beyond the end of aminoglycoside exposure (Esterhai, et al., 1986; Magnusson & Padoan 1991; Moore, et al., 1984; Lerner, et al., 1986). Similarly, in other experimental animals there are numerous reports in the literature indicating that aminoglycosides may continue to induce functional and structural hair cell damage for days or weeks after the termination of treatment (e.g., Tucci & Rubel, 1990; Beaubien, et al., 1990; Shepherd & Martin, 1995).

In other systems, delayed onset of hair cell death has been attributed in part to drug administration route and transit time (reviewed in Nakashima, 2000). However, in the lateral line system, hair cells are immediately exposed to aminoglycosides added to fish medium. Experiments with fluorescently tagged aminoglycosides revealed the presence of drug within hair cells after a 1 min drug exposure (Santos, et al., 2006). These observations suggest that delays in damage are due to intrinsic properties of the cell death process. Either hair cells have been damaged early and are already destined to die, but are still present at the times examined, or drugs like gentamicin (or its downstream toxic metabolites) are still present intracellularly and continue to cause damage beyond the time of washout. Aran et al. (1993) reported that gentamicin, given at a non-toxic concentration, remains in hair cells for up to 11 months after treatment. These alternatives are not mutually exclusive or resolvable by the current studies.

Implications for clinical treatment

Clinical treatment of gram-negative infections with aminoglycosides has been largely curtailed in the United States except in recalcitrant cases, but remains prevalent worldwide. Historically, aminoglycosides as a class were assessed for those that retained bactericidal or bacteriostatic action and minimized oto- or nephrotoxicity. Our observations that many of the aminoglycosides exhibit a second wave of hair cell loss with prolonged administration in

the zebrafish is concordant with observations in other systems of hearing loss occurring subsequent to the end of aminoglycoside treatment (e.g., Beaubien, et al., 1995; Taylor, et al., 2008). The difference we see between aminoglycosides in zebrafish suggest that some aminoglycosides, such as neomycin, may have little if any hair cell loss after initial impact while others, such as gentamicin and kanamycin, may have more of a biphasic impact. These results may have important implications for treatments and protection of the cochlea, and need to be carefully tested in the mammalian inner ear.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank our colleague Kelly Tremblay for contributions to the early experimental design and analyses. Support was provided by the Department of Speech and Hearing Sciences, the Virginia Merrill Bloedel Hearing Research Center and NIH grants DC04661; DC05987; DC06998; DC00018.

REFERENCES

- Aran JM, Erre JP, Guilhaume A, Aurousseau C. The comparative ototoxicities of gentamicin, tobramycin and dibekacin in the guinea pig. A functional and morphological cochlear and vestibular study. *Acta Otolaryngol., Suppl.* 1982; 390:1–30. [PubMed: 6299052]
- Aran JM, Dulon D, Hiel H, Erre JP, Aurousseau C. Ototoxicity of aminosides: recent results on uptake and clearance of gentamicin by sensory cells of the cochlea. *Rev. Laryngol. Otol. Rhinol. (Bord.)*. 1993; 114(2):125–128. [PubMed: 8284554]
- Au S, Weiner ND, Schacht J. Aminoglycoside antibiotics preferentially increase permeability in phosphoinositide-containing membranes: a study with carboxyfluorescein in liposomes. *Biochim. Biophys. Acta*. 1987; 902(1):80–86. [PubMed: 3038190]
- Bang PI, Sewell WF, Malicki JJ. Morphology and cell type heterogeneities of the inner ear epithelia in adult and juvenile zebrafish (*Danio rerio*). *J. Comp. Neurol.* 2001; 438:173–190. [PubMed: 11536187]
- Battaglia A, Pak K, Brors D, Bodmer D, Frangos JA, Ryan AF. Involvement of ras activation in toxic hair cell damage of the mammalian cochlea. *Neuroscience*. 2003; 122(4):1025–1035. [PubMed: 14643769]
- Beaubien AR, Desjardins S, Ormsby E, Bayne A, Carrier K, Cauchy MJ, Henri R, Hodgen M, Salley J, St. Pierre A. Delay in hearing loss following drug administration. A consistent feature of amikacin ototoxicity. *Acta Otolaryngol.* 1990; 109(5-6):345–352. [PubMed: 2360443]
- Beaubien AR, Karpinski K, Ormsby E. Toxicodynamics and toxicokinetics of amikacin in the guinea pig cochlea. *Hear. Res.* 1995; 83(1-2):62–79. [PubMed: 7607992]
- Birmingham-McDonogh O, Stone JS, Reh TA, Rubel EW. FGFR3 expression during development and regeneration of the chick inner ear sensory epithelia. *Dev. Biol.* 2001; 238(2):247–259. [PubMed: 11784008]
- Bodmer D, Brors D, Pak K, Gloddek B, Ryan A. Rescue of auditory hair cells from aminoglycoside toxicity by *Clostridium difficile* toxin B, an inhibitor of the small GTPases Rho/Rac/Cdc42. *Hear. Res.* 2002; 172(1-2):81–86. [PubMed: 12361869]
- Borovinskaya MA, Pai RD, Zhang W, Schuwirth BS, Holton JM, Hirokawa G, Kaji H, Kaji A, Cate JH. Structural basis for aminoglycoside inhibition of bacterial ribosome recycling. *Nat. Struct. Mol. Biol.* 2007; 14(8):727–732. [PubMed: 17660832]
- Borovinskaya MA, Shoji S, Fredrick K, Cate JH. Structural basis for hygromycin B inhibition of protein biosynthesis. *RNA*. 2008; 14(8):1590–1599. [PubMed: 18567815]
- Chang JS, Popper AN, Saidel WM. Heterogeneity of sensory hair cells in a fish ear. *J. Comp. Neurol.* 1992; 324(4):621–640. [PubMed: 1430341]

- Cheng AG, Cunningham LL, Rubel EW. Hair cell death in the avian basilar papilla: characterization of the *in vitro* model and caspase activation. *J. Assoc. Res. Otolaryngol.* 2003; 4(1):91–105. [PubMed: 12417974]
- Cheng AG, Cunningham LL, Rubel EW. Mechanisms of hair cell death and protection. *Curr. Opin. Otolaryngol. Head Neck Surg.* 2005; 13(6):343–348. [PubMed: 16282762]
- Chiu LL, Cunningham LL, Raible DW, Rubel EW, Ou HC. Using the zebrafish lateral line to screen for ototoxicity. *J. Assoc. Res. Otolaryngol.* 2008; 9(2):178–190. [PubMed: 18408970]
- Chung WH, Pak K, Lin B, Webster N, Ryan AF. A PI3K pathway mediates hair cell survival and opposes gentamicin toxicity in neonatal rat organ of Corti. *J. Assoc. Res. Otolaryngol.* 2006; 7(4): 373–382. [PubMed: 17053865]
- Coombs, S.; Montgomery, JC. The enigmatic lateral line system. In: Fay, RR.; Popper, AN., editors. *Comparative hearing. Fish and amphibians.* Springer; New York: 1999.
- Cunningham LL, Cheng AG, Rubel EW. Caspase activation in hair cells of the mouse utricle exposed to neomycin. *J. Neurosci.* 2002; 22(19):8532–8540. [PubMed: 12351727]
- Cunningham LL, Matsui JI, Warchol ME, Rubel EW. Overexpression of Bcl-2 prevents neomycin-induced hair cell death and caspase-9 activation in the adult mouse utricle *in vitro*. *J. Neurobiol.* 2004; 60(1):89–100. [PubMed: 15188275]
- Cunningham LL, Brandon CS. Heat shock inhibits both aminoglycoside- and cisplatin-induced sensory hair cell death. *J. Assoc. Res. Otolaryngol.* 2006; 7(3):299–307. [PubMed: 16794914]
- Dai CF, Steyger PS. A systemic gentamicin pathway across the stria vascularis. *Hear. Res.* 2008; 235(1-2):114–124. [PubMed: 18082985]
- Dambly-Chaudière C, Sapède D, Soubiran F, Decorde K, Gompel N, Ghysen A. The lateral line of zebrafish: a model system for the analysis of morphogenesis and neural development in vertebrates. *Biol. Cell.* 2003; 95(9):579–587. [PubMed: 14720460]
- Day AS, Lue JH, Yang TH, Young YH. Effect of intratympanic application of aminoglycosides on click-evoked myogenic potentials in Guinea pigs. *Ear Hear.* 2007; 28(1):18–25. [PubMed: 17204896]
- Dodson HC. Loss and survival of spiral ganglion neurons in the guinea pig after intracochlear perfusion with aminoglycosides. *J. Neurocytol.* 1997; 26(8):541–556. [PubMed: 9350806]
- Dulon D, Aran JM, Zajic G, Schacht J. Comparative uptake of gentamicin, netilmicin, and amikacin in the guinea pig cochlea and vestibule. *Antimicrob. Agents Chemother.* 1986; 30(1):96–100. [PubMed: 3489440]
- Esterhai JL Jr, Bednar J, Kimmelman CP. Gentamicin-induced ototoxicity complicating treatment of chronic osteomyelitis. *Clin. Orthop. Relat. Res.* 1986; 209:185–188. [PubMed: 3488158]
- Fischel-Ghodsian N. Mitochondrial deafness. *Ear Hear.* 2003; 24(4):303–313. [PubMed: 12923421]
- Forge A, Li L. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. *Hear. Res.* 2000; 139(1-2):97–115. [PubMed: 10601716]
- Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol. Neurotol.* 2000; 5(1):3–22. [PubMed: 10686428]
- Gale JE, Marcotti W, Kennedy HJ, Kros CJ, Richardson GP. FM1-43 dye behaves as a permeant blocker of the hair-cell mechanotransducer channel. *J. Neurosci.* 2001; 21:7013–7025. [PubMed: 11549711]
- Guan MX, Yan Q, Li X, Bykhovskaya Y, Gallo-Teran J, Hajek P, Umeda N, Zhao H, Garrido G, Mengesha E, Suzuki T, del Castillo I, Peters JL, Li R, Qian Y, Wang X, Ballana E, Shohat M, Lu J, Estivill X, Watanabe K, Fischel-Ghodsian N. Mutation in TRMU related to transfer RNA modification modulates the phenotypic expression of the deafness-associated mitochondrial 12S ribosomal RNA mutations. *Am. J. Hum. Genet.* 2006; 79(2):291–302. [PubMed: 16826519]
- Harris JA, Cheng AG, Cunningham LL, MacDonald G, Raible DW, Rubel EW. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (*Danio rerio*). *J. Assoc. Res. Otolaryngol.* 2003; 4(2):219–234. [PubMed: 12943374]
- Hawkins JE Jr, Lurie MH. The ototoxicity of streptomycin. *Trans. Am. Otol. Soc.* 1952; 40:133–153. [PubMed: 13064693]
- Hernández PP, Olivari FA, Sarrazin AF, Sandoval PC, Allende ML. Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker *sox2* and proliferation-dependent

- and - independent mechanisms of hair cell renewal. *Dev. Neurobiol.* 2007; 67(5):637–654. [PubMed: 17443814]
- Hirose K, Hockenbery DM, Rubel EW. Reactive oxygen species in chick hair cells after gentamicin exposure *in vitro*. *Hear. Res.* 1997; 104(1-2):1–14. [PubMed: 9119753]
- Hutchin T, Cortopassi G. Proposed molecular and cellular mechanism for aminoglycoside ototoxicity. *Antimicrob. Agents Chemother.* 1994; 38(11):2517–2520. [PubMed: 7872740]
- Izumikawa M, Batts SA, Miyazawa T, Swiderski DL, Raphael Y. Response of the flat cochlear epithelium to forced expression of Atoh1. *Hear. Res.* 2008; 240(1-2):52–56. [PubMed: 18430530]
- Jiang H, Sha SH, Schacht J. Kanamycin alters cytoplasmic and nuclear phosphoinositide signaling in the organ of Corti *in vivo*. *J. Neurochem.* 2006; 99(1):269–276. [PubMed: 16903869]
- Kotecha B, Richardson GP. Ototoxicity *in vitro*: effects of neomycin, gentamicin, dihydrostreptomycin, amikacin, spectinomycin, neamine, spermine, and poly-L-lysine. *Hear. Res.* 1994; 73:173–184. [PubMed: 7514588]
- Lahne M, Gale JE. Damage-induced activation of ERK1/2 in cochlear support cells is a hair cell death-promoting signal that depends on extracellular ATP and calcium. *J. Neurosci.* 2008; 28(19):4918–4928. [PubMed: 18463245]
- Leake PA, Hdrarek GT, Bonham BH, Snyder RL. Topography of auditory nerve projections to the cochlear nucleus in cats after neonatal deafness and electrical stimulation by a cochlear implant. *J. Assoc. Res. Otolaryngol.* 2008; 9(3):349–372. [PubMed: 18574634]
- Lerner SA, Schmitt BA, Seligsohn R, Matz GJ. Comparative study of ototoxicity and nephrotoxicity in patients randomly assigned to treatment with amikacin or gentamicin. *Am. J. Med.* 1986; 80(6B):98–104. [PubMed: 3524221]
- Lynch SR, Puglisi JD. Structural origins of aminoglycoside specificity for prokaryotic ribosomes. *J. Mol. Biol.* 2001; 306(5):1037–1058. [PubMed: 11237617]
- Ma EY, Rubel EW, Raible DW. Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *J. Neurosci.* 2008; 28(9):2261–2273. [PubMed: 18305259]
- Magnet S, Blanchard JS. Molecular insights into aminoglycoside action and resistance. *Chem. Rev.* 2005; 105(2):477–498. [PubMed: 15700953]
- Magnusson M, Padoan S. Delayed onset of ototoxic effects of gentamicin in treatment of Menière's disease. Rationale for extremely low dose therapy. *Acta Otolaryngol.* 1991; 111(4):671–676. [PubMed: 1950529]
- Mangiardi DA, McLaughlin-Williamson K, May KE, Messana EP, Mountain DC, Cotanche DA. Progression of hair cell ejection and molecular markers of apoptosis in the avian cochlea following gentamicin treatment. *J. Comp. Neurol.* 2004; 475(1):1–18. [PubMed: 15176081]
- Marcotti W, van Netten SM, Kros CJ. The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the mechano-electrical transducer channels. *J. Physiol.* 2005; 567:505–521. [PubMed: 15994187]
- Matsui JI, Cotanche DA. Sensory hair cell death and regeneration: two halves of the same equation. *Curr. Opin. Otolaryngol. Head Neck Surg.* 2004; 12(5):418–425. [PubMed: 15377955]
- Matsui JI, Gale JE, Warchol ME. Critical signaling events during the aminoglycoside-induced death of sensory hair cells *in vitro*. *J. Neurobiol.* 2004; 61(2):250–266. [PubMed: 15389694]
- Montgomery J, Carton G, Voigt R, Baker C, Diebel C. Sensory processing of water currents by fishes. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 2000; 355(1401):1325–1327. [PubMed: 11079424]
- Moore RD, Smith CR, Lietman PS. Risk factors for the development of auditory toxicity in patients receiving aminoglycosides. *J. Infect. Dis.* 1984; 149(1):23–30. [PubMed: 6693788]
- Murakami SL, Cunningham LL, Werner LA, Bauer E, Pujol R, Raible DW, Rubel EW. Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*). *Hear. Res.* 2003; 186(1-2):47–56. [PubMed: 14644458]
- Nagy I, Bodmer M, Brors D, Bodmer D. Early gene expression in the organ of Corti exposed to gentamicin. *Hear. Res.* 2004; 195(1-2):1–8. [PubMed: 15350274]
- Nakashima T, Teranishi M, Hibi T, Kobayashi M, Umemura M. Vestibular and cochlear toxicity of aminoglycosides—a review. *Acta Otolaryngol.* 2000; 120(8):904–911. [PubMed: 11200584]

- Nelson EG, Hinojosa R. Presbycusis: a human temporal bone study of individuals with downward sloping audiometric patterns of hearing loss and review of the literature. *Laryngoscope*. 2006; 116(9 Pt 3 Suppl 112):1–12. [PubMed: 16946668]
- Ou HC, Raible DW, Rubel EW. Cisplatin-induced hair cell loss in zebrafish (*Danio rerio*) lateral line. *Hear. Res.* 2007; 233(1-2):46–53. [PubMed: 17709218]
- Owens KN, Cunningham DE, MacDonald G, Rubel EW, Raible DW, Pujol R. Ultrastructural analysis of aminoglycoside-induced hair cell death in the zebrafish lateral line reveals an early mitochondrial response. *J. Comp. Neurol.* 2007; 502(4):522–543. [PubMed: 17394157]
- Owens KN, Santos F, Roberts B, Linbo T, Coffin AB, Knisely AJ, Simon JA, Rubel EW, Raible DW. Identification of genetic and chemical modulators of zebrafish mechanosensory hair cell death. *PLoS Genet.* 2008; 4(2):e1000020. [PubMed: 18454195]
- Parks, TN.; Rubel, EW.; Popper, AN.; Fay, RR., editors. *Plasticity of the Auditory System*. Vol. Vol. 23. Springer-Verlag; New York: 2004. Series: Springer Handbook of Auditory Research
- Pirvola U, Xing-Qun L, Virkkala J, Saarman M, Murakata C, Camoratto AM, Walton KM, Ylikoski J. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J. Neurosci.* 2000; 20(1):43–50. [PubMed: 10627579]
- Platt C. Zebrafish inner ear sensory surfaces are similar to those in goldfish. *Hear. Res.* 2003; 65:133–140. [PubMed: 8458746]
- Prezant TR, Agapian JV, Bohlman MC, Bu X, Oztas S, Qui WQ, Anos KS, Cortopassi GA, Jaber L, Rotter JJ, Shohat M, Fishel-Ghodesian N. Mitochondrial ribosomal RNA mutation associated with antibiotic-induced and non-syndromic deafness. *Nat. Genet.* 1993; 4(3):289–294. [PubMed: 7689389]
- Raible DW, Kruse GJ. Organization of the lateral line system in embryonic zebrafish. *J. Comp. Neurol.* 2000; 421(2):189–198. [PubMed: 10813781]
- Rauch SD, Velazquez-Villaseñor L, Dimitri PS, Merchant SN. Decreasing hair cell counts in aging humans. *Ann. N.Y. Acad. Sci.* 2001; 942:220–227. [PubMed: 11710464]
- Rizzi MD, Hirose K. Aminoglycoside ototoxicity. *Curr. Opin. Otolaryngol. Head Neck Surg.* 2007; 15(5):352–357. [PubMed: 17823553]
- Rybak LP, Whitworth CA. Ototoxicity: therapeutic opportunities. *Drug Discov. Today.* 2005; 10(19):1313–1321. [PubMed: 16214676]
- Rybak LP, Whitworth CA, Mukherjee D, Ramkumar V. Mechanisms of cisplatin-induced ototoxicity and prevention. *Hear. Res.* 2007; 226(1-2):157–167. [PubMed: 17113254]
- Santos F, MacDonald G, Rubel EW, Raible DW. Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*). *Hear. Res.* 2006; 213(1-2):25–33. [PubMed: 16459035]
- Saunders JC, Cohen YE, Szymko YM. The structural and functional consequences of acoustic injury in the cochlea and peripheral auditory system: a five year update. *J. Acoust. Soc. Am.* 1991; 90(1):136–146. [PubMed: 1880281]
- Schacht J. Biochemical basis of aminoglycoside ototoxicity. *Otolaryngol. Clin. North Am.* 1993; 26(5):845–856. [PubMed: 8233493]
- Schacht J. Biochemistry and pharmacology of aminoglycoside-induced hearing loss. *Acta Physiol. Pharmacol. Ther. Latinoam.* 1999; 49(4):251–256. [PubMed: 10797868]
- Schmitt, N.; Wang, J.; Rubel, EW.; Lenoir, M.; Raible, DW.; Puel, J-L. Rapid hearing loss and hair cell degeneration following acute intracochlear perfusion of neomycin in guinea pig. *Assoc. Res. Otolaryngol. Midwinter meeting*; 2009.
- Selimoglu E, Kalkandelen S, Erdogan F. Comparative vestibulotoxicity of different aminoglycosides in the Guinea pigs. *Yonsei Med. J.* 2003; 44(3):517–522. [PubMed: 12833591]
- Sha SH, Schacht J. Antioxidants attenuate gentamicin-induced free radical formation *in vitro* and ototoxicity *in vivo*: D-methionine is a potential protectant. *Hear. Res.* 2000; 142(1-2):34–40. [PubMed: 10748326]
- Shakil S, Khan R, Zarrilli R, Khan AU. Aminoglycosides versus bacteria--a description of the action, resistance mechanism, and nosocomial battleground. *J. Biomed. Sci.* 2008; 15(1):5–14. [PubMed: 17657587]

- Shepherd RK, Martin RL. Onset of ototoxicity in the cat is related to onset of auditory function. *Hear. Res.* 1995; 92(1-2):131–142. [PubMed: 8647736]
- Smith CR, Baughman KL, Edwards CQ, Rogers JF, Lietman PS. Controlled comparison of amikacin and gentamicin. *N. Engl. J. Med.* 1977; 296(7):349–353. [PubMed: 319355]
- Song J, Yan HY, Popper AN. Damage and recovery of hair cells in fish canal (but not superficial) neuromasts after gentamicin exposure. *Hear. Res.* 1995; 91(1-2):63–71. [PubMed: 8647725]
- Steyger PS, Peters SL, Rehling J, Hordichok A, Dai CF. Uptake of gentamicin by bullfrog saccular hair cells in vitro. *J Assoc. Res. Otolaryngol.* 2003; 4(4):565–578. [PubMed: 14605921]
- Sugahara K, Rubel EW, Cunningham LL. JNK signaling in neomycin-induced vestibular hair cell death. *Hear. Res.* 2006; 221(1-2):128–135. [PubMed: 17005344]
- Suzuki M, Ushio M, Yamasoba T. Time course of apoptotic cell death in guinea pig cochlea following intratympanic gentamicin application. *Acta Otolaryngol.* 2008; 128(7):724–731. [PubMed: 18568512]
- Tabuchi K, Pak K, Chavez E, Ryan AF. Role of inhibitor of apoptosis protein in gentamicin-induced cochlear hair cell damage. *Neuroscience.* 2007; 149(1):213–222. [PubMed: 17869439]
- Taleb M, Brandon CS, Lee FS, Lomax MI, Dillmann WH, Cunningham LL. Hsp70 inhibits aminoglycoside-induced hair cell death and is necessary for the protective effect of heat shock. *J. Assoc. Res. Otolaryngol.* 2008; 9(3):277–289. [PubMed: 18512096]
- Taylor RR, Nevill G, Forge A. Rapid hair cell loss: A mouse model for cochlear lesions. *J. Assoc. Res. Otolaryngol.* 2008; 9(1):44–64. [PubMed: 18057986]
- Tucci DL, Rubel EW. Physiological status of regenerated hair cells in the avian inner ear following aminoglycoside ototoxicity. *Otolaryngol. Head Neck Surg.* 1990; 103(3):443–450. [PubMed: 2122376]
- Wanamaker HH, Slepecky NB, Cefaratti LK, Ogata Y. Comparison of vestibular and cochlear ototoxicity from transtympanic streptomycin administration. *Am. J. Otol.* 1999; 20(4):457–464. [PubMed: 10431887]
- Wang BM, Weiner ND, Takada A, Schacht J. Characterization of aminoglycoside-lipid interactions and development of a refined model for ototoxicity testing. *Biochem Pharmacol.* 1984; 33(20):3257–3262. [PubMed: 6487373]
- Wang J, Van De Water TR, Bonny C, de Ribaupierre F, Puel J-L, Zine A. A peptide inhibitor of c-Jun N-terminal kinase protects against both aminoglycoside and acoustic trauma-induced auditory hair cell death and hearing loss. *J. Neurosci.* 2003; 23(24):8596–8607. [PubMed: 13679429]
- Wang J, Ruel J, Ladrech S, Bonny C, van de Water TR, Puel J-L. Inhibition of the c-Jun N-terminal kinase-mediated mitochondrial cell death pathway restores auditory function in sound-exposed animals. *Mol. Pharmacol.* 2007; 71(3):654–666. [PubMed: 17132689]
- Westerfield, M. *The Zebrafish book. A guide for the laboratory use of zebrafish (Danio rerio).* University of Oregon Press; Eugene, (OR): 2000.
- Wei X, Zhao L, Liu J, Dodel RC, Farlow MR, Du Y. Minocycline prevents gentamicin-induced ototoxicity by inhibiting p38 MAP kinase phosphorylation and caspase 3 activation. *Neuroscience.* 2005; 131(2):513–521. [PubMed: 15708492]
- Wersall J. The ototoxic potential of netilmicin compared with amikacin. An animal study in guinea pigs. *Scand. J. Infect. Dis. Suppl.* 1980; 23:104–113. [PubMed: 6970981]
- Yan Q, Li X, Faye G, Guan MX. Mutations in MTO2 related to tRNA modification impair mitochondrial gene expression and protein synthesis in the presence of a paromomycin resistance mutation in mitochondrial 15 S rRNA. *J. Biol. Chem.* 2005; 280(32):29151–29157. [PubMed: 15944150]

Fig 1.

Neomycin and gentamicin cause different amounts of damage dependent on the time of exposure. Loss of DASPEI staining in neuromasts as a function aminoglycoside concentration is shown by the lines and solid symbols as compared to mock-treated controls: neomycin (solid line, squares); gentamicin (dashed line, circles). For the 50 and 200 μM exposures, the corresponding hair cell counts (right ordinate) are shown as open squares (neomycin) or open circles (gentamicin) and are staggered slightly to the left on the X-axis for clarity. **A**, Hair cell loss as monitored by DASPEI staining after 30 min drug exposure followed by a one hour recovery period. Note that the hair cell damage and loss induced by gentamicin is attenuated in comparison to that induced by neomycin. Counts of individual hair cells confirm differences observed by DASPEI staining. **B**, Hair cell loss as monitored by DASPEI staining after 6 hour continual drug exposure to either neomycin or gentamicin. Gentamicin is just as effective as neomycin after chronic exposure. Hair cell counts confirm DASPEI results. Error bars show standard deviations. 2-way ANOVA revealed significant main effects of drug and concentration and a significant interaction ($p < 0.0001$).

Fig 2.

Changes in hair cell number differ after acute or chronic treatment to neomycin or gentamicin. Numbers of hair cells remaining after acute or chronic exposure to neomycin (solid lines, squares), gentamicin (dashed lines, circles) or mock-treatment (dotted lines, triangles) are shown. Hair cells of 5 dpf zebrafish were pre-labeled with FM1-43FX, exposed to drug acutely or chronically, euthanized, fixed and counted. **A,B**, 50 μ M aminoglycoside; **C,D**, 200 μ M aminoglycoside. Bars indicate standard deviations. **A,C**, Chronic treatment. Animals were exposed to drug or mock-treated in embryo medium for 1.5, 3, 6, or 24 hrs. No significant hair cell loss was observed with neomycin between 3 hours and 24 hours while additional hair cell loss was observed with gentamicin from over this period (one-way ANOVA, $p < 0.0001$). **B,D**, Acute treatment. Animals were exposed to drug for 30 min, rinsed in embryo medium four times, and held in the last embryo medium rinse for 0.5 to 23.5 hrs for a total treatment time of 1.5, 3, 6, or 24 hrs. No significant increase in hair cell loss was induced by neomycin after 1.5 hr with 50 μ M or after 3 hr with 200 μ M neomycin (2-way ANOVA, $p > 0.05$). In contrast, additional loss of hair cells was induced by gentamicin (2-way ANOVA; main effect of time and concentration, p 's < 0.0001).

Fig 3.

sentinel mutants are resistant to acute gentamicin-induced hair cell loss but are not resistant to chronic gentamicin exposure. **A**, Acute exposure to gentamicin (50 and 200 μ M) for 30 min followed by a one hour recovery in fresh embryo medium. A significant main effect of genotype and gentamicin concentration and a significant interaction were observed (2-way ANOVA, p 's<0.0001). **B**, Chronic exposure to gentamicin for 6 hr. Both *snl*+ siblings (solid line, squares) and *snl* homozygotes (dashed line, circles) are sensitive to chronic gentamicin (p <0.0001). There is no significant difference between mutants and siblings without drug or with chronic treatment with 200 μ M gentamicin (Bonferroni post-hoc comparisons, p >0.05). Bars denote standard deviations.

Fig 4.

The small molecule protectant PROTO-1 completely attenuates hair cell death induced acutely by gentamicin and only partially protects hair cells from chronic gentamicin exposure. Zebrafish larvae were treated with PROTO1 or mock-treated for 1 hr prior to and coincident with gentamicin exposure. Gentamicin only (solid lines, squares), Gentamicin and PROTO1 (dashed lines, circles). **A**, Acute exposure for 30 min followed by 1 hr recovery. Hair cell death is inhibited in the presence of PROTO1 (p 's<0.0001). **B**, Chronic gentamicin exposure for 6 hr reveals that PROTO1 provides a small but significant amount of protection at intermediate gentamicin concentrations (2-way ANOVA, p <0.0001). Bonferroni post-hoc comparisons indicate PROTO1 protection only at 200 μM gentamicin (p <0.001). Bars denote standard deviations.

Fig 5.

The mechanosensory channel blocker amiloride prevents hair cell death induced by either acute or chronic treatment with either neomycin or gentamicin. **A**, Zebrafish larvae were pre-treated 1 mM amiloride then exposed concurrently to 200 μ M neomycin or gentamicin acutely (30 min aminoglycoside exposure followed by 1 hour washout). Controls were treated with aminoglycoside without amiloride exposure. There is a significant reduction in hair cell death in the presence of amiloride for both aminoglycosides (2-way ANOVA, $p < 0.0001$). Treatment with amiloride alone does not lead to hair cell loss as compared to mock treatment. **B**, Zebrafish larvae were treated with variable concentrations amiloride 15 min prior to and during 6 hour exposure to neomycin (100 μ M) or gentamicin (50 μ M) in the continued presences of amiloride. Amiloride significantly reduced the hair cell loss induced by chronic exposure to either neomycin or gentamicin in a dose-dependent manner (2-way ANOVA, $p < 0.0001$). Bars show standard deviations.

Fig 6.

Hair cell loss in the zebrafish lateral line induced by different aminoglycosides. Most aminoglycosides show differences between acute and chronic treatment. Graphs show results of DASPEI staining analysis of hair cells following acute (dashed lines circles) or chronic exposure (solid lines, squares) to: **A**, Amikacin; **B**, Kanamycin; **C**, Streptomycin; or **D**, Tobramycin. Bars show standard deviations. Analyses by 2-way ANOVA revealed significant main effects of drug concentration and treatment condition and a significant interaction with each aminoglycoside (p 's < 0.0001).