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Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*)

Setsuko L. Murakami [a,b,c], Lisa L. Cunningham [c,d], Lynne A. Werner [b], Eric Bauer [c,d], Rémy Pujol [c,e], David W. Raible [c,f], Edwin W. Rubel [c,d,*]

[a] *Graduate Program in Audiology, University of Washington, Seattle, WA 98195, USA*
[b] *Department of Speech and Hearing Sciences, University of Washington, Seattle, WA 98195, USA*
[c] *Virginia Merrill Bloedel Hearing Research Center, University of Washington, Box 357923, Seattle, WA 98195-7923 USA*
[d] *Department of Otolaryngology-Head and Neck Surgery, University of Washington, Seattle, WA 98195, USA*
[e] *INSERM, University Montpellier, Montpellier, France*
[f] *Department of Biological Structure, University of Washington, Seattle, WA 98195-7293 USA*

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Abstract

Mechanosensory hair cells of the inner ear are susceptible to death when exposed to a variety of drugs including aminoglycoside antibiotics. During avian and mammalian development, there is a period of relative insensitivity to aminoglycoside-induced hair cell death. This study was designed to test the hypothesis that zebrafish (*Danio rerio*) have developmental differences in sensitivity to aminoglycoside-induced hair cell death in the lateral line neuromasts. Larval zebrafish of various ages were exposed to several concentrations of neomycin, and their hair cells were examined using the potentiometric vital dye, DASPEI. Results indicate that zebrafish larvae aged 4 days post-fertilization are relatively insensitive to aminoglycoside-induced hair cell death compared to older fish. Thus zebrafish hair cells show developmental differences in sensitivity to aminoglycoside-induced death similar to those reported for inner ear hair cells of birds and mammals.

Key words: Ototoxicity; Critical period; Apoptosis; Hearing loss; Aminoglycoside; Deafness

1. Introduction

The majority of permanent hearing loss is due to the death of inner ear sensory hair cells. Auditory hair cells are sensitive to a wide variety of insults, including aging, noise trauma, and exposure to certain chemotherapeutic agents including aminoglycoside antibiotics. The dose-limiting factors in aminoglycoside use are their ototoxicity and renal toxicity. Aminoglycosides kill hair cells in all vertebrates tested including humans.

Aminoglycosides vary in the amount of hair cell death they cause and the inner ear organs they affect (Forge and Schacht, 2000). Aminoglycoside-induced cochleotoxicity is characterized clinically by hearing loss initially affecting high frequencies, progressing to low frequencies with continued exposure. This corresponds to initial hair cell damage in the basal portion of the cochlea, progressing to damage in the more apical regions of the cochlea.

There is tremendous individual variability in susceptibility to hair cell death resulting from age, noise exposure, and ototoxicity. Some of this variability is genetic in humans (Gates et al., 1999) as well as other species. As many as 17% of patients with extreme sensitivity to aminoglycoside ototoxicity carry a mutation in position 1555 of the mitochondrial ribosomal RNA (Forge and Schacht, 2000). This is the only known genetic mutation that influences sensitivity to aminoglyco-

* Corresponding author. Tel.: +1 (206) 543-8360; Fax: +1 (206) 221-5685.
E-mail address: rubel@u.washington.edu (E.W. Rubel).

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side-induced hair cell death. The experiments presented here are part of a larger research program aimed at utilizing the powerful genetic potential of the zebrafish to discover genes that influence susceptibility and resistance to hair cell death.

The zebrafish (*Danio rerio*) lateral line represents an exciting model system to study genetic variations in susceptibility of hair cells to ototoxic agents. Zebrafish have a short generation time (birth to sexual maturity) of 3–4 months and produce large numbers of progeny, two features that facilitate large-scale genetic screening (Patton and Zon, 2001). In addition, this system facilitates examination of hair cells in the living animal. Like most fish, zebrafish have typical vertebrate inner ears with both hearing organs and vestibular organs (reviewed by Popper, 2000). In addition to the inner ear, fish have lateral line organs, comprised of a series of clusters of hair cells and support cells called neuromasts. Each neuromast contains 10–20 hair cells that are surrounded by support cells. In larval zebrafish at the ages studied, 20–30 neuromasts reside in a stereotypical pattern on the surface of the fish's head and body. The stereocilia of these lateral line hair cells protrude into the surrounding water. The adult lateral line system is much more complex, consisting of several hundred neuromasts, some of which are on the body surface (free neuromasts) while others are below the surface in bony canals (Metcalfe, 1989). The lateral line system is used to detect water-borne mechanical stimuli as well as the motion of the fish's body in the water (Coombs and Montgomery, 1999; Montgomery et al., 2000). The hair cells of the zebrafish inner ear and lateral line are remarkably similar in both structure and function to the hair cells in the human inner ear (Haddon and Lewis, 1996). Lateral line hair cells are susceptible to aminoglycoside-induced hair cell death, and they can be easily studied in living zebrafish (Song et al., 1995; Williams and Holder, 2000; Harris et al., 2003).

Developmental changes in sensitivity to aminoglycoside-induced hair cell death have been studied in birds and in a variety of mammals (rats, mice, guinea pigs, and cats). Prior to the onset of auditory function, auditory hair cells are relatively insensitive to aminoglycoside-induced death. The cochlea is affected by aminoglycosides as soon as it begins to function as a mechanosensory receptor (Friedmann and Bird, 1961), which in some cases is in utero (Raphael et al., 1983). It has also been shown that between the onset of cochlear function and full maturation, the cochlea passes through a period of heightened sensitivity to aminoglycosides relative to its sensitivity in adulthood. This sensitive period is often referred to as a critical period (Forge and Schacht, 2000).

Examples of developmental differences in sensitivity to aminoglycoside-induced ototoxicity can be found in various mammalian inner ears. For example, the rat cochlea is relatively insensitive to kanamycin-induced damage prior to the 8th postnatal day. From the onset of cochlear potentials on the 8th postnatal day, the sensitivity of the rat cochlea to aminoglycoside-induced damage increases significantly (Marot et al., 1980). In the guinea pig, kanamycin has a heightened ototoxic effect, causing elevated hair cell loss during the third trimester of gestation, when the guinea pig's cochlea undergoes rapid functional maturation, compared to the first two trimesters (Raphael et al., 1983). Based on studies such as these, the existence of the same type of critical period in human development has been assumed (Rasmussen, 1969; Bernard et al., 1980; Bernard, 1981; Pujol, 1986).

Similar developmental differences in sensitivity to aminoglycoside-induced ototoxicity have also been described in birds. A study examining chicks suggested that gentamicin may not be as effective at damaging hair cells before they are fully mature (Duckert and Rubel, 1990).

The current study was designed to test the hypothesis that hair cells of the larval zebrafish lateral line neuromasts show developmental differences in sensitivity to aminoglycoside-induced death. This hypothesis was tested by examining the relationship between neomycin concentration and hair cell death at various stages of zebrafish larval development.

2. Materials and methods

2.1. Animals

Zebrafish (*D. rerio*) embryos were produced by paired matings of adult fish maintained at 28.5°C (Westerfield, 2000) in the University of Washington zebrafish facility. Larvae typically hatched 3 or 4 days post-fertilization (dpf) and were then moved to a larger (0.4-l) tank in the zebrafish facility. Beginning at 4 dpf, larvae were fed live paramecia (Westerfield, 2000). Fish were tested at 4, 5, 6, 7, 8, or 13 dpf. Larvae were maintained at a density of 30–50 per 100-mm^2 Petri dish in embryo medium (1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4, 1 mM $CaCl_2$, 500 μM KCl, 15 μM NaCl, and 500 μM $NaHCO_3$ in dH_2O, Westerfield, 2000) in a tissue culture incubator at 28.5°C. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Neomycin treatment

Neomycin sulfate (Pharma-Tek, Huntington, NY,

USA) was prepared as a 100 mM stock solution in dH_2O. This stock was diluted in embryo medium to final concentrations of 50 μM, 100 μM, 150 μM, 200 μM, and 400 μM in each well of a six-well tissue culture plate. Age 4 dpf fish that had not hatched by the time of testing were manually dechorionated using a plastic pipet. Live, free-swimming zebrafish larvae were placed into a 50-ml conical tube with one end cut off and a mesh cover at the bottom that was used as a transfer device. Larvae were transferred from control (neomycin-free) embryo medium to neomycin-containing medium and incubated for 1 h. After 1 h, larvae were rinsed three times quickly in fresh medium and returned to the incubator in neomycin-free medium for an additional 3 h. A previous study has shown that this protocol results in hair cell death by 4 h after initial neomycin exposure (Harris et al., 2003).

2.3. DASPEI staining

The fluorescent vital dye 2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide (DASPEI; Molecular Probes, Eugene, OR, USA) was used to stain zebrafish larval hair cells within neuromasts (Jorgensen, 1989; Balak et al., 1990). DASPEI is a voltage-sensitive dye that specifically labels the hair cells of the lateral line neuromasts and the nasal epithelium of the fish. During the final 15 min of incubation in neomycin-free medium, DASPEI was added to the medium to make a final dilution of 0.005%. During the last 5 min of this 15-min interval, the larvae were anesthetized in MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma, St. Louis, MO, USA, 8 μg/ml). Larvae were then rinsed once in fresh embryo medium and analyzed under an epifluorescent dissecting microscope equipped with a DASPEI filter set (Leica MZFl111; excitation 450–490 nm and barrier 515 nm). Images of live zebrafish larvae stained with DASPEI were captured on a cooled CCD camera (Leica, DFC 350 F).

2.4. Transmission electron microscopy

Neuromasts of control and neomycin-exposed (500 μM neomycin) zebrafish larvae were examined using transmission electron microscopy in order to visualize ultrastructural changes in neuromasts exposed to neomycin. Larvae at age 5 dpf were immobilized in ice water and immersion fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4)+0.001% $CaCl_2$ for 1 h. Specimens were then placed in fresh fixative and held overnight at 4°C.

Larvae were washed in 0.1 M cacodylate buffer and post-fixed in 1% OsO_4 in 0.1 M cacodylate+0.001% $CaCl_2$ for 1 h on ice. Fish were again washed in cacodylate and then dehydrated through graded ethanols, infiltrated and embedded in Spurr's epoxy resin via propylene oxide. Larvae were oriented to obtain cross-sections in a rostral to caudal manner. Semi-thin sections (2 μm) were collected and stained with 1% toluidine blue to locate appropriate neuromasts. Ultrathin sections (90 nm) were cut on a Leica Ultracut S microtome and mounted on 200-mesh Athene thin-bar grids and contrasted with uranyl acetate and lead citrate. Grids were examined using a JEOL JEM 1200 EX transmission electron microscope.

Low magnification (2000×) survey micrographs were made to record the entire neuromast. Higher magnification (6000–10 000×) micrographs were made to document ultrastructural details of neuromast hair cells and supporting cells.

2.5. Examination of neuromasts

By age 4 dpf, zebrafish larvae have 18 head and nine trunk neuromasts on each side of the body in several lines that together comprise the larval lateral line system (Raible and Kruse, 2000). In the present study we scored 10 of the head neuromasts (SO1, SO2, SO3, IO4, O2, MI1, MI2, IO1, IO2, and IO3) of each larva stained with DASPEI. Each neuromast was scored individually according to the following criteria: a score of 2 indicated normal DASPEI staining; a score of 1 indicated reduced DASPEI staining; and a score of 0 indicated the absence of DASPEI staining. Total DASPEI scores were then tabulated for one randomly chosen side of each fish with a maximum possible score of 20 (or 10 neuromasts multiplied by the high score of 2), indicating normal staining of each neuromast. The DASPEI scoring method has been validated by comparison of hair cell counts in fixed tissue stained with phalloidin and anti-acetylated tubulin (Harris et al., 2003). Scores from all of the groups of fish ($n = 15$–25 fish/concentration) were expressed as a proportion of this maximum possible DASPEI score of 20. Scores were then subjected to a two-way factorial analysis of variance (ANOVA) to identify overall and interaction effects of age and neomycin concentration on DASPEI scores. Scores from each age were separately subjected to a simple linear regression to establish the effects of varying neomycin concentrations on DASPEI scores. The slopes of the regression lines were compared using analysis of covariance (Snedecor and Cochran, 1980). From each of the simple linear regressions, the concentration of neomycin that resulted in a DASPEI score of 10 (50% of maximum possible DASPEI score of 20) was calculated for each age, and this value was referred to as the lethal dose resulting in 50% hair cell death (LD_{50}).

2.6. Twelve-hour neuromast scoring and data analysis

In order to examine the possibility of rapid hair cell recovery following neomycin exposure, additional groups of 5 dpf zebrafish larvae ($n = 24$–50 fish/concentration) were treated with 0 μM, 50 μM, 100 μM, 150 μM, and 200 μM neomycin using the method described above. One-half of these larvae were analyzed using the DASPEI scoring method 4 h after initial neomycin exposure as above. The other half were allowed to recover for 12 h in neomycin-free medium before being analyzed using DASPEI. Scores from both 4- and 12-h groups of 5 dpf fish were expressed as a proportion of the maximum possible DASPEI score of 20. Scores were subjected to a two-way factorial ANOVA to examine the effects of the number of hours after initial neomycin exposure, neomycin concentration, and their interaction on DASPEI scores. Dose–response curves were generated by plotting DASPEI scores versus neomycin concentrations for both 4- and 12-h recovery periods. Data were also subjected to multiple paired *t*-tests comparing the two time periods after exposure to various concentrations.

2.7. Immunohistochemistry

In order to more closely examine hair cells following 12 h of recovery post neomycin exposure, a separate group of 5 dpf larvae were treated with 0 or 200 μM neomycin sulfate ($n = 5$–7 fish/treatment group) and fixed 12 h later. Hair cells were labeled using a monoclonal antibody directed against acetylated tubulin, and stereocilia were stained for F-actin with phalloidin. Twelve hours after initial neomycin exposure, larvae were anesthetized with MS222 (10 μg/ml) in embryo medium. They were then fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). Larvae were then washed in 1% bovine serum albumin and 0.05% Triton X-100 in phosphate-buffered saline (PBST). Non-specific binding was blocked by incubating larvae in 10% normal goat serum in PBST for 1 h at room temperature. They were then incubated overnight at 4°C in anti-acetylated tubulin (Sigma) diluted to 1:1000 in blocking serum. Larvae were then washed in PBST and incubated for 5 h at room temperature in Alexa 594-conjugated goat anti-mouse IgG (1:400 in blocking serum; Molecular Probes). Larvae were rinsed in PBST and incubated for 3 h in Alexa 488-conjugated phalloidin (Molecular Probes) diluted 1:200 in PBS/1% bovine serum albumin. After a final wash, larvae were mounted in Fluoromount G (Southern Biotechnology Associates, Birmingham, AL, USA) and coverslipped. Specimens were examined and photographed using a confocal laser scanning microscope (Bio-Rad 1024).

3. Results

3.1. Lateral line hair cell death caused by neomycin

DASPEI is a voltage-sensitive dye that preferentially labels the hair cells of lateral line neuromasts as well as the nasal epithelium. Fig. 1A shows the stereotypical distribution of neuromasts in a living control 5 dpf zebrafish stained with DASPEI. With the low level of magnification illustrated in Fig. 1A, individual hair cells within a neuromast cannot be visualized. Neomycin exposure resulted in significant loss of DASPEI staining in neuromasts. Fig. 1B demonstrates that a 1-h exposure to 200 μM neomycin eliminated DASPEI staining in lateral line hair cells in all neuromasts when viewed 3 h later. In addition to hair cells, DASPEI staining is present in the nasal epithelium in both conditions (large arrows in Fig. 1A,B, Alexandre and Ghysen, 1999). The

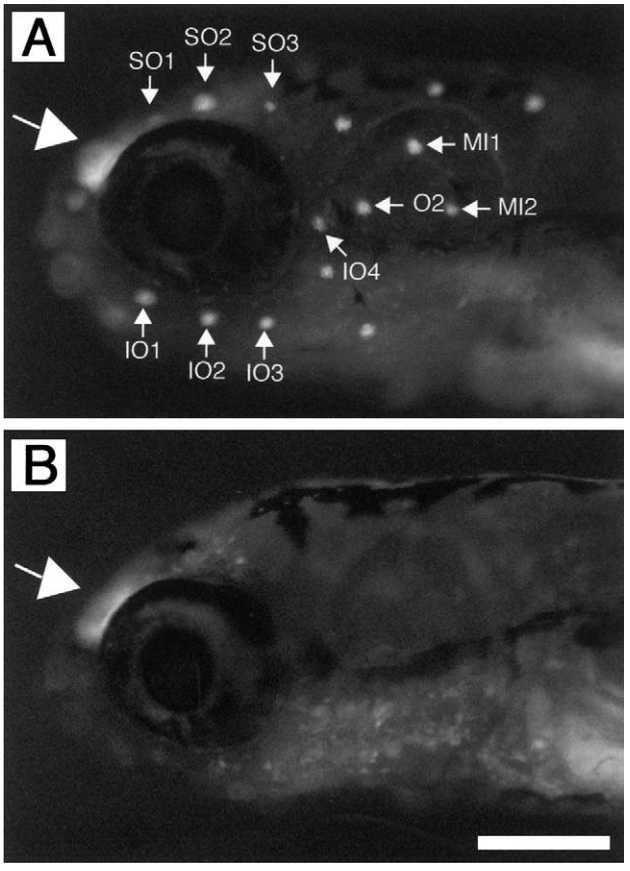

Fig. 1. Neomycin exposure eliminates DASPEI staining in neuromasts of zebrafish larvae. (A) A 5 dpf control zebrafish larva stained with the fluorescent vital dye DASPEI. Neuromasts appear as punctate dots in this low-magnification view. Arrows point to neuromasts used for this study. (B) A 5 dpf zebrafish larva treated with 200 μM neomycin for 1 h and then stained with DASPEI 4 h later. Neomycin exposure eliminates DASPEI staining in the neuromasts. DASPEI staining of the nasal epithelium (large arrows in both A and B) is unaffected by neomycin exposure. Scale bar in B = 0.5 mm and applies to both A and B.

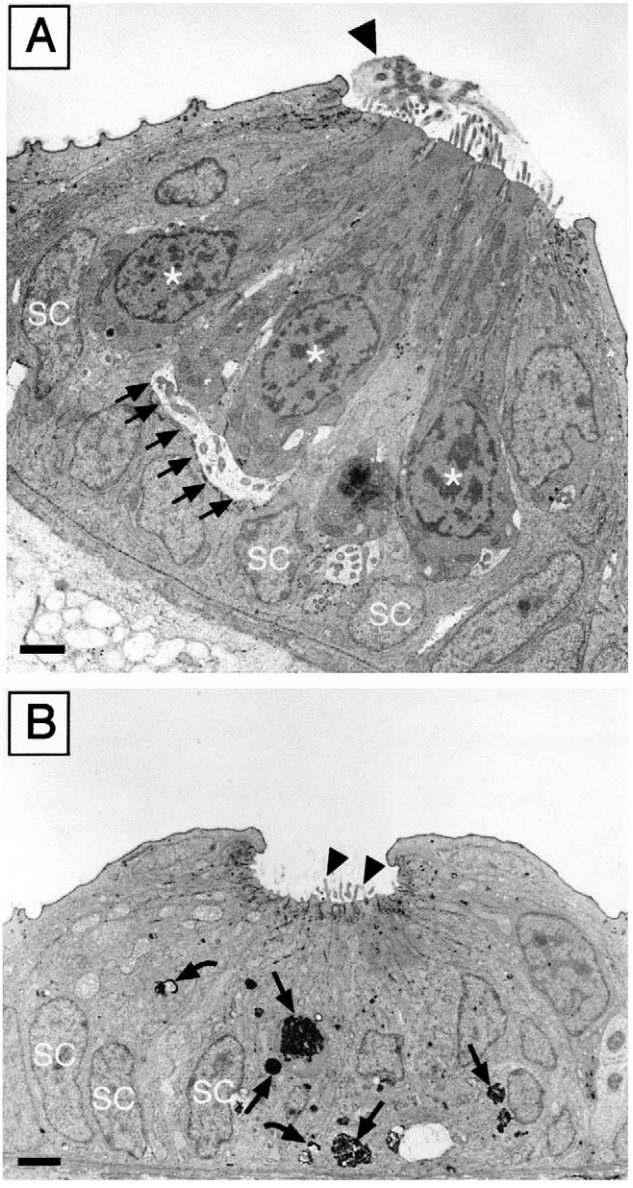

Fig. 2. Transmission electron microscopy of control and neomycin-treated neuromasts. (A) TEM of a control neuromast of a 5 dpf zebrafish larva. Several hair cells are clearly visible in this section: at least three of them (asterisks) are cut through their nucleus. Their cuticular plates bear stereocilia. The kinocilium from each hair cell is embedded in the remnants of the cupula and bent onto the top of stereocilia (arrowhead). At the base of hair cells, nerve endings are recognizable; arrows point to an afferent nerve ending which contacts two adjacent hair cells. SC = support cells. (B) TEM of a neuromast of a zebrafish larva exposed for 1 h to 500 μM neomycin. This exposure results in an almost complete destruction of the hair cells: only remnants of what appear to be stereocilia (arrowheads) remain visible. Support cells remain present (SCs). In the neuromast, apoptotic debris (arrows) as well as excitotoxic afferent nerve endings (curved arrows) are present. Note: panels A and B are at different magnifications. Scale bar in A = 2 μm. Scale bar in B = 2.5 μm.

staining of the nasal epithelium was unaffected by this level of neomycin exposure, and thus served as an internal control for the reliability of DASPEI staining.

Fig. 2 shows transmission electron micrographs (TEMs) of neuromasts from control and neomycin-treated larvae. As compared to control neuromasts (Fig. 2A), neuromasts from neomycin-treated larvae (Fig. 2B) showed marked damage to hair cells and nerve endings. An exposure to 500 μM neomycin for 1 h resulted in an almost complete disappearance of normal hair cells, although remnants of what appeared to be stereocilia remained visible. Numerous apoptotic bodies and excitotoxic profiles of nerve endings were visible. Support cells appeared normal.

3.2. *Dose–response relationships at 4 and 12 h post neomycin exposure*

Two different control experiments were conducted to evaluate the potential contribution of sublethal hair cell damage to the loss of DASPEI staining due to neomycin exposure. In one experiment, 5 dpf larvae were exposed to varying concentrations of neomycin for 1 h and then allowed to survive in normal medium for either 4 or 12 h. The survival of lateral line hair cells was then assessed by DASPEI labeling using the scoring system described above. The assumption made here was that if neomycin exposure, at the concentrations used, temporarily disrupts DASPEI labeling but allows

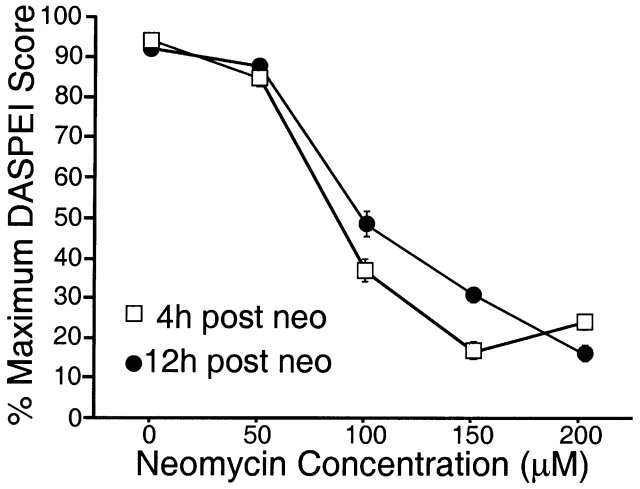

Fig. 3. DASPEI scores remain low 12 h after neomycin exposure. 5 dpf zebrafish larvae were exposed to various concentrations of neomycin and examined either 4 or 12 h later ($n = 12$–25 larvae/group). Mean (±S.E.M.) DASPEI scores (relative to the total possible DASPEI score of 20) are shown for each group. For both recovery periods, increasing neomycin concentration resulted in decreased hair cell survival. Note that in the groups exposed to 100 μM and 150 μM neomycin there was a small increase in hair cell survival at 12 h recovery ($P < 0.01$) and slightly less hair cell survival at 12 h after exposure to 200 μM ($P < 0.05$). Where error bars are not visible, the size of the error bar is smaller than the symbol.

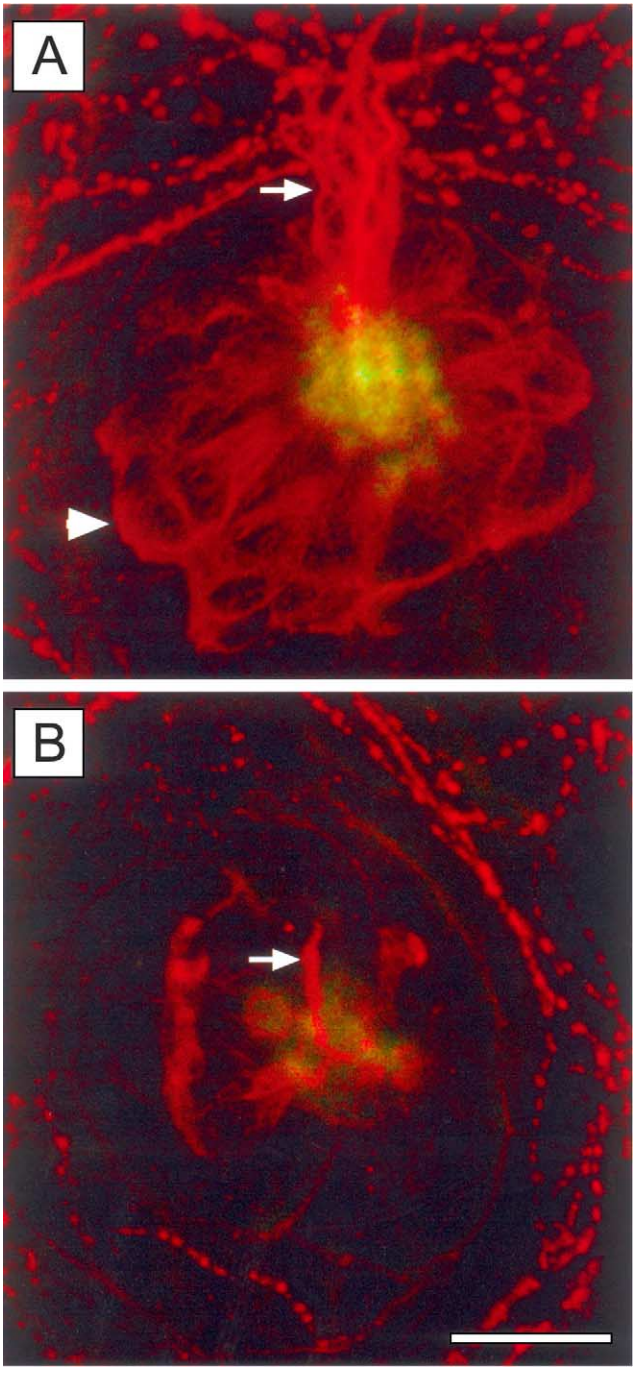

Fig. 4. Immunohistochemistry of control and neomycin-treated neuromasts. (A) Control neuromast of a 5 dpf zebrafish larva. Neuromasts were double-labeled using phalloidin (green) and an antibody directed against acetylated tubulin (red). Phalloidin-labeled stereocilia bundles are visible on the tops of the hair cells. Anti-acetylated tubulin labels the kinocilium (arrow) of each hair cell as well as the hair cell bodies (arrowhead). (B) Neuromast of a 5 dpf zebrafish larva 12 h after exposure to 200 μM neomycin. Anti-acetylated tubulin reveals very few or no hair cell bodies, although one or two kinocilia remain (arrow). Phalloidin labeling of stereocilia is greatly diminished compared to the control neuromast. Scale bar in B = 10 μm and applies to both A and B.

hair cell survival, recovery may have occurred by 8 h later. This assumption is based on the fact that zebrafish lateral line hair cell regeneration begins by 12 h post neomycin exposure (Harris et al., 2003), suggesting that by this time the hair cells have had sufficient time to complete the life-or-death transition and initiate either recovery or degeneration. Fig. 3 demonstrates that DASPEI scores decrease with increasing neomycin concentration for both the 4-h and 12-h recovery periods. More specifically, the relationship between neomycin concentration and hair cell damage is very similar at the two survival times. When exposed to intermediate concentrations (100 and 150 μM), DASPEI scores at 12 h were slightly higher than scores observed at 4 h. When exposed to 200 μM neomycin, the opposite was true. A two-way factorial ANOVA revealed a significant main effect for neomycin concentration ($P < 0.001$), a significant main effect for survival time ($P < 0.01$), and a significant interaction term ($P < 0.01$). Individual comparisons between the two survival periods at each concentration indicated that there was a small but significant amount of recovery between 4 and 12 h in the 100 and 150 μM neomycin concentration groups ($P < 0.01$ for each). In addition, the apparent increase in hair cell loss seen at 12 h after exposure to 200 μM neomycin exposure was marginally significant ($P < 0.05$). To further examine the integrity of hair cells in the absence of DASPEI labeling, fixed neuromasts were also examined using immunohistochemistry 12 h after neomycin exposure. Fig. 4 shows hair cell stereocilia labeled for F-actin with phalloidin, and hair cells within each neuromast labeled with anti-acetylated tubulin. In the control neuromast (Fig. 4A), anti-acetylated tubulin labels the hair cell cytoplasm as well as the kinocilia. Stereocilia are labeled with phalloidin. There was a marked decrease in both phalloidin and acetylated tubulin labeling in neuromasts of neomycin-exposed larvae (Fig. 4B) relative to control larvae. Thus the immunohistochemistry data indicate that hair cells are missing from the neuromasts of fish exposed to 200 μM neomycin and allowed to recover for 12 h.

3.3. *Dose–response relationships during zebrafish development*

To test the hypothesis that zebrafish have developmental differences in sensitivity to aminoglycoside-induced lateral line hair cell death, zebrafish larvae at ages 4 dpf, 5 dpf, 6 dpf, 7 dpf, 8 dpf, and 13 dpf were exposed to various concentrations of neomycin for 1 h and analyzed 4 h later using the DASPEI scoring system. Results are shown in Fig. 5. At every age examined, neomycin exposure resulted in a dose-dependent decrease in hair cell survival. Larvae aged 4 dpf showed significantly less sensitivity to neomycin-in-

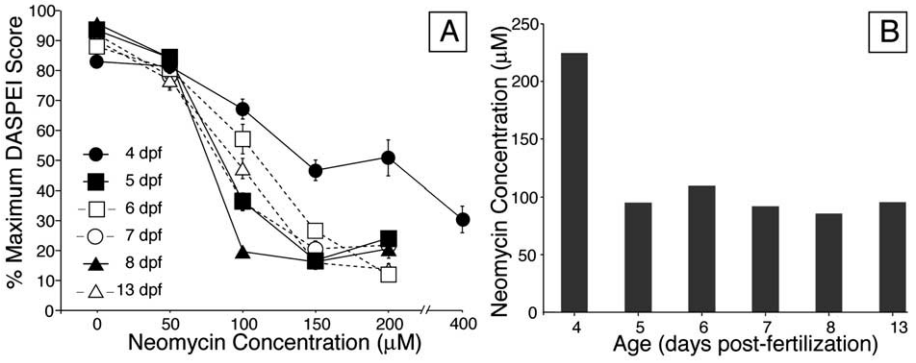

Fig. 5. Developmental differences in sensitivity to neomycin-induced hair cell death. (A) Dose–response relationships. Zebrafish larvae at ages 4, 5, 6, 7, 8, 13 dpf were exposed to various concentrations of neomycin and examined 4 h later ($n = 15$–25 larvae/group). Mean (±S.E.M.) DASPEI scores (as a percentage of the maximum possible DASPEI score of 20) are shown for each group. For all ages, increasing neomycin concentration resulted in decreased hair cell survival. Neuromasts of 4 dpf zebrafish were significantly less sensitive to neomycin-induced hair cell death than all other ages. Where error bars are not visible, the size of the error bar is smaller than the symbol. (B) LD_{50}. The neomycin concentration that resulted in 50% reduction in DASPEI score was calculated for each age tested (4, 5, 6, 7, 8, 13 dpf). The neomycin concentration required to destroy 50% of 4 dpf zebrafish hair cells was significantly higher than the concentrations required for all other ages.

duced hair cell death than did older larvae. Because the DASPEI scores for 4 dpf fish remained high even at 200 μM neomycin, an additional group of 4 dpf larvae was examined at 400 μM neomycin (Fig. 5A). Even at this dose, DASPEI labeling of 4 dpf larvae was not eliminated, although DASPEI scores were decreased relative to the scores at 200 μM neomycin. An analysis of covariance comparing simple linear regressions of each age (not including asymptotic data; 4 dpf: 50–400 μM neomycin, all other ages: 50–150 μM neomycin) to one another showed that DASPEI scores of 4 dpf fish were significantly higher than DASPEI scores of all other ages of fish. These results indicate a remarkable lack of sensitivity to neomycin-induced lateral line hair cell death in the 4 dpf zebrafish larvae. In addition, these results demonstrate a very rapid increase in sensitivity to neomycin-induced hair cell death in the 24 h between 4 dpf and 5 dpf.

Fig. 5A also shows some differences in sensitivity among the other ages tested, although none of them as pronounced as the difference in the 4 dpf group. For example, although 6 dpf fish are more sensitive than 4 dpf fish, they appear slightly less sensitive than all other ages of fish. A two-way factorial ANOVA (age × neomycin concentration) showed a significant interaction effect ($P < 0.01$). Analysis of covariance also showed that DASPEI scores of 6 dpf larvae were slightly (but significantly) higher than DASPEI scores of 5, 7, 8, and 13 dpf larvae ($P < 0.05$).

The data were also analyzed to determine the neomycin concentration required to reduce the DASPEI score by 50% at each age (Fig. 5B). This dose is reported as the LD_{50}. These values were calculated using the slope and intercept values from simple linear regressions of the dose–response curve at each age. Fig. 5B shows that a much higher concentration of neomycin is required in order to produce a 50% reduction in DASPEI scores of neuromasts from 4 dpf larvae than is required for any of the older ages, and that the other five groups are remarkably similar.

Two additional analyses were performed in order to examine potential causes for the observed differences in hair cell susceptibility between 4 dpf and 5 dpf. First, we examined whether the lack of sensitivity to neomycin-induced damage in 4 dpf larvae could be attributed to any individual neuromasts. We reasoned that 'younger' (later-developing) neuromasts might show less sensitivity than 'older' (earlier-developing) neuromasts. A two-way ANOVA of 4 dpf fish (individual neuromast location × neomycin concentration) indicated that specific individual neuromasts differed from each other in their susceptibility to neomycin. Further analysis revealed that the SO3 neuromast showed reduced DASPEI labeling in control (no neomycin treatment) 4 dpf larvae and at all concentrations of neomycin. This finding was surprising since SO3 is among the earliest neuromasts to appear (Raible and Kruse, 2000). Still, SO3 was the only neuromast to differ from all other neuromasts in the control condition, so a two-way factorial ANOVA (SO3 inclusion or exclusion × neomycin concentration) was performed, but did not show a significant interaction effect. When SO3 DASPEI scores were not included with the total DASPEI scores for the 4 dpf fish, the entire 4 dpf dose–response curve was shifted to the right, showing an even greater difference between 4 dpf and the other ages. From this additional analysis, it appears that there is a significant difference between individual neuromast scores, but this difference does not account for the lack of sensitivity to neomycin-induced hair cell death seen in 4 dpf larvae.

The second set of additional analyses was aimed at examining whether individual hair cells within a given

neuromast were insensitive at 4 dpf. We reasoned that if the neuromasts of 4 dpf larvae contained a subset of insensitive hair cells, those neuromasts would receive significantly more DASPEI scores of 1 than of 0 or 2, since the sensitive hair cells would not be labeled with DASPEI and the insensitive ones would. On the other hand, if all the hair cells in a given neuromast had similar sensitivities to neomycin-induced hair cell death, we would expect to see a range of scores (0, 1, and 2) that was comparable to the range of scores seen in the older fish. The percentage of DASPEI scores of 0, 1, and 2 was examined for each neuromast across all concentrations of neomycin for all ages (data not illustrated). This analysis revealed that 4 dpf neuromasts received a range of DASPEI scores that was similar to that received by older fish. Thus it appears that the lack of sensitivity to neomycin-induced hair cell death is not attributable to the presence of a subset of insensitive hair cells within each neuromast. It is more likely that all (or most) of the hair cells in a given neuromast from 4 dpf larvae are relatively insensitive to neomycin-induced death.

4. Discussion

4.1. Zebrafish lateral line hair cells demonstrate developmental differences in sensitivity to aminoglycoside-induced hair cell death

The primary goal of these experiments was to determine whether the zebrafish lateral line neuromasts, like the inner ears of other vertebrates, demonstrate developmental differences in sensitivity to aminoglycoside-induced hair cell death. The primary finding is that 4 dpf zebrafish larvae are significantly less sensitive to neomycin-induced hair cell death than older larvae. For all ages tested (4 dpf, 5 dpf, 6 dpf, 7 dpf, 8 dpf, and 13 dpf), exposure to higher concentrations of neomycin resulted in decreasing hair cell survival (as measured by DASPEI scores). While all of the experiments presented here were performed independently of those in our previous paper, the dose–response relationships reported here are in good agreement with the earlier study (Harris et al., 2003).

Zebrafish larvae aged 4 dpf showed a remarkable lack of sensitivity to neomycin-induced hair cell death relative to the sensitivity of older fish. This lack of sensitivity was demonstrated both by the comparisons of the dose–response relationships at various ages and by the calculation of the LD_{50} for each age. In addition, our results demonstrate a rapid increase in sensitivity to neomycin-induced hair cell death between 4 dpf and 5 dpf. Thus our data indicate that developing zebrafish have a period of relative insensitivity to aminoglycoside-induced hair cell death similar to that found in studies of mammalian inner ear development (Marot et al., 1980; Bernard, 1981; Raphael et al., 1983).

4.2. The lack of sensitivity to hair cell death in 4 dpf zebrafish may relate to the onset of mechanotransduction

Several previous investigations have suggested that inner ear hair cells are insensitive to aminoglycoside-induced death prior to the onset of mechanosensory transduction (Marot et al., 1980; Bernard, 1981; Raphael et al., 1983). Thus, the simplest explanation for our finding that 4 dpf larvae are relatively insensitive to neomycin-induced hair cell death may be that zebrafish larvae aged 4 dpf are not yet utilizing the hair cells of the lateral line neuromasts as mechanotransducers. All 10 of the neuromasts examined in the current experiments appear between 24 and 72 h post fertilization (Raible and Kruse, 2000). By 4 dpf, all of the neuromasts studied are innervated (Raible and Kruse, 2000). Thus all of the neuromasts examined in this study had formed at least 24 h prior to the earliest time point we examined (4 dpf). However, relatively little is known about the onset of zebrafish lateral line hair cell function. Blaxter and Fuiman (1989) examined the escape responses of larvae of several other teleost species and showed that they can respond to mechanical stimuli from a probe in the dark. Very young larvae were often able to respond to the probe before it touched them. These responses were diminished by treatment with either streptomycin or turbulence, indicating that the responses were mediated by the free (surface) neuromasts of the larval lateral line system (Blaxter and Fuiman, 1989). Zebrafish larvae adopt an upright swimming position at about 72 h post fertilization. In addition, zebrafish larvae aged 72 h post fertilization demonstrate an acoustic/vibrational startle reflex in response to a tap on the Petri dish in which they are housed (Nicolson et al., 1998). These data suggest that there are functional hair cells in the lateral line neuromasts of the 4 dpf zebrafish larvae. However, there is some overlap in the frequency sensitivities of the lateral line hair cells and those of the inner ears of zebrafish. Therefore, it is somewhat difficult to separate the functions of the two systems using behavioral tests. As a result, it is difficult to determine the exact time that lateral line hair cells of larval zebrafish begin to function as mechanotransducers in order to compare this time to the onset of increased susceptibility found in this study at age 5 dpf.

Our finding that 4 dpf larvae are not as susceptible to neomycin-induced hair cell death relative to older larvae is in contrast with the findings of Harris et al. (2003), who reported that 4 dpf and 5 dpf zebrafish did not differ significantly in their susceptibility to neo-

mycin-induced hair cell death. We believe the explanation for this difference is that Harris et al. (2003) used only those 4 dpf zebrafish that had naturally hatched and were already free-swimming. In the present study, some 4 dpf zebrafish were manually dechorionated in order to more closely represent the full range of development of larvae at this age. Thus the 4 dpf zebrafish used in the Harris et al. (2003) study were, on average, developmentally slightly more advanced than those used in the current study. This explanation again suggests that the lack of sensitivity in the 4 dpf larvae may be related to absence of mechanotransduction, since 4 dpf larvae that have not yet hatched may not be utilizing the hair cells of the lateral line neuromasts as mechanotransducers.

4.3. *Very little hair cell recovery occurs immediately following neomycin exposure*

Our experiments showed that DASPEI labeling is lost with exposure to neomycin, which is consistent with results of Harris et al. (2003). Transmission electron microscopy was performed in order to determine whether loss of DASPEI labeling was indicative of death of hair cells. Results showed that exposure to a high concentration of neomycin resulted in complete destruction of the hair cells in the neuromast. This finding confirms that loss of DASPEI staining reflects hair cell death and not merely loss of the mitochondrial membrane potential.

In order to determine whether damaged lateral line hair cells readily recover after exposure to lower concentrations of neomycin, larvae were treated with varying concentrations of neomycin and examined either 4 or 12 h later. The dose–response curves for zebrafish examined 4 and 12 h after exposure showed remarkable similarity. However, 12 h after neomycin exposure, DASPEI scores of larvae exposed to 100 μM or 150 μM neomycin were marginally (but significantly) higher than the scores at 4 h post neomycin exposure. This result suggests that while the majority of hair cells do not recover from neomycin exposure, some hair cells may recover within this time period. Immunohistochemistry results confirmed the interpretation that most hair cells had undergone cell death by 12 h after exposure to 200 μM neomycin. These results are in agreement with the transmission electron microscopy data obtained at 500 μM neomycin.

Two alternative explanations for higher DASPEI scores at 12 h versus 4 h relate to the fact that neuromasts are continuously adding new hair cells (Corwin, 1981, 1985). First, since immature hair cells may not be as susceptible to aminoglycoside-induced death (Rubel, 1978; Duckert and Rubel, 1990; Hashino and Salvi, 1996), the cells displaying DASPEI labeling at 12 h that were not labeled at 4 h may be those that differentiated over this period. The second potential alternative explanation is that hair cells have regenerated by 12 h. This explanation is eliminated by the observation that regenerated zebrafish lateral line hair cells are not observed until 24–48 h following neomycin exposure (Harris et al., 2003). Thus, examining hair cells 12 h after neomycin exposure allowed some time for hair cells to recover without the possibility of regeneration.

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