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Research paper

Lateral line hair cell maturation is a determinant of aminoglycoside susceptibility in zebrafish (*Danio rerio*)

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Abstract

Developmental differences in hair cell susceptibility to aminoglycoside-induced cell death has been observed in multiple species. Increased sensitivity to aminoglycosides has been temporally correlated with the onset of mechanotransduction-dependent activity. We have used in vivo fluorescent vital dye markers to further investigate the determinants of aminoglycoside induced hair cell death in the lateral line of zebrafish (*Danio rerio*). Labeling hair cells of the lateral line in vivo with the dyes FM 1-43, To-Pro-3, and Yo-Pro-1 served as reliable indicators of hair cell viability. Results indicate that hair cell maturation is a determinant of developmental differences in susceptibility. The age dependent differences in susceptibility to aminoglycosides are independent of the onset of mechanotransduction-dependent activity as measured by FM 1-43 uptake and independent of hair cell ability to take up fluorescently conjugated aminoglycosides.
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1. Introduction

Aminoglycosides are clinically used drugs that cause dose-dependent sensorineural hearing loss (Smith et al., 1977) and are known to kill hair cells in the mammalian inner ear (Theopold, 1977). The mechanisms of aminoglycoside-induced hair cell toxicity are an area of active investigation. Free radical formation (Priuska and Schacht, 1995; Clerici et al., 1996; Hirose et al., 1997) and subsequent activation of apoptotic pathways (Torchinsky et al., 1999; Forge and Li, 2000; Pirvola et al., 2000; Cunningham et al., 2002; Matsui et al., 2002; Cheng et al., 2003; Cunningham et al., 2004; Mangiardi et al., 2004; Matsui et al., 2003) has been shown to play a role in this process in both in vitro and in vivo preparations.

Among the model systems used to understand the mechanisms of hair cell toxicity, the zebrafish is a relative newcomer. The lateral line of zebrafish is a mechanosensory organ composed of a collection of neuromasts along the head and body containing hair cells and supporting cells (Metcalfe et al., 1985; Raible and Kruse, 2000; Gompel et al., 2001). Hair cells of the larval zebrafish lateral line, located superficially, are visualized and manipulated more readily than the hair cells of the mammalian inner ear. Gene expression, protein localization, morphological and behavioral assay tools available in the zebrafish make their larvae appealing subjects for toxicological studies.

Zebrafish lateral line hair cells show significant structural, functional and molecular similarities to the mammalian inner ear hair cells (Coombs and Montgomery, 1999). Shared structures include a TRPA1 mechanotransduction channel (Corey et al., 2004), and Cadherin 23-containing tip links (Sollner et al., 2004; Siemens et al., 2004.) Genetic

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mutations in Myosin VIIA, known to cause hearing disorders such as Usher 1B syndrome, DFNB2 and DFNA11 in humans, show morphological abnormalities in zebrafish similar to those in murine models (Ernest et al., 2000). The hair cells of the lateral line also share features with mammalian vestibular hair cells including kinocilia and, in midshipman fish, high frequency response (Weeg and Bass, 2002). Significant differences include the role of TRPN1 (NompC), a presumed mechanotransduction channel to date found in zebrafish but not mammals (Sidi et al., 2003; Corey et al., 2004).

Zebrafish lateral line hair cells are sensitive to aminoglycoside antibiotics (Williams and Holder, 2000; Harris et al., 2003) and exhibit the morphological characteristics of apoptotic death (Williams and Holder, 2000; Murakami et al., 2003). Analysis of zebrafish with genetically altered mechanosensory transduction has shed some light on the dynamics of aminoglycoside hair cell toxicity. Specifically, zebrafish mutants with mechanotransducive and hair cell morphology abnormalities show decreased sensitivity to streptomycin (Seiler and Nicolson, 1999). The streptomycin resistant fish show abnormal microphonic potentials and decreased uptake of the fluorescent vital dye FM 1-43, well established as a marker of hair cell mechanotransduction (Gale et al., 2001; Meyers et al., 2003). Blocking expression of the putative zebrafish mechanosensory channels TRPN1 (Sidi et al., 2003) and TRPA1 (Corey et al., 2004) by morpholino-mediated knock down suppressed both microphonic potential and the uptake of FM 1-43, confirming that this dye is a good marker of hair cell mechanotransduction in zebrafish as in other systems. Taken together, these data suggest a link between FM 1-43 dye uptake, aminoglycoside sensitivity, and mechanotransduction in zebrafish.

We previously described developmental differences in sensitivity to neomycin in the lateral line of zebrafish; at 4 days post-fertilization (dpf), larvae are relatively insensitive to neomycin-induced hair cell death when compared to their older counterparts (Murakami et al., 2003). Developmental differences in sensitivity have also been observed in rat inner ear, where kanamycin susceptibility is not observed until post-natal day eight, closely corresponding to the onset of cochlear potentials (Marot et al., 1980). Correlated timing of aminoglycoside sensitivity onset with the onset of hearing has also been observed in chick (Friedmann and Bird, 1961).

Studies presented in this report sought to directly determine if there is a link between developmental insensitivity to aminoglycosides and mechanotransduction-dependent activity. Taking advantage of the transparency of zebrafish, we have imaged aminoglycoside-induced hair cell death in vivo using fluorescent vital dyes as markers of hair cell viability. In this report, we show that hair cell age, independent of the developmental onset of mechanotransduction, is a determinant of observed susceptibility differences.

2. Materials and methods

2.1. Animals

Zebrafish embryos of the AB wildtype strain were produced by paired matings of adult fish in the University of Washington zebrafish facility. Beginning at 4 days post-fertilization (dpf), larvae were fed live paramecia. Larvae were maintained at a density of 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) in a tissue incubator at 28.5 °C. The University of Washington Institutional Animal Care and Use Committee approved all animal procedures.

2.2. Vital dye staining

Larvae were placed in a transfer device fashioned from a 50 ml conical tube with one end cut off and a mesh cover at the bottom. Lateral line hair cells were labeled with FM 1-43 (*n*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)-styryl) pyridinium dibromide; Invitrogen Molecular Probes, Eugene, OR) by immersing free swimming larvae in 3 μM FM 1-43 in embryo medium for 30 s, followed by three rinses in embryo medium. Using this procedure, FM 1-43 is restricted to hair cells in neuromasts (Seiler and Nicolson, 1999). In Xenopus hair cells, FM 1-43 labels mitochondria and rough endoplasmic reticulum throughout the cytoplasm and its entry is inhibited by cation channel blockers including neomycin and amiloride (Nishikawa and Sasaki, 1996).

Hair cell nuclei in lateral line neuromasts were labeled using either To-Pro-3 or Yo-Pro-1 (invitrogen molecular probes) at 2 μM for 1 h followed by three rinses. To-Pro-3 and Yo-Pro-1 are cyanine monomer dyes that stain nucleic acid with different excitation-emission spectra, (642/661) and (491/509), respectively. Although cyanine dyes such as To-Pro-3 or Yo-Pro-1 are usually cell-impermeant, they can pass through large non-selective channels, such as P2X7 receptors.

To identify hair cells of different maturational ages within a single neuromast, 4 dpf larvae were labeled with FM 1-43, and then allowed to grow for 24 h to 5 dpf at which time they were labeled with To-Pro-3. Hair cells already present at 4 dpf were therefore labeled with both dyes. Hair cells that became permeable to To-Pro-3 during the 24-h period were labeled with only the To-Pro-3 dye. As the use of the fluorescent dyes is qualitative, age differences are not determined by differences in dye uptake but rather age is determined by the described sequential labeling: the older hair cell population is double labeled and the younger hair cell population is single labeled.

2.3. Neomycin treatment

Neomycin sulfate from a 50 mM stock solution in dH_2O (Sigma, St. Louis, MO) was diluted in embryo medium to final concentrations of either 25, 50, 100, 200 and 400 μM in each well of a six-well culture plate. Following vital dye staining, free-swimming 5 dpf larvae were transferred from control (neomycin-free) embryo medium to neomycin-containing medium and incubated for 1 h.

2.4. Immobilization for imaging

Zebrafish were anesthetized with MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma, 8 μg/ml) and immobilized. Immobilization is achieved by creating a clot from bovine plasma and thrombin. The clot is lifted from a glass slide and the larvae together with the neomycin-containing embryo media is slid underneath and effectively contained under the clot (Langenberg et al., 2003).

2.5. Image capture and analysis

Fish were imaged using an inverted epifluorescent Zeiss Axiovert 200M microscope with an automated stage and 40× or 63× water immersion objective. To capture the full depth of the hair cells, multiple optical

sections were taken at each interval. Images were collected with Slidebook 4.0 software (Intelligent Imaging Innovations, Denver, CO) and processed for deconvolution using the Nearest Neighbor method. Alternatively, neuromasts were imaged on a Zeiss LSM 510 Pascal confocal microscope. Larval viability was confirmed by examination of cardiac contractility at the conclusion of the assay.

Hair cells within neuromasts of the supraorbital (SO1 and SO2), otic (O1) and occipital (OC1) lateral lines (Raible and Kruse, 2000) were analyzed. These neuromasts were selected as they are all readily imaged without the need to reposition the fish during image capture. The total number of hair cells of the SO1, SO2, O1 and OC1 neuromasts were counted in each animal for all experimental and control conditions.

2.6. Gentamicin-Texas Red conjugation

For studies of aminoglycoside entry into lateral line hair cells, 4.4 ml of gentamicin sulfate (GT) (Sigma, St. Louis, MO, 50 mg/ml) and 0.6 ml succinimidyl esters of Texas Red (TR) (Molecular Probes, Eugene, OR; 2 mg/ml in dimethyl formamide) were agitated overnight to produce the conjugate solution (Steyger et al., 2003). The conjugated solution was diluted in embryo media for a final concentration of 200 μM gentamicin. Larvae were exposed to the gentamicin Texas Red solution (GTTR) for 1 min, rinsed in embryo medium and observed immediately.

3. Results

In order to determine if double labeling of lateral line hair cells in vivo could be used to record hair cell survival, we incubated zebrafish larvae using a combination of the FM 1-43 and To-Pro-3/Yo-Pro-1 dyes. FM 1-43 selectively labels active hair cells when the hair cells are exposed to the dye for a brief period in many animals, including zebrafish (Seiler and Nicolson, 1999). To-Pro-3 and Yo-Pro-1 are cyanine dyes that upon entry into the cell bind to DNA and become fluorescent. We find that To-Pro-3 and Yo-Pro-1 selectively label neuromast hair cells in living larvae (Fig. 1A). All hair cells that rapidly labeled (30 s) with FM 1-43 were also labeled with the To-Pro-3/Yo-Pro-1 dye (Fig. 1B–D). To determine the extent of double labeling, we assayed the staining of hair cells in neuromasts SO1, SO2, O1 and OC1 (Fig. 1; Raible and Kruse, 2000) at 4 and 5 dpf (Table 1). We find that all hair cells that take up a nuclear dye (Yo-Pro-1 or To-Pro-3) are labeled by FM 1-43 and vice versa. The combination of the nuclear dyes and FM 1-43 labeling allows us to unambiguously assay the number of functional hair cells per neuromast in live animals as development proceeds (Table 2).

We investigated the toxic effects of the aminoglycoside antibiotic neomycin by pre-labeling hair cells with nuclear dyes and FM 1-43 (Fig. 2). After 20 min in 200 μM neomycin, labeled hair cells display cytoplasmic shrinking, nuclear condensation and fragmentation (Fig. 2B). After treatment with 200 μM neomycin for 1 h, almost all of the hair cells are eliminated (Fig. 2C). The remaining bright puncta of FM 1-43 staining are likely cell fragments, similar to those observed in TEM analysis 1 h after neomycin treatment (Murakami et al., 2003). We were not able to conclusively determine if dying hair cells were removed by expulsion, phagocytoses or other mechanisms. However, 1–2 hair cells remain after treatment, consistent with our previous results assaying hair cell loss using acetylated tubulin antibodies and phalloidin staining (Harris et al., 2003). Surviving hair cells could not be distinguished a priori by staining pattern, morphological characteristics or

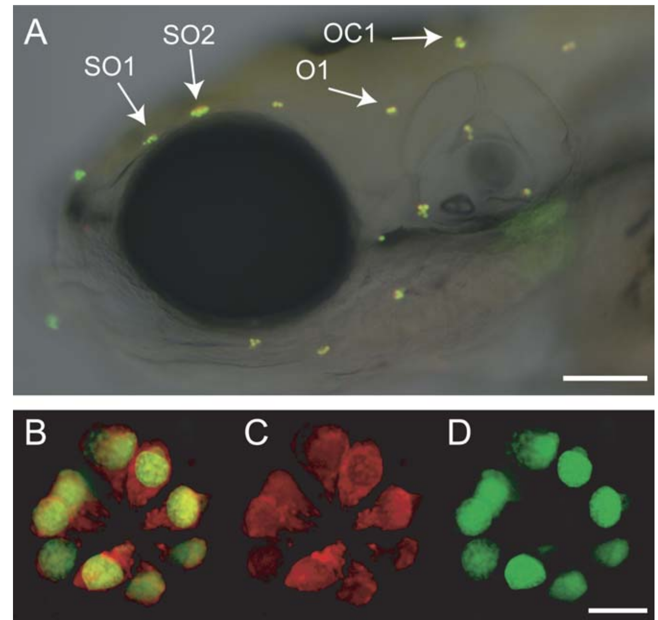

Fig. 1. Staining of neuromasts with FM 1-43 and Yo-Pro-1. (A) Head of 5 dpf larvae with supraorbital (SO1 and SO2), otic (O1) and occipital (OC1) neuromasts indicated. These neuromasts were chosen for the relative ease of imaging when larvae are immobilized on an imaging slide. (B) Simultaneous staining of hair cells within an O1 neuromast by FM 1-43 (red) and Yo-Pro-1 (green). All FM 1-43 positive cells (C) are also Yo-Pro-1 positive (D). Scale bar = 50 μm (A); 10 μm (B–D).

Table 1
Total number of hair cells in the SO1, SO2, O1, and OC1 neuromasts identified by To-Pro-3 and FM 1-43 labeling of zebrafish larvae 4 and 5 dpf ($N = 10$ larvae per group)

Neuromast	4 dpf To-Pro-3+	4 dpf FM 1-43+	5 dpf To-Pro-3+	5 dpf FM 1-43+	No. Larvae
SO1	44	44	57	57	10
SO2	60	60	79	79	10
O1	77	77	77	77	10
OC1	50	50	52	52	10

All hair cells identified by To-Pro 3 labeling in the 4 and 5 dpf larvae rapidly take up FM 1-43 (30 s).

Table 2
Development of FM 1-43 staining

Neuromast	2 dpf	3 dpf	4 dpf	5 dpf
SO1	0 ± 0	2 ± 3	4 ± 1	6 ± 2
SO2	0 ± 0	5 ± 3	6 ± 2	8 ± 2
O1	0 ± 0	7 ± 2	8 ± 1	8 ± 2
OC1	0 ± 0	3 ± 1	5 ± 1	5 ± 1

The number of hair cells positive for dye uptake at each day post-fertilization (dpf) is shown for the neuromast specified.
Ten animals were assayed on each day.

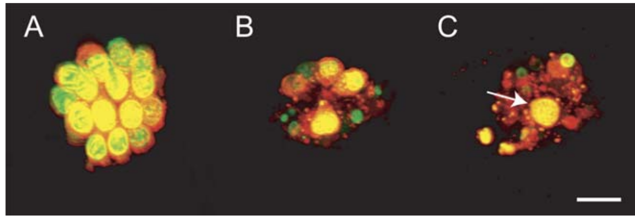

Fig. 2. Five dpf neuromast exposed to 200 μM neomycin. (A) Living hair cells are double labeled with FM 1-43 (red) and Yo-Pro-1 (green) in SO2 neuromast. (B) Damage is evident after treatment with 200 μM neomycin for 20 min including nuclear condensation, in O1 neuromast. (C) Hair cell loss is evident 1 h after treatment with 200 μM neomycin in O1 neuromast. However, some hair cells show resistance to aminoglycoside treatment (arrows). Scale bar = 10 μm.

location within the neuromast. Loss of labeled hair cells was dependent on neomycin dose (Fig. 3), in agreement with previous results (Harris et al., 2003). Taken together, these results validate the use of FM 1-43 and To-Pro-3/Yo-Pro-1 as a reliable method to identify viable lateral line hair cells in living animals.

3.1. *Young larvae show resistance to neomycin-induced lateral line hair cell death*

We compared the susceptibility of individual hair cells to neomycin in 4 and 5 dpf larvae. Larvae from each age group were double labeled with the fluorescent dyes, treated with different concentrations of neomycin for 1 h, and then hair cells counts were obtained. Four dpf zebrafish were less susceptible to neomycin compared to 5 dpf larvae (Fig. 4). A two-way factorial ANOVA (age X neomycin concentration) showed a highly significant main effect of neomycin concentration ($p < 0.001$) and the interaction term ($p < 0.001$). This analysis included total number of hair cells in 4 and 5 dpf control animals as well as the experimental groups. The main effect of age on total number of hair cells was not significant. Individual comparisons using Tukey's method revealed significantly less hair cell loss at 4 dpf than 5 dpf when larvae were exposed to 200 or 400 μM neomycin concentrations ($p < 0.01$). These data demonstrate age-dependent susceptibility differences at the single cell level and confirm our previous study that used the potentiometric dye DASPEI to score neuromast viability (Murakami et al., 2003).

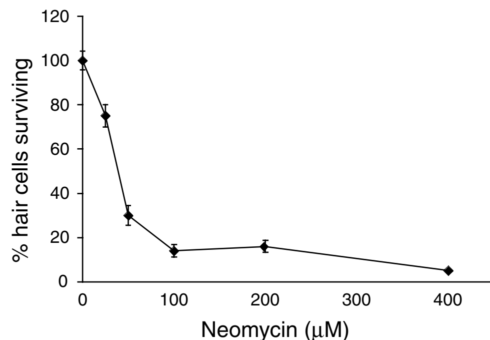

Fig. 3. Neomycin dose–response relationship. Zebrafish larvae at 5 dpf were exposed to the concentrations of neomycin indicated on the abscissa and were examined 1 h after exposure for surviving FM 1-43/To-Pro-3 labeled hair cells ($N = 10$ larvae/group examining the SO1, SO2, O1 and OC1 neuromasts). The percentage of hair cells surviving compared to control (±1 SEM) are shown for each group. Error bars smaller than symbol are not visible.

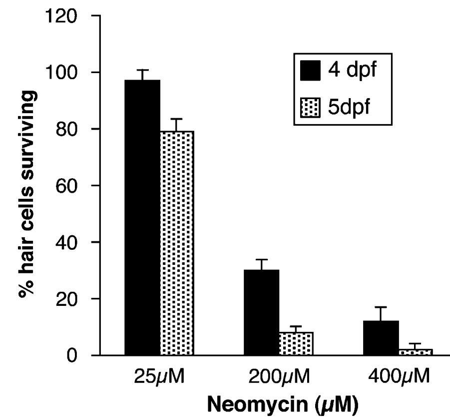

Fig. 4. Lateral line hair cells of 4 dpf larvae are less susceptible to neomycin than are 5 dpf larvae. Hair cells were double labeled with FM 1-43 and To-Pro-3 then exposed to neomycin for 1 h ($N = 24$–30 larvae per dose/age examining the SO1, SO2, O1 and OC1 neuromasts). Percentages are of control ±1 SEM.

3.2. *Onset of mechanotransduction-dependent activity measured by FM 1-43 uptake*

One possible reason for age-dependent differences in neomycin susceptibility is that hair cells found in younger animals may not have active mechanotransduction or a smaller percentage of neuromast hair cells may have mechanotransduction capability. To address this possibility, hair cells identified by To-Pro-3 labeling were exposed to FM 1-43 for 30 s at 4 and 5 dpf. Results of this experiment are shown in Table 1. At 4 dpf all hair cells that are labeled with To-Pro-3 are rapidly labeled with FM 1-43. At 5 dpf the same results are observed: all hair cells that label with To-Pro-3 also label rapidly with FM 1-43. There was no statistically significant difference between groups. While there may be differences in the kinetics of mechanotransduction that cannot be measured by this assay, our results suggest that differences in the onset of mechanotransduction are not sufficient to explain the observed developmental insensitivity to aminoglycoside hair cell toxicity.

3.3. *Measurement of aminoglycoside uptake*

Another possible reason for age-dependent differences in neomycin susceptibility is that hair cells in younger larvae may not efficiently take up aminoglycosides. To evaluate this possibility, 4 and 5 dpf larvae ($N = 10$ larvae per test

condition) were exposed to gentamicin conjugated to Texas Red (GTTR). Larvae were prelabeled with To-Pro-3 and then incubated with 200 μM GTTR for 30 s and observed for 1 h. GTTR fluorescence was detected in the cytoplasm of all To-Pro-3 positive hair cells at both ages (Fig. 5). GTTR fluorescence was not detected in cells not labeled with To-Pro-3. Several controls suggest that cellular fluorescence reflects aminoglycoside uptake. Larvae treated with 200 μM GTTR and a 40-fold excess of unconjugated gentamicin showed more variable uptake with some but not all hair cells exhibiting double labeling, suggesting that the fluorescently conjugated gentamicin competed with the unconjugated gentamicin for uptake. Larvae treated with 200 μm gentamicin and unconjugated TR (not shown), or TR alone (Fig. 5C and D), showed no uptake of fluorescent label into hair cells. These results indicate that under conditions of rapid exposure to the drug, aminoglycoside uptake by lateral line hair cells does not differ between zebrafish larvae at 4 and 5 dpf.

3.4. Developmental differences in hair cell susceptibility

A third hypothesis to explain the developmental differences in aminoglycoside susceptibility with larval age is that differences in susceptibility depend upon the maturity of the hair cell rather than the age of the animal per se. To explore this question, younger and older hair cells were differentially labeled with fluorescent vital dyes in 5 dpf larvae and their response to 200 μM neomycin was compared in vivo. To identify hair cells of different ages within a single neuromast of 5 dpf larvae, hair cells were first labeled with FM 1-43 in larvae at 4 dpf. The same fish were labeled one day later (at 5 dpf) with To-Pro-3, immediately prior to aminoglycoside exposure. This procedure meant that functioning hair cells present at 4 dpf (i.e., hair cells capable of taking up FM 1-43 rapidly) were labeled with both dyes when examined at 5 dpf. Hair cells in which the transduction channels matured between 4 and 5 dpf (after the initial FM 1-43 exposure) were labeled only with To-Pro-3. As expected, there were many fewer ‘younger’ (single labeled) hair cells than ‘older’ (double labeled) hair cells (Fig. 6), with about 0.5 hair cells added to each neuromast between 4 and 5 dpf, consistent with our counts of total hair cells reported above (Table 2).

Animals labeled to distinguish younger and older hair cells were exposed to either control solution or 200 μM neomycin for 1 h, and neuromasts were imaged in vivo immediately following treatment. The remaining number of hair cells of each population following exposure to 200 μM neomycin is shown in Fig. 6 (neo). While double-labeled (older) hair cells showed a 70% reduction after neomycin exposure, virtually all of the single-labeled (young) hair cells survived. By unpaired *t*-test there was no statistically significant difference between the numbers of young hair cells with or without neomycin treatment ($p > 0.8$), while the loss of older hair cells was highly significant ($p < 0.0001$). These results demonstrate that the maturation of individual hair cells, rather than overall larval maturation, is a determinant in the observed stage-dependent differences in susceptibility to neomycin.

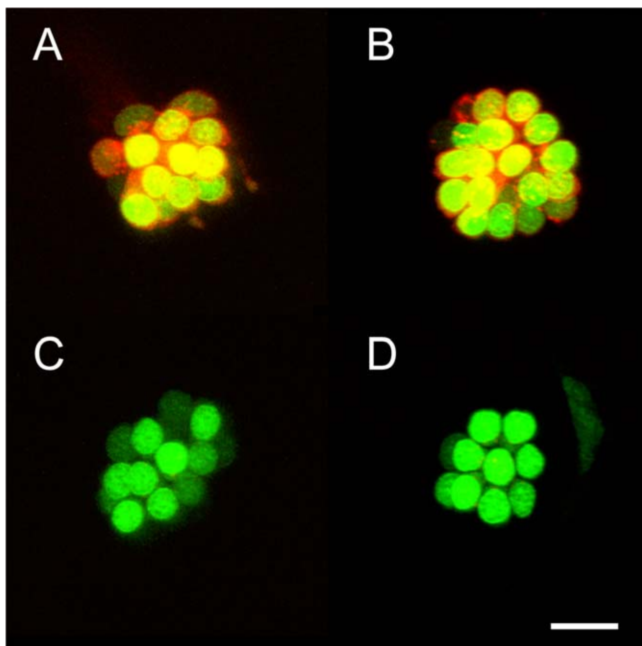

Fig. 5. Entry of Texas Red conjugated gentamicin (GTTR) into sensory hair cells. Hair cells were first labeled with a 1 h treatment with Yo-Pro-1 (green). Entry of GTTR (red) is evident by resulting color of all hair cells after a 1-min exposure in SO2 neuromasts from 4 dpf (A) or 5 dpf (B) larvae. Exposure to unconjugated Texas Red does not label hair cells (C, 4 dpf; D, 5 dpf). Scale bar = 10 μm.

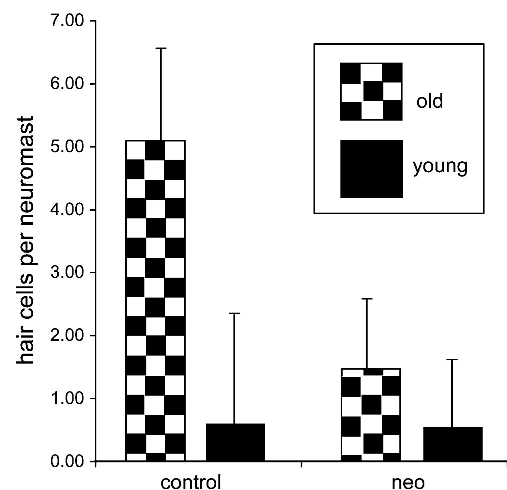

Fig. 6. Lateral line hair cell age is a determinant of neomycin susceptibility. The average number of younger and old hair cells per neuromast in 5 dpf larvae following 0 μM vs. 200 μM neomycin treatment. $N = 10$ larvae per test condition examining the SO1, SO2, O1 and OC1 neuromasts. Error bars show standard deviations. Hair cells were identified by sequential labeling with FM 1-43 and To-Pro-3.

4. Discussion

In this study, we used in vivo labeling methods to examine the morphological dynamics regulating aminoglycoside-induced hair cell death and survival. Age-dependent differences in susceptibility to aminoglycosides were previously described in the lateral line hair cells of zebrafish using the mitochondrial potentiometric dye DASPEI (Murakami et al., 2003). We confirmed and extended these results to the single cell level using the fluorescent vital dyes FM 1-43 and the cyanine monomeric dyes To-Pro-3 and Yo-Pro-1 to label lateral line hair cells in living zebrafish specimens. Once inside the cell, To-Pro-3 and Yo-Pro-1 bind DNA and thus specifically label hair cell nuclei, making them useful additions to other vital dyes such as DASPEI, 4-di-2-asp and FM 1-43 that specifically label zebrafish hair cells (Collazo et al., 1994; Seiler and Nicolson, 1999; Harris et al., 2003). The mechanism of entry of these dyes is unknown. While they are considered to be cell-impermeant, To-Pro and Yo-Pro dyes can enter through large non-selective channels, such as P2X7 receptors. Since zebrafish hair cells express large non-selective mechanotransduction channels such as TRPA1 and TRPN1 that can likely be permeated by a number of fluorescent dyes (Gale et al., 2001; Meyers et al., 2003; Sidi et al., 2003; Corey et al., 2004; Hellwig et al., 2004), it is reasonable to hypothesize that these channels provide entry to To-Pro and Yo-Pro dyes. Alternatively, To-Pro and Yo-Pro dyes might be taken up by hair cells by endocytosis, a process active at the apical ends of mechanosensory hair cells (Forge and Richardson, 1993; Kachar et al., 1997). While further experiments will be useful in order to address this issue, they are outside the scope of the experiments presented above.

There is ample evidence to suggest that mechanotransduction is a prerequisite for normal susceptibility of aminoglycoside toxicity to hair cells. Mutations that block mechanotransduction in mouse and zebrafish inner ear hair cells confer resistance to aminoglycosides (Richardson et al., 1997; Seiler and Nicolson, 1999). In addition, activation of mechanotransduction by acoustic stimulation exacerbates toxicity (Hayashida et al., 1989). Several investigations have also correlated the onset of auditory function with the onset of hair cell aminoglycoside sensitivity (Friedmann and Bird, 1961; Marot et al., 1980; Bernard, 1981; Raphael et al., 1983). We report here evidence for maturation-dependent factors in addition to the onset of transduction that influence lateral line susceptibility to aminoglycoside toxicity. Despite clear evidence for stage-dependent differences in susceptibility to aminoglycosides between 4 and 5 dpf, we found no age-dependent differences in the uptake of FM 1-43 dye, an indicator of mechanotransduction, during this time period. One caveat of our analysis is the reliance on FM 1-43 as an indicator of mechanotransduction. However, the evidence for the dependence of FM 1-43 uptake on functional mechanotransduction is supported by electrophysiological and pharmacological studies (Gale et al., 2001; Meyers et al., 2003), developmental studies (Geleoc and Holt, 2003; Si et al., 2003), and genetic loss-of-function studies in zebrafish (Seiler and Nicolson, 1999; Sidi et al., 2003; Corey et al., 2004). While our data suggest that the onset of mechanotransduction does not underlie the age-dependent differences in aminoglycoside susceptibility, future experiments will be needed to determine whether subtle differences in the mechanotransduction process that change with age might alter aminoglycoside susceptibility.

After neomycin treatment at any age, we always find a few resistant hair cells remain (e.g., Fig. 3), consistent with our previous results (Harris et al., 2003). One possible explanation is that these surviving cells represent a distinct type of hair cell, such as the type I-like or type II-like hair cells that have been described in the adult lateral line (Song et al., 1995). However, under the experimental test conditions used, we could not detect distinct morphological characteristic in surviving cells. On the other hand, lateral line neuromasts continue to add hair cells during the stages assayed (Williams and Holder, 2000; Harris et al., 2003), suggesting that the surviving hair cells represent a developmentally immature, aminoglycoside insensitive subpopulation of hair cells. To determine whether cell maturity rather than larval age was a determinant of aminoglycoside susceptibility, we conducted an assay using sequential labeling with two hair cell specific fluorescent dyes, allowing us to identify a young and older hair cell population. We found that younger hair cells in comparison to older hair cells in 5 dpf larvae were markedly resistant to neomycin. These neomycin resistant cells are indistinguishable from sensitive cells in their ability to rapidly take up FM 1-43, consistent with the conclusion that other factors besides mechanotransduction play a role in age-dependent aminoglycoside susceptibility.

Although younger hair cells demonstrated FM 1-43 uptake, indicative of active mechanotransduction, it was still possible that they differentially exclude aminoglycosides. For example, newly regenerating hair cells show resistance to the aminoglycoside kanamycin until they are functionally mature enough to take up the drug (Hashino and Salvi, 1997). To test this idea, we sought to determine whether these younger, less susceptible, hair cells could take up fluorescently labeled aminoglycosides by comparing 4 and 5 dpf larvae. All hair cells at both ages were exposed to a 200 μM Texas Red conjugated gentamicin solution (Steyger et al., 2003). All hair cells rapidly labeled with the conjugated gentamicin at both ages. The rapid uptake of labeled gentamicin is consistent with recent reports suggesting that aminoglycosides can enter mammalian cells through large non-selective channels, including the mechanotransduction channel (Gale et al., 2001; Marcotti et al., 2005; Myrdal et al., 2005; Myrdal and Steyger, 2005). We have not tested whether zebrafish hair cells can also take up gentamicin by a slower endocytotic mechanism, as also postulated in other systems (de Groot et al., 1990; Hashino and Shero, 1995). Our results suggests that the resistance of the less mature hair cells is not due to failure of aminoglycoside uptake.

Our conclusions raise important questions about what developmental differences in hair cells might account for differences in injury responses and available cell death or survival pathways. Aminoglycosides have been shown to catalyze formation of reactive oxygen species (Priuska and Schacht, 1995), aminoglycoside exposure causes a rapid increase in free radicals (Hirose et al., 1997) and there is evidence that antioxidant therapy attenuates hair cell damage (Song et al., 1998; Conlon et al., 1999; Santos et al., 2005). Reactive oxygen species may be less abundant in immature hair cells. Intrinsic differences in the vulnerability to reactive oxygen species has been postulated to underlie the differential susceptibility of outer hair cells at different basal to apical positions along the basilar membrane (Sha et al., 2001). Alternatively, as production of reactive oxygen species by aminoglycoside-induced damage has been isolated in renal mitochondria (Walker and Shah, 1987) there may be a difference in mitochondrial number or activity in younger hair cells. Mitochondrial ribosomes are structurally similar to the prokaryotic ribosomal target of aminoglycosides and have been postulated to underlie aminoglycoside ototoxicity (Cortopassi and Hutchin, 1994); hence fewer mitochondria in younger hair cell may decrease the chance of toxicity. Future experiments are needed to explore differential developmental expression of cell death and survival pathway molecules. Understanding intracellular differences that promote survival or death may in turn serve as useful tools in devising strategies to prevent ototoxic mediated hair cell death.

There are a number of similarities between hair cell death in the mammalian inner ear and zebrafish lateral line. As in mice, the morphology of hair cell death in zebrafish includes apoptotic changes including pyknosis and cytoplasmic shrinking. Compounds known to be ototoxic in humans such as aminoglycosides and cisplatinin cause dose-dependent selective loss of hair cells in zebrafish lateral line (Harris et al., 2003; Ton and Parng, 2005; Ou, Raible and Rubel, unpublished). In addition, the attenuation of hair cell death in mammals by caspase inhibitors (Forge and Li, 2000; Cunningham et al., 2002) and antioxidants (Rybek and Kelly, 2003) and c-Jun kinase inhibitors (e.g., Pirvola et al., 2000) has also been suggested in zebrafish (Williams and Holder, 2000; Ton and Parng, 2005; Santos et al., 2005). While there are shared ototoxicity profiles between mammalian species and zebrafish lateral line hair cells, the kinetics of drug mediated hair cell death may be quite different, lateral line hair cell death may be accelerated. We find that in the zebrafish lateral line, dose-dependent hair cell death has occurred 1 h after drug exposure. Although damage has been observed from similar doses on a similar time scale in cultured mouse cochlea (Kotecha and Richardson, 1994), cultured cochlear guinea pig hair cells show viability at 6 h after high dose exposure (Dulon et al., 1986); these differences may be species-specific or due to differences in culture conditions. Variation in the timing of hair cell death is also observed in vivo depending factors such as dose and activity (Hayashida et al., 1989; Aran et al., 1999); differences in these studies likely reflect the different ways hair cells may die in vivo, including caspase-independent pathways (Jiang et al., 2006). It will be important to further elucidate the variations in hair cell pathophysiology between species, ages and experimental conditions. Our observations, elucidating the mechanism of susceptibility and ultimately the genetic regulation of lateral line hair cell death, will be informative in this respect and may add to our understanding of general principles of hair cell biology and pathophysiology.

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