



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

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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 min, regardless of concentration or exposure time. Gentamicin-induced loss of half of the hair cells within 90 min and substantial additional loss, which was prolonged and cumulative over exposure times up to at least 24 h. 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.

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1. 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 and 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 and Schacht, 2000).

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In bacteria, aminoglycosides bind rRNA subunits and interfere with translation by affecting ribosome translocation, peptide release, mRNA coding and ribosome recycling (Magnet and 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 and Puglisi, 2001), leading to the proposal that the mitochondrial ribosome may be an aminoglycoside target (Hutchin and Cortopassi, 1994). Furthermore, mutations of mitochondrial 12S rRNA and tRNAs have been associated with aminoglycoside-associated hearing loss in humans (Fischel-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 and 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 and 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 and Lurie, 1952; Rizzi and Hirose, 2007; Forge and 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 US 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 and 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., Tucci and Rubel, 1990; Bermingham-McDonogh et al., 2001; Parks et al., 2004; Matsui and Cotanche, 2004; Hernández et al., 2007; Ma et al., 2008; Izumikawa et al., 2008; Leake 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 and Montgomery, 1999; Dambly-Chaudière 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.

2. Materials and methods

2.1. 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, pH 7.2) in a six 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.

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

2.2. Drug exposure

Stock solutions of aminoglycosides were diluted in EM to final concentrations of 0.1–400 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, and 23.5 h (total treatment times of 1.5, 3, 6, and 24 h). For chronic treatments, larvae were treated with drug or embryo media (mock-treated) for 1.5, 3, 6, or 24 h 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 1 h recovery or chronically for 6 h continuously. For experiments with the otoprotective agent PROTO1 (Owens et al., 2008), larvae were pre-treated for 1 h in embryo medium supplemented with 10 μM PROTO1, then neomycin or gentamicin was added and fish were treated acutely (30 min, followed by 1 h recovery in fresh EM without PROTO1) or chronically (6 h 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 pre-treated for 15 min with 1 mM amiloride (Sigma) in embryo medium, transferred to 200 μM neomycin or gentamicin plus 1 mM amiloride for 30 min, washed four times in embryo medium and held in the final rinse for 1 h prior to evaluation of hair cells with DASPEI. For chronic exposure in the presence of amiloride, larvae were treated with 100–500 μM amiloride and either 100 μM neomycin or 50 μM gentamicin for 6 h prior to evaluation of hair cells with DASPEI.

2.3. 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 10 neuromasts (SO1, SO2, IO1–4, M2, MI1, MI2 and O2; Raible and 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.

2.4. FM1-43 labeling of hair cells

For counts, hair cells were pre-labeled with the mechanotransduction marker FM1-43 FX (3 μM in EM; Molecular Probes, Eugene, OR) for 45–60 s. The larvae were then quickly rinsed four

times with EM and held in the final rinse for 20 min prior to drug treatment. Following drug treatment, animals were euthanized and fixed in cold 4% paraformaldehyde for 1 h, fresh cold fixative overnight, and then washed three times with 0.1 M PBS (pH 7.2). For examination, larvae were mounted in Fluoromount-G on double bridge coverslips (created by gluing two 22 × 22 mm squares at each end of a 24 × 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 40× objective. Ten to thirty 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 were 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 Fig. 1.

3. Results

3.1. 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 h 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 μM 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 μM 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 's < 0.0001). Substantial additional damage was observed after continuous treatment with gentamicin for 6 h (Fig. 1B). This result was also supported by 2-way ANOVA with significant main effects of drug, concentration and an interaction (p 's < 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 1.5–24 hours to detect cumulative effects. In the second protocol (hereafter called “acute” exposure), zebrafish larvae were exposed to aminoglycoside for 30 min, rinsed with fresh embryo medium and held for varying lengths of time (1–24 h 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 4 neuromasts (see Materials and methods). By pre-labeling hair cells before treatment, we avoided counting regenerated hair cells, which begin to arise by 24 h 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

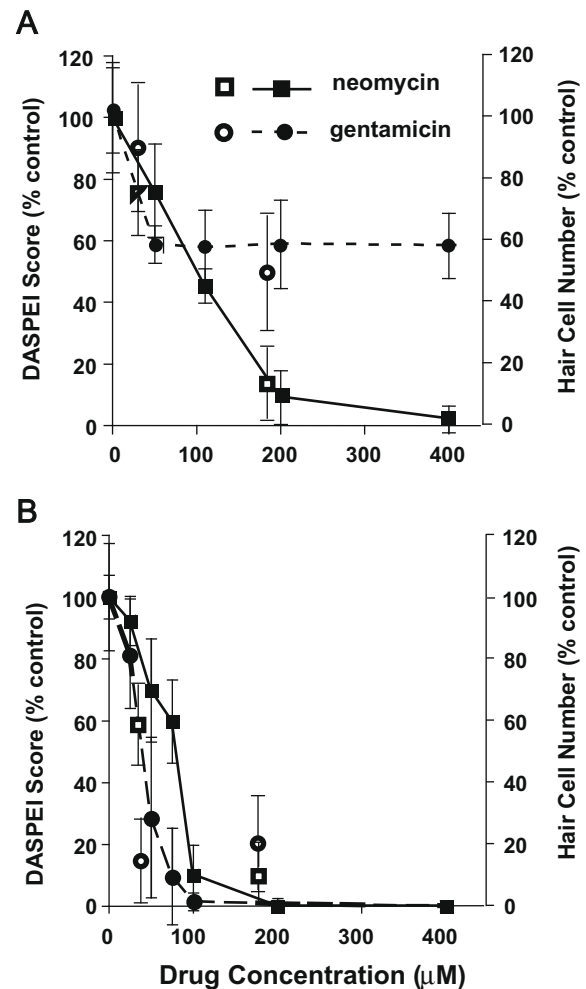


Fig. 1. Neomycin and gentamicin cause different amounts of damage dependent on the time of exposure. Loss of DASPEI staining in neuromasts as a function aminoglycoside concentration is shown by the lines and solid symbols as compared to mock-treated controls: neomycin (solid line, squares); gentamicin (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 1 h 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 h 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 one standard deviation. 2-way ANOVA revealed significant main effects of drug and concentration and a significant interaction (p 's < 0.0001).

cells to 50 or 200 μM neomycin were essentially unchanged with longer drug exposure. No significant additional hair cell death was observed between 3 and 24 h (Fig. 2A and C, solid lines; 1-way ANOVA). Similarly, 100 μM and 400 μM neomycin show no additional hair cell death between 1.5 and 24 h post-treatment (not shown). 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 h exposure time (1-way ANOVA, p < 0.0001). Hair cell loss due to chronic gentamicin exposure was relatively concentration independent. That is, chronic exposure to a modest, 50 μM (Fig. 2A) concentration of gentamicin elicited levels of cell death similar to concentrations 4 to 8-fold higher (200 μM, Fig. 2C; 400 μM, not shown; 2-way ANOVA, p > 0.05). Moreover, low and

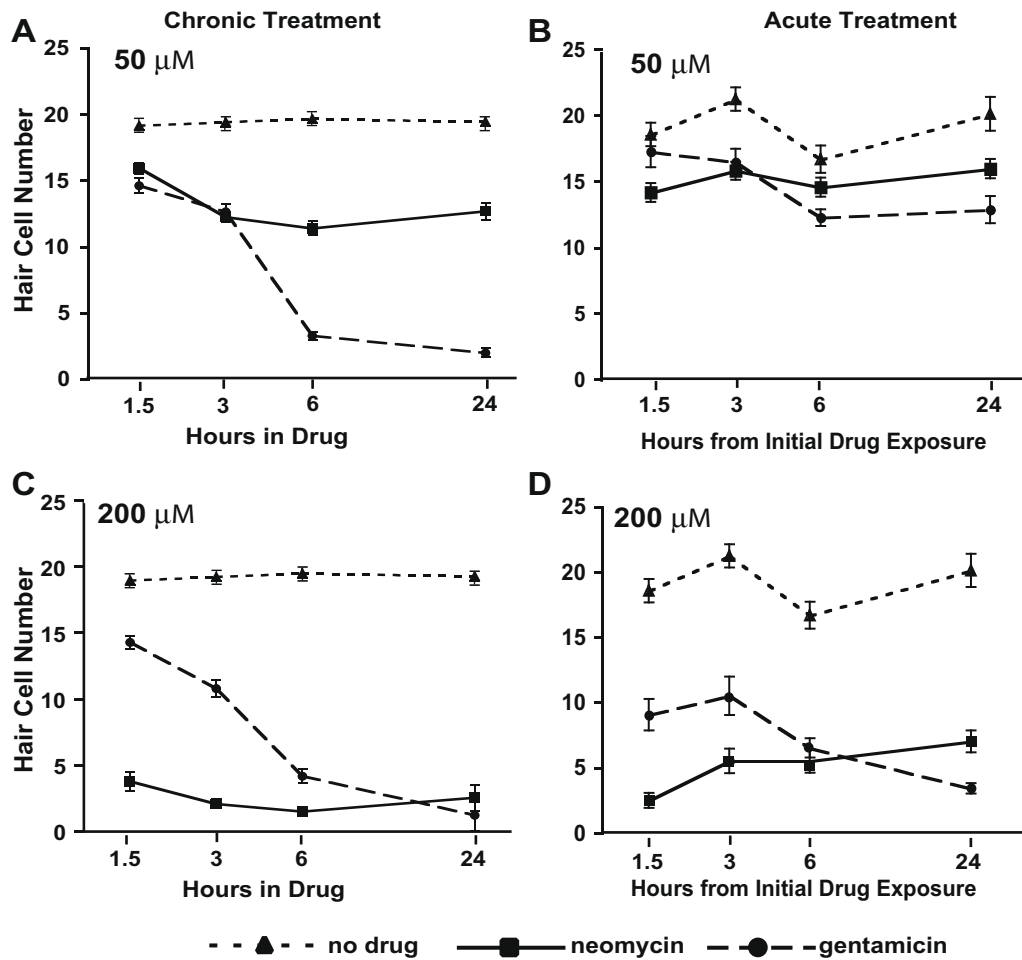


Fig. 2. Changes in hair cell number differ after acute or chronic treatment to neomycin or gentamicin. Numbers of hair cells remaining after acute or chronic exposure to neomycin (solid lines, squares), gentamicin (dashed lines, circles) or mock-treatment (dotted lines, triangles) are shown. Hair cells of 5 dpf zebrafish were pre-labeled with FM1-43FX, exposed to drug acutely or chronically, euthanized, fixed and counted. (A and B) Aminoglycoside, 50 μ M; (C and D) 200 μ M aminoglycoside. Bars indicate one standard deviation. (A and C) Chronic treatment. Animals were exposed to drug or mock-treated in embryo medium for 1.5, 3, 6, or 24 h. No significant hair cell loss was observed with neomycin between 3 and 24 h while additional hair cell loss was observed with gentamicin from over this period (1-way ANOVA, $p < 0.0001$). (B and 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 0.5–23.5 h for a total treatment time of 1.5, 3, 6, or 24 h. No significant increase in hair cell loss was induced by neomycin after 1.5 h with 50 μ M or after 3 h 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).

high concentrations affected hair cell loss on a similar time scale, predominantly by 3 and 6 h.

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 or 200 μ M aminoglycoside for 30 min, rinsed and then held in fresh embryo media for up to 24 h. Loss of hair cells was unchanged with longer recovery time following exposure to neomycin (Fig. 2B and D, 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 μ M neomycin, there was no evidence of increased loss at any time after 1.5 h ($p > 0.05$) and for 200 μ M neomycin there was no significant difference after 3 h ($p > 0.5$). In contrast, additional loss of hair cells occurred with time following exposure to gentamicin (Fig. 2B and D, 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 μ M gentamicin exposure. While hair cells continue to be lost 6 h after acute exposure, a substantial number remain. In comparison, almost all hair cells are lost after a 6-h 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 h) and a later, slower process (between 1.5 and 24 h). 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.

3.2. Protective mutants and drugs block the 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 h 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 h, *snl* mutants responded similarly to their wildtype siblings (Fig. 3B), with concentration-dependent loss of DASPEI labeling that was indistinguishable from that of 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 μM gentamicin are not significant ($p > 0.05$).

Pre-treatment 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 h recovery), animals treated with 10 μM 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 μM 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.

3.3. 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 and 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 1 mM amiloride when animals were treated with either 200 μM neomycin or gentamicin acutely (Fig. 5A, 30 min exposure with washout and 1 h 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 h 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.

3.4. 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 1 h recovery. For

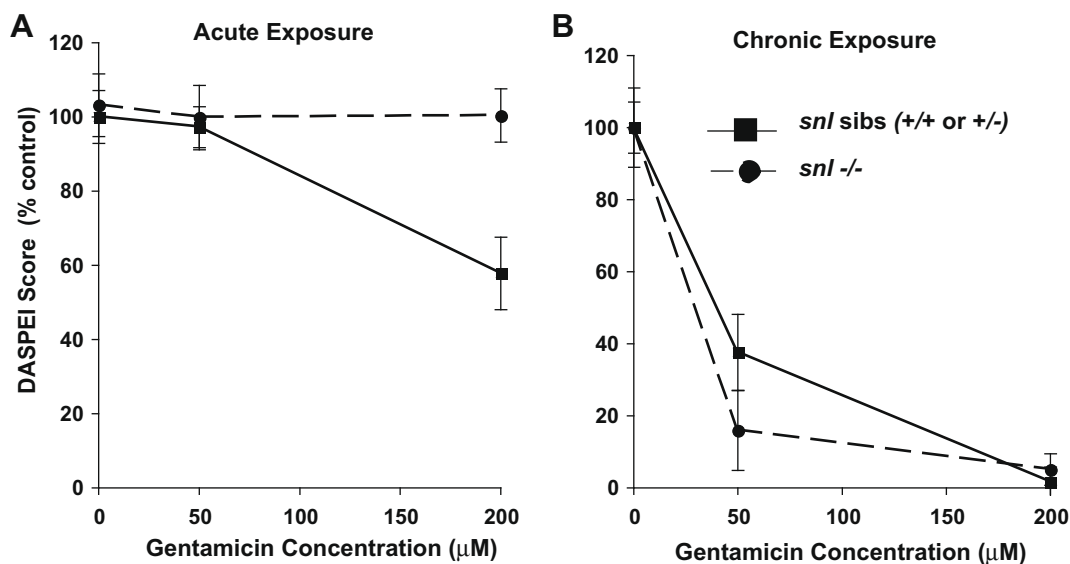


Fig. 3. *Sentinel* mutants are resistant to acute gentamicin-induced hair cell loss but are not resistant to chronic gentamicin exposure. (A) Acute exposure to gentamicin (50 and 200 μM) for 30 min followed by a 1 h recovery in fresh embryo medium. A significant main effect of genotype and gentamicin concentration and a significant interaction were observed (2-way ANOVA, p 's < 0.0001). (B) Chronic exposure to gentamicin for 6 h. 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 μM gentamicin (Bonferroni post-hoc comparisons, $p > 0.05$). Bars denote one standard deviation.

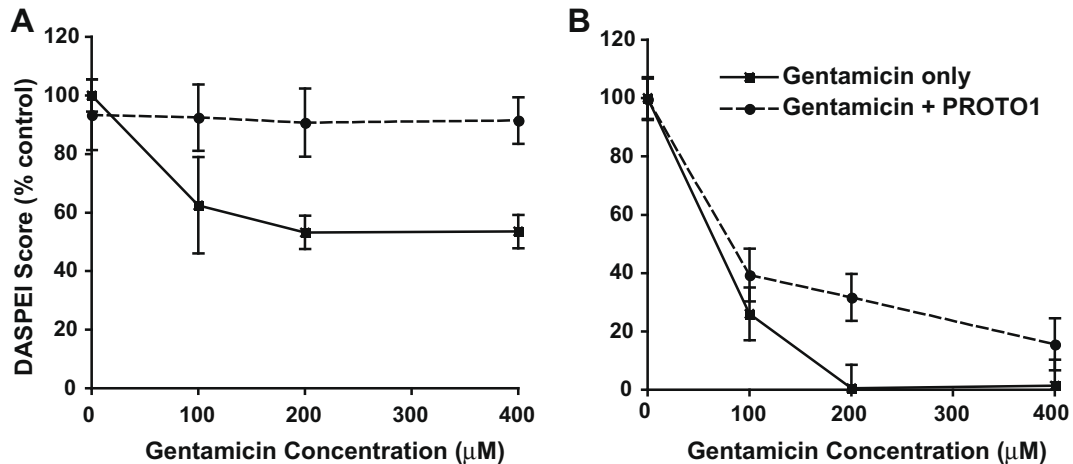


Fig. 4. The small molecule protectant PROTO1 completely attenuates hair cell death induced acutely by gentamicin and only partially protects hair cells from chronic gentamicin exposure. Zebrafish larvae were treated with PROTO1 or mock-treated for 1 h prior to and coincident with gentamicin exposure. Gentamicin only (solid lines, squares), Gentamicin and PROTO1 (dashed lines, circles). (A) Acute exposure for 30 min followed by 1 h recovery. Hair cell death is inhibited in the presence of PROTO1 ($p < 0.0001$). (B) Chronic gentamicin exposure for 6 h 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 one standard deviations.

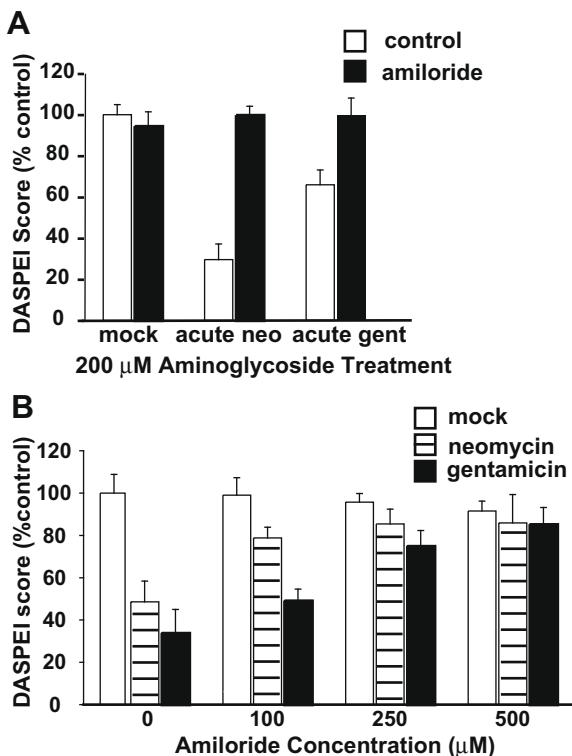


Fig. 5. The mechanosensory channel blocker amiloride prevents hair cell death induced by either acute or chronic treatment with either neomycin or gentamicin. (A) Zebrafish larvae were pre-treated with 1 mM amiloride then exposed concurrently to 200 µM neomycin or gentamicin acutely (30 min aminoglycoside exposure followed by 1 h 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 of amiloride 15 min prior to and during 6 h exposure to neomycin (100 µM) or gentamicin (50 µM) 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.

chronic exposure, animals were exposed to drug continually for 6 h. 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. 6, 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.

4. Discussion

4.1. Aminoglycosides differentially induce hair cell death

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 min and, second, a slower process (or processes) that kills most of the remaining hair cells if exposure time is sufficient (3–6 h). Rapid hair cell death is observed with all of the aminoglycosides tested, although the amount of death induced by a particular aminoglycoside differs. Neomycin

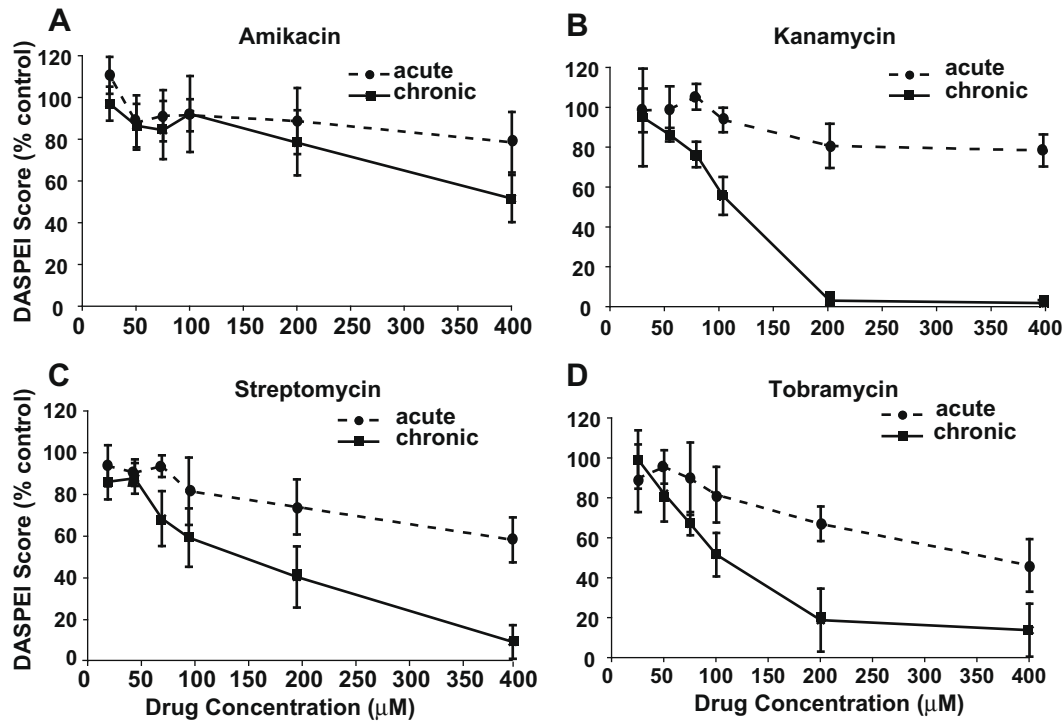


Fig. 6. Hair cell loss in the zebrafish lateral line induced by different aminoglycosides. Most aminoglycosides show differences between acute and chronic treatment. Graphs show results of DASPEI staining analysis of hair cells following acute (dashed lines, circles) or chronic exposure (solid lines, 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).

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 min and a second mechanism that is first evident between 3–6 h and continues for at least 24 h, 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 PROTO1 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. Marcotti et al. (2005) demonstrated that dihydrostreptomycin acted as a permeant blocker of the mouse transduction channel.

Furthermore, Gale et al. (2001) showed that the mechanotransduction channel blocker FM1-43 reduces neomycin hair cell toxicity in mouse. Previous observation that gentamicin tagged with the fluorophore Texas Red enters hair cells in 30 min (Steyger et al., 2003). Rapid entry of FM1-43 in murine hair cells occurs in <1 min (Gale et al., 2001) indicating that entry via the mechanotransduction channel is rapid. Fluorescently-labeled gentamicin also enters hair cells of the zebrafish lateral line within 1 min. (Santos et al., 2006; Owens et al., 2008) perhaps consistent with entry via the mechanotransduction channel. 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 aminoglycosides 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 1–24 h 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 and Blanchard, 2005; Borovinskaya et al., 2007, 2008).

4.2. 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 h of aminoglycoside exposure in rat Organ of Corti (Wei et al., 2005; Nagy et al., 2004; Lahne and Gale, 2008) and within 6 h of gentamicin exposure in guinea pig or gerbil utricle (Forge and Li, 2000). Ototoxic damage to mouse cochlear cultures has been reported with aminoglycosides following 1 h treatment (Kotecha and Richardson, 1994). With *in vivo* aminoglycoside exposure in mammalian systems (introduced systemically or intratympanically), the onset of hair cell death is often reported as taking 24 h or more. Intracochlear perfusion of gentamicin in guinea pigs induced damage to hair cells in a basal-to-apical gradient at 1 h 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 h 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 h post-treatment and subsequent shifts in hearing thresholds by 18 h 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 (Wang 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 min.

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. (1984) with *in vitro* assays looking at phospholipid binding. Kotecha and 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 the oscar, 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 the age studied here, 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; Fischel-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 and Brandon, 2006; Taleb et al., 2008) are partially protective against aminoglycoside-induced hair cell death. Overexpression of the anti-apoptotic factor, Bcl-2, and inhibition of intrinsic caspase cascades (cas9, cas3) attenuates aminoglycoside-induced hair cell death in chick and rodent (Cunningham et al., 2002; 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 and 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.

4.3. 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 (Moore et al., 1984; Lerner et al., 1986; Esterhai et al., 1986; Magnusson and Padoan, 1991). 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 and Rubel, 1990; Beaubien et al., 1990; Shepherd and 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 et al., 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.

4.4. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.heares.2009.03.001.

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