JARO 04: 219–234 (2003)
DOI: 10.1007/s10162-002-3022-x

JARO
Journal of the Association for Research in Otolaryngology

Neomycin-Induced Hair Cell Death and Rapid Regeneration in the Lateral Line of Zebrafish (*Danio rerio*)

Julie A. Harris,[1,2] Alan G. Cheng,[1] Lisa L. Cunningham,[1] Glen MacDonald,[1,4] David W. Raible,[2,3] and Edwin W Rubel[1,2]

[1]*Virginia Merrill Bloedel Hearing Research Center and Department of Otolaryngology–Head and Neck Surgery, University of Washington, Seattle, WA 98195, USA*

[2]*Graduate Program in Neurobiology and Behavior, University of Washington, Seattle, WA 98195, USA*

[3]*Department of Biological Structure, University of Washington, Seattle, WA 98195, USA*

[4]*Core for Communication Research, University of Washington, Box 357923, Seattle, WA 98195, USA*

Received: 16 May 2002; Accepted: 5 October 2002; Online publication: 6 May 2003

ABSTRACT

Mechanoreceptive hair cells are extremely sensitive to aminoglycoside antibiotics, including neomycin. Hair cell survival was assessed in larval wild-type zebrafish lateral line neuromasts 4 h after initial exposure to a range of neomycin concentrations for 1 h. Each of the lateral line neuromasts was scored in live fish for the presence or absence of hair cells using the fluorescent vital dye DASPEI to selectively label hair cells. All neuromasts were devoid of DASPEI-labeled hair cells 4 h after 500 μM neomycin exposure. Vital DASPEI staining was proportional to the number of hair cells per neuromast identified in fixed larvae using immunocytochemistry for acetylated tubulin and phalloidin labeling. The time course of hair cell regeneration in the lateral line neuromasts was also analyzed following neomycin-induced damage. Regenerated hair cells were first observed using live DASPEI staining 12 and 24 h following neomycin treatment. The potential role of proliferation in regenerating hair cells was analyzed. A 1 h pulse-fix protocol using bromodeoxyuridine (BrdU) incorporation was used to identify S-phase cells in neuromasts. BrdU incorporation in neomycin-damaged neuromasts did not differ from control neuromasts 4 h after drug exposure but was dramatically upregulated after 12 h. The proliferative cells identified during a 1 h period at 12 h after neomycin treatment were able to give rise to new hair cells by 24–48 h after drug treatment. The results presented here provide a standardized preparation for studying and identifying genes that influence vertebrate hair cell death, survival, and regeneration following ototoxic insults.

Keywords: hair cell death, lateral line, aminoglycosides, hair cell regeneration, zebrafish, genetic screening

INTRODUCTION

The degeneration of mechanosensory hair cells of the inner ear sensory epithelia is the primary cause of both deafness and balance disorders. Hair cells are commonly lost through aging, as well as noise- and drug-induced trauma. In particular, hair cells are very susceptible to aminoglycosides. While the exact mechanisms of aminoglycoside ototoxicity remain unknown, it appears to involve both known apoptotic cell death pathways and the formation of free radicals (for review see Forge and Schacht 2000). For example, the apoptotic cell death proteases, caspases, are activated in hair cells following aminoglycoside

Correspondence to: Edwin W Rubel • University of Washington • VMB Hearing Research Center • Box 357923 • Seattle, WA 98195-7293. Telephone: (206) 543-8360; fax: (206) 221-5685; email: rubel@u.washington.edu

exposure and inhibiting caspase activation reduces hair cell death (Li et al. 1995; Forge and Li 2000; Williams and Holder 2000; Cheng et al. 2001; Cunningham et al. 2002; Matsui et al. 2002). In addition, the c-Jun N-terminal kinase (JNK) signaling cascade, implicated in apoptotic death following a variety of insults including oxidative stress, is activated in auditory hair cells following aminoglycoside treatment (Pirvola et al. 2000; Ylikoski et al. 2002).

One approach to conserve sensory function following ototoxicity is to identify molecules necessary for inducing hair cell regeneration. Hair cells are not replaced in mammalian auditory organs following ototoxic insults (Schuknecht 1974; Roberson and Rubel 1994; Chardin and Romand 1995). Nonmammalian vertebrate hair cells, however, can regenerate following noise- or drug-induced hair cell death, and regeneration restores sensory function (Cotanche 1987; Cruz et al. 1987; Weisleder and Rubel 1993; Cotanche et al. 1994; Carey et al. 1996; Smolders 1999; Stone and Rubel 2000a). A second approach toward reducing hearing and balance disorders is to identify molecules that can protect, or prevent, hair cells from undergoing death following ototoxic stimuli. *In vitro* preparations are useful for applying candidate protective molecules, such as caspase inhibitors, directly to vertebrate hair cells, but for the most part they lack the capacity to identify novel endogenous genes involved in cell death and survival. It is well known that there is a genetic contribution to aminoglycoside-induced deafness, and, therefore, it is of importance to identify the candidate genes that increase (or decrease) susceptibility to ototoxicity (Fischel–Ghodsian 1999).

The zebrafish (*Danio rerio*) is emerging as a powerful preparation for identifying essential vertebrate genes and their functions. Following random mutagenesis, phenotypes can be rapidly analyzed and mutations assigned to specific chromosomal locations using molecular mapping techniques. In addition to the inner ear, fish have a second mechanosensory organ, the lateral line. The lateral line consists of neuromasts that reside along the head and body. Each neuromast contains a group of hair cells that function to detect water current relative to the animal's body via movement of their stereocilia (Kalmijn 1989; Montgomery et al. 2000). Lateral line hair cells of zebrafish larvae are easily accessed making them ideal for experimental manipulation.

The present study was designed to provide a standardized model for studying lateral line hair cell death and regeneration. First, a dose–response relationship between lateral line hair cell survival and neomycin concentration was established in zebrafish larvae. Second, the time frame of lateral line hair cell regeneration in larval zebrafish following aminoglycoside-induced damage was assessed. The long-term goal of this research is to understand the interaction of genetic factors and environmental insults such as ami-noglycoside antibiotics on hair cell loss and renewal.

MATERIALS AND METHODS

Animals

Zebrafish embryos (*Danio rerio*) were produced by paired matings of fish raised at 28.5°C (Westerfield 2000). Animals were maintained at 28.5°C until four or five days postfertilization (p4 and p5) when the experiments were conducted. All animal procedures were approved by the University of Washington Institutional Animal Care Committee.

Dose–response relationship

Aminoglycoside treatment. Figure 1A shows the experimental paradigm for this study. Neomycin sulfate (Pharma-Tek, Huntington, NY) was added to 6 ml of embryo medium [1 mM $MgSO_4$, 120 μM KH_2PO_4, 74 μM Na_2HPO_4 1 mM $CaCl_2$, 500 μM KCl, 15 mM NaCl, and 500 μM $NaHCO_3$ in dH_2O (Westerfield 2000)] in each well of a six-well tissue culture plate to make final concentrations of 0, 10, 50, 100, 125, 250, 300, or 500 μM. Zebrafish larvae ($n \geq 6$/well/trial) were placed into a 50 ml tube which had one end cut off and a mesh cover at the bottom to be used as a transfer device. Larvae were moved from fresh medium, immersed in neomycin-containing medium, and incubated (28.5°C) for 1 h. Larvae of different ages (p4 or p5) were treated separately. After 1 h, animals were rinsed three times quickly in fresh medium and returned to the incubator in normal embryo medium for three additional hours at 28.5°C. Each treatment group was replicated 2–5 times (n = 18–45 fish/treatment group, n = 232 zebrafish total).

Neuromast staining. The fluorescent dye 2-[4-(dimethylamino)styryl]-N-ethylpyridinium iodide (DASPEI; Molecular Probes, Eugene, OR) was used as a vital dye to stain hair cells within neuromasts. Larvae were incubated in embryo medium containing 0.005% DASPEI for 15 min. The larvae were anesthetized in MS222 (10 μg/ml, 3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma, St. Louis, MO) for 5 min. The zebrafish were then rinsed once in fresh embryo medium and analyzed under an epifluorescence dissecting microscope (Leica MZ-12 FL111) equipped with a DASPEI filter set (excitation 450–490 nM and barrier 515 nM; Chroma Technologies, Brattleboro, VT).

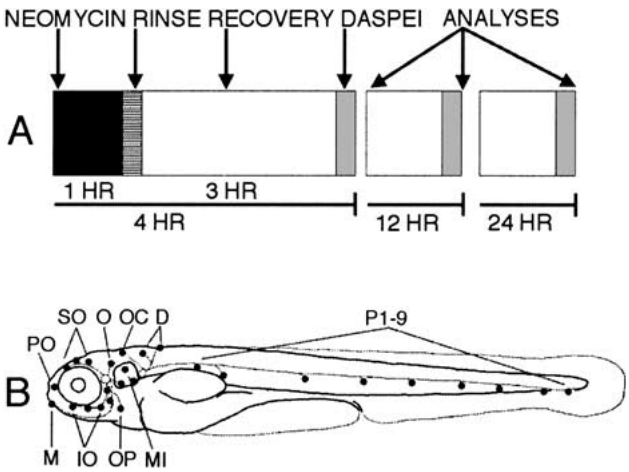

FIG. 1. Experimental protocol. **A**. Larvae were exposed to neomycin for 1 h. Neuromast hair cell survival was analyzed 4, 12, and 24 h following initial neomycin exposure. **B**. The stereotyped positions of lateral line neuromasts in p4 and p5 zebrafish larvae (adapted from Metcalfe et al. 1985; Raible and Kruse 2000). The position names are based on the head and trunk lateral lines. Abbreviations: D = dorsal trunk, IO = infraorbital, M = mandibular, MI = middle, O = otic, OC = occipital, OP = opercular, P = posterior, PO = preoptic, SO =supraorbital.

Neuromast scoring and data analysis. At four and five days postfertilization, larvae are free swimming and have 18 head and 9 trunk neuromasts on each side of the body in the several lines that make up the lateral line system. The location of each of these 27 neuromasts is shown in Figure 1B (Metcalfe et al. 1985; Raible and Kruse 2000). This schematic was used as the data sheet for scoring neuromast hair cell survival. Individual neuromasts were scored as having normal staining indicating hair cells are present (DASPEI score = 2), reduced staining (DASPEI score = 1), or absent staining (DASPEI score = 0). Total neuromast scores were tabulated for one side of each fish. The maximum score possible was 54, or 27 neuromasts multiplied by the high score of 2 which indicates normal staining of each neuromast. The average score from 37 untreated animals was 52.3. The scores from the treated groups of fish were expressed as a proportion of this average neuromast score from untreated fish. Data were subjected to a one-way or two-way analysis of variance (Statview 5.0 for Macintosh, Abacus Concepts, Berkeley, CA) to assess the effects of neomycin concentration, age, or both on neuromast survival. The scores were also analyzed separately for each neuromast to determine if individual neuromasts showed differential sensitivities to neomycin.

Hair cell regeneration

Neuromast scoring and data analysis. The time frame of hair cell regeneration in p4 and p5 zebrafish was investigated using the aminoglycoside treatment schedule described above (Fig. 1A) but with additional time points of assessment at 12 and 24 h after initial neomycin exposure. These experiments included groups treated with 0, 125, 250, and 500 μM neomycin. At 4 h after initial drug treatment, 2–3 fish per group were scored using DASPEI as a positive control for the damage protocol. Remaining fish were scored at either 12 or 24 h. These groups were replicated 2 times (12 h; $n = 15$–24 fish/treatment group) or 3 times (24 h; $n = 11$–29 fish/treatment group). Total neuromast scores based on DASPEI staining were subjected to analysis of variance for overall and interaction effects of time and neomycin concentration on hair cell regeneration.

Cell proliferation and analysis. A pulse-fix protocol using 5-bromo-2-deoxyuridine (BrdU; Sigma) was used to identify S-phase cells in lateral line neuromasts. Larvae were either untreated (0 μM neomycin, $n = 19$) or treated (500 μM neomycin $n = 17$) for 1 h and then allowed to recover for 4, 12, or 24 h after initial exposure as described above. At these times, larvae were placed into 10 mM BrdU with 1% dimethylsulfoxide (DMSO) in embryo medium for 1 h at 28.5°C. They were then anesthetized in MS222 and fixed with 4% paraformaldehye (PFA) in phosphate-buffered saline (PBS) for 2 h at room temperature (RT).

BrdU incorporation was detected by immunocytochemistry. The fixed larvae were washed in PBS with 1% DMSO and 0.1% Tween-20 (PBDT) and placed in methanol for 1 h at −20°C. Larvae were then rehydrated in a graded methanol series and washed in PBDT for 20 min. Following rehydration, all larvae were digested with Proteinase K (10 μg/ml; GIBCO, Carlsbad, CA) for 20 min, washed in PBDT, and refixed in 4% PFA for 20 min. After another washing in PBDT, the larvae were placed into 2N HCl for 1 h at RT. Larvae were washed again in PBDT and nonspecific binding was blocked in 10% normal goat serum in PBDT for 1 h at RT. The larvae were incubated in mouse anti-BrdU IgG in blocking serum (1:100; Becton Dickinson, San Jose, CA) overnight at 4°C. Larvae were washed for 1 h in PBDT and incubated in Alexa 594-conjugated goat antimouse IgG for 5 h at RT (1:200; Molecular Probes). The larvae were whole mounted and coverslipped with Vectashield (Vector, Burlingame, CA). BrdU-labeled cells in neuromasts were counted on a fluorescence light microscope using a 40X objective and Nomarski optics (Leitz Aristoplan). These counts were subjected to a two-way analysis of variance for effects of recovery time and neomycin treatment on the number of proliferative cells.

To determine if cells labeled with BrdU at 12 h after neomycin treatment could develop into mature hair cells, larvae were treated as above but then sur-

TABLE 1

Mean number of hair cells in neuromasts of the lateral line 96–120 h postfertilization

Neuromast position	*n*	*Hair cell number (mean ± SD)*	*Approximate time of appearance (h)*
Head			
02	17	12.6 ± 2.12	34
01	14	14.9 ± 3.18	37
MI1	18	13.2 ± 3.39	41
SO3	10	3.4 ± 2.60	41
SO2	7	14.7 ± 4.03	50
M2	18	10.1 ± 2.10	50
IO4	16	9.1 ± 2.68	50
OC1	11	9.7 ± 1.27	60
IO2	11	9.5 ± 2.77	60
IO3	15	8.6 ± 2.17	60
M1	9	10.0 ± 1.32	65
IO1	10	11.2 ± 3.19	65
MI2	13	7.0 ± 2.94	72
SO1	8	7.6 ± 1.92	72
OP1	20	13.3 ± 2.94	72
Trunk			
PO	7	7.4 ± 2.57	
D1	10	11.7 ± 1.77	
D2	5	8.2 ± 1.48	
P1	19	10.4 ± 1.74	
P2	13	9.5 ± 2.15	
P3	13	9.1 ± 2.90	
P4	11	10.0 ± 2.00	
P5	9	10.3 ± 2.24	
P6	6	11.5 ± 1.64	
P7	12	8.6 ± 2.28	
P8	12	9.0 ± 2.09	
P9	12	9.1 ± 1.88	

[a]Approximate time of appearance from Raible and Kruse (2000). Times were estimated based on DASPEI detection in live larvae for head neuromasts only. Neuromast location abbreviations are described in the Figure 1 legend.

vived for 12 or 36 h after the 1 h BrdU exposure. In this protocol, if cells that are in S-phase during the BrdU exposure (at 12 h after neomycin) have differentiated into hair cells, they will be double-labeled for BrdU and a hair cell-specific protein. It is important to recognize that only a portion of the new hair cells are expected to be double-labeled for BrdU because the BrdU is available for only a short time (1 h), and we do not expect that the mitotic divisions of the precursor population will be precisely synchronized. Whole mounts were double-immunolabeled for BrdU and acetylated tubulin. The fixed larvae were rinsed in PBS and incubated in acetone for 7 min at −20°C. They were then rehydrated in distilled H_2O and PBS followed by 3 rinses in 1% bovine serum albumin, 1% DMSO, and 0.1% TritonX-100 in PBS (PBT). Nonspecific binding was blocked by incubating larvae in 10% normal goat serum in BSA/DMSO/PBT for 1 h at RT. Larvae were incubated in mouse antiacetylated tubulin IgG (1:1000, Sigma) in blocking solution overnight at 4°C. They were rinsed again in BSA/DMSO/PBT for 2 h and incubated in Alexa 594 conjugated goat antimouse IgG for 5 h at RT (1:300, Molecular Probes). The same procedure described above for BrdU labeling was then followed except that rat-anti-BrdU (1:100, Accurate Chemical & Scientific Corp. Westbury, NY) was used in the double-labeling experiment followed by the secondary antibody Alexa 488 conjugated goat antirat IgG (Molecular Probes). Double labeling was assessed on a confocal microscope (Bio-Rad MRC-1024UV).

Hair cell counts

Number of hair cells within normal neuromasts of p4 and p5 zebrafish larvae. To determine the average number of hair cells located within individual neuromasts of the larval lateral line at p4 and p5, hair cell stereocilia were stained for F-actin with fluorescently conjugated phalloidin (Whitfield et al. 1996; Haddon et al. 1999; Ernest et al. 2000; Williams and Holder 2000; Bang et al. 2001). Larvae were anesthetized with MS222 and fixed in 4% PFA for 2 h at RT. The fixed larvae were rinsed 3 times in PBS with 0.5% TritonX-100. Whole zebrafish larvae were then incubated in rhodamine-conjugated phalloidin (1:100, Molecular Probes) for 2 h at RT, followed by 3 rinses in PBS.

Larvae were mounted onto glass slides using Vectashield (Vector) and coverslipped. Hair cell counts were made in a total of 326 neuromasts. Each neuromast position was analyzed in 5 to 20 fish (see Table 1) under epifluorescent illumination using a 63X oil immersion objective (Leitz Aristoplan).

Correspondence of DASPEI, actin, and acetylated tubulin staining

To verify that the DASPEI score that was assigned to specific neuromasts in the neomycin toxicity experiments was proportional to the number of hair cells present after aminoglycoside treatment, hair cell ciliary bundles were stained for F-actin with phalloidin in the same animals that were scored by DASPEI. Animals from all treatment groups at 4 h after neomycin exposure were included for analysis. The number of phalloidin-stained hair cell bundles in individual neuromasts ($n = 388$) on each of 31 zebrafish were counted. The analysis was blinded. The counts were subjected to analysis of variance as a function of DASPEI scoring group (0, 1, or 2).

Hair cell somata were immunolabeled with antiacetylated tubulin to compare cell survival with DASPEI staining after aminoglycoside treatment. For this analysis, animals from control and 500 μM treatment groups ($n = 5$/treatment group) were scored using DASPEI under an epifluorescence dissecting microscope. The fish were then immediately anesthetized and fixed. Hair cells were labeled with mouse antiacetylated tubulin as described above. Immunoreactive cells were assessed using a Bio-Rad MRC-1024UV confocal microscope.

In an additional experiment to quantitatively compare DASPEI scores with the number of hair cells present in a neuromast, larvae ($n = 9$–20 per group) were exposed to a range of neomycin doses (0, 125, 250, and 500 μM) for 1 h. Four hours after the initial drug exposure, head neuromasts were scored by labeling with DASPEI. The larvae were then anesthetized and fixed and hair cells were double-labeled first for acetylated tubulin and second with phalloidin, similar to methods described above except acetone was omitted from the procedure and Alexa 488 conjugated phalloidin was used (Molecular Probes). Hair cell counts were made independently using tubulin and phalloidin labeling in the same head neuromasts that were previously scored live using DASPEI. All hair cell counts were conducted with the experimenter ''blinded'' with respect to treatment group and to scores using the other two methods. Each neuromast was analyzed under epifluorescent illumination using a 60X oil immersion objective.

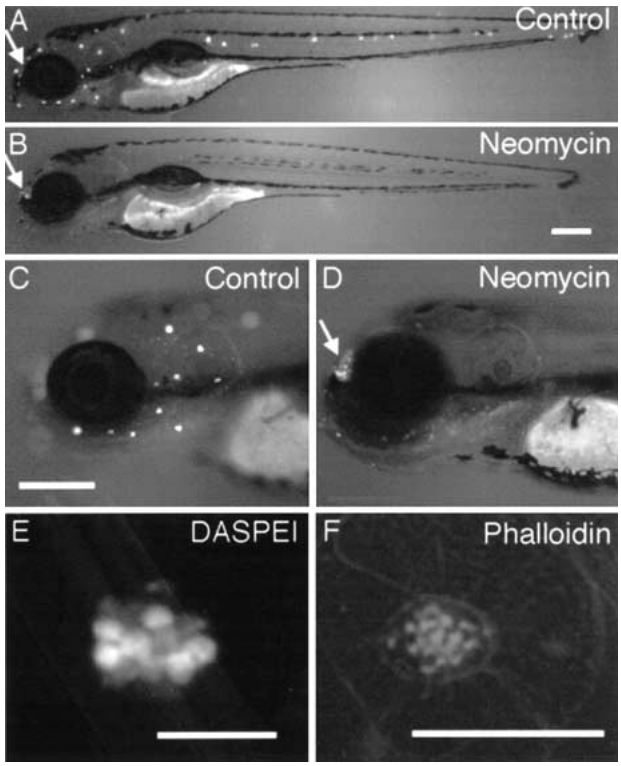

FIG. 2. Lateral line neuromasts stained with DASPEI. **A, C.** Live untreated p5 zebrafish larvae. **B, D**. Representative live p5 larvae 4 h after initial treatment with 500 μM neomycin for 1 h. A–D are lateral views with anterior to the left and dorsal up. The nasal epithelium is also stained with DASPEI in both groups (arrows). **E.** A higher-magnification view of a DASPEI-stained neuromast from a live control animal shows the rosette pattern of hair cells. **F**. A neuromast from a fixed larva containing hair cells labeled for f-actin with phalloidin. The neuromasts shown in E and F are not identical neuromasts and thus may have a different average number of hair cells (see Table 1). Scale bars = 0.5 mm (A and B in **B**; C and D in **C**) and 25 μm (E and F).

The hair cell counts and DASPEI scores were then subjected to a two-way ANOVA for analysis type and neomycin concentration.

Scanning electron microscopy

Anesthetized larvae were immersion fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) and 8 mM $CaCl_2$ on a shaker table for 1 h at 25°C and then overnight at 4°C. Following three 10-min washes in 0.1 M cacodylate buffer, larvae were post-fixed in 1% OsO_4 in 0.1 M cacodylate and 8 mM $CaCl_2$ on ice for 1 h on the shaker table. Larvae were washed again three times at 10 min each and dehydrated through a graded ethanol series: 35%, 70%, 95%, 100%, and 100% for 10 min each, then critically point dried using CO_2. Zebrafish were then mounted onto SEM specimen mounts using double-sided carbon adhesive tape and sputter-coated with Au/Pd. Specimens were examined and photographed with a

JEOL JSM 6300F field emission scanning electron microscope.

RESULTS

Lateral line hair cell labeling in larval zebrafish

The voltage-sensitive dye DASPEI specifically labels sensory hair cells of the lateral line following 15 min of incubation. DASPEI has previously been shown to label nerve fibers and neurons which contact the hair cells with longer incubation times and at higher concentrations than used here (Alexandre and Ghysen 1999). In addition to labeling hair cells, DASPEI is also selectively taken up by the nasal epithelium (Alexandra and Ghysen 1999). Figures 2A and C show the stereotypical distribution of neuromasts (schematized in Fig. 1B) in representative live untreated zebrafish larvae incubated in DASPEI. At the level of magnification shown in Figures 2A–D, individual hair cells within a given neuromast cannot be visualized. At higher magnification, individual DASPEI-stained hair cells are centrally located in the neuromast structures and easily observable, as illustrated in Figure 2E.

To quantify the number of hair cells present in lateral line neuromasts at these ages, hair cell ciliary bundles were labeled with phalloidin, as shown in Figure 2F. On average, there are 10 hair cells per neuromast in the lateral line of untreated p4 and p5 larvae. However, the mean numbers (± SD) of hair cells contained in each neuromast range from 3.4 ± 2.60 to 14.7 ± 4.03 and depend on the neuromast location in the lateral line system (Table 1). Neuromasts that appear earlier in development, such as O1, O2, and MI1 (see Fig. 1B), generally contained higher numbers of hair cells per neuromast at this age than neuromasts that develop later, although this relationship was not true for all neuromasts (Raible and Kruse 2000).

Neomycin toxicity of lateral line hair cells

Neomycin exposure induced lateral line hair cell death in p4 and p5 zebrafish larvae in a dose-dependent manner. Figures 2B and D illustrate that a high dose of 500 μM neomycin for 1 h effectively eliminated DASPEI-stained lateral line hair cells 4 h later in all neuromasts, regardless of their position. Staining of the sensory nasal epithelium was unaffected by exposure to this concentration of neomycin; this served as an internal control for the reliability of DASPEI labeling.

Having established a dose sufficient to eliminate lateral line hair cells stained with DASPEI at 4 h after exposure, we determined the dose–response relationship by analyzing DASPEI labeling of neuromasts 4 h after initial exposure to concentrations of neomycin ranging from 0 to 500 μM. Figure 3 shows the data from all treatment groups expressed as a percentage of the average DASPEI score from an untreated control group. DASPEI scores were 60% of the control group score following 125 μM neomycin treatment reflecting significant hair cell loss. Hair cell survival dropped close to 0% of control after 300 and 500 μM. The overall decrease in neuromast hair cell survival with increasing neomycin concentration was highly significant ($p < 0.001$).

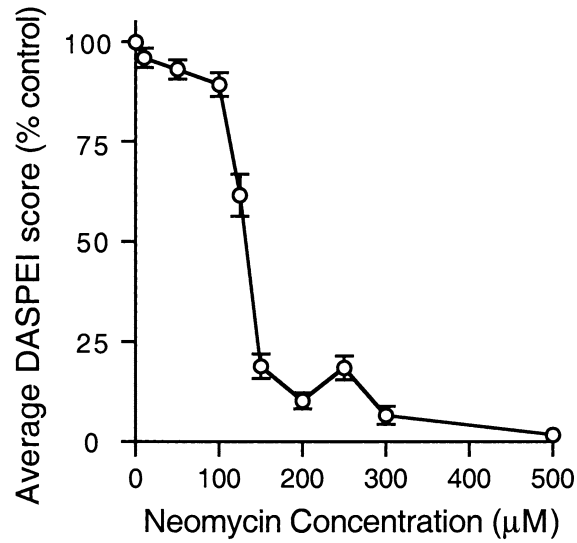

FIG. 3. The dose–response relationship between neuromast hair cell survival as indicated by DASPEI staining in live fish and neomycin concentration. Zebrafish larvae ($n = 232$; 18–41/treatment group) treated for 1 h with various doses of neomycin were examined for the viability of hair cells in neuromasts stained with DASPEI. The doses used were 0, 10, 50, 100, 125, 150, 200, 250, 300, and 500 μM neomycin in embryo medium. DASPEI staining first significantly decreased after 50 μM neomycin. Average DASPEI scores were approximately 60 of control levels in larvae exposed to 125 μM neomycin. An exposure level of 500 μM neomycin eliminated DASPEI-labeled hair cells in neuromasts. The overall decrease in average DASPEI score with increasing neomycin concentrations was highly significant ($p < 0.001$).

Hair cells are added in neuromasts throughout the life of fish and amphibians, including the zebrafish (Metcalfe et al. 1985). Therefore, an additional control it was important to compare the dose–response relationships of the younger (p4) versus older (p5) animals. Qualitatively there were no striking differences in susceptibility of p4 and p5 lateral line neuromast hair cells to neomycin. Both ages showed a similar shape and trend of the dose–response relationship (data not shown). However, older larvae (p5) had quantitatively more DASPEI–stained neuromasts indicating greater hair cell survival than p4 larvae in most treatment groups (10, 50, 100, 150,

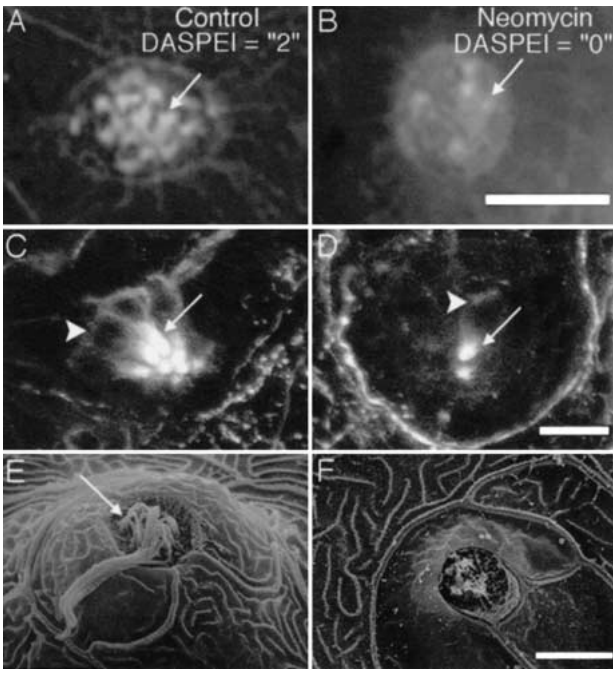

FIG. 4. DASPEI scores are proportional to the number of hair cells present within a neuromast analyzed three ways. In panels A and B, hair cell stereocilia bundles were labeled with fluorescent phalloidin. **A**. Representative phalloidin-labeled hair cells from a neuromast given the DASPEI score of 2. **B.** There are fewer phalloidin-labeled hair cells in a representative neuromast given the DASPEI score of 0. In panels C and D, neuromast hair cell bodies and stereocilia were labeled with antiacetylated tubulin. **C.** A representative control neuromast containing acetylated tubulin-positive hair cells (DASPEI score = 2). **D.** A representative neuromast from an animal fixed 4 h after initial treatment with 500 μM neomycin for 1 h contains fewer acetylated tubulin-labeled hair cells (DASPEI score = 0). Lateral line neuromasts were examined qualitatively using scanning electron microscopy. **E.** SEM of a representative neuromast from an untreated larva (DASPEI score = 2) showing intact kinocilia and stereocilia bundles of its hair cells. **F.** A damaged neuromast (DASPEI score = 0) from a larva 4 h after being treated with 500 μM neomycin. Kinocilia and stereocilia bundles were eliminated. Arrows indicate stereocilia or cuticular plate in A–D. Arrow in E is pointing to kinocilia. Arrowheads in C and D indicate hair cell bodies. Scale bars = 10 μm (A and B in **B**, C and D in **D**) and 5 μm (E and F in **F**).

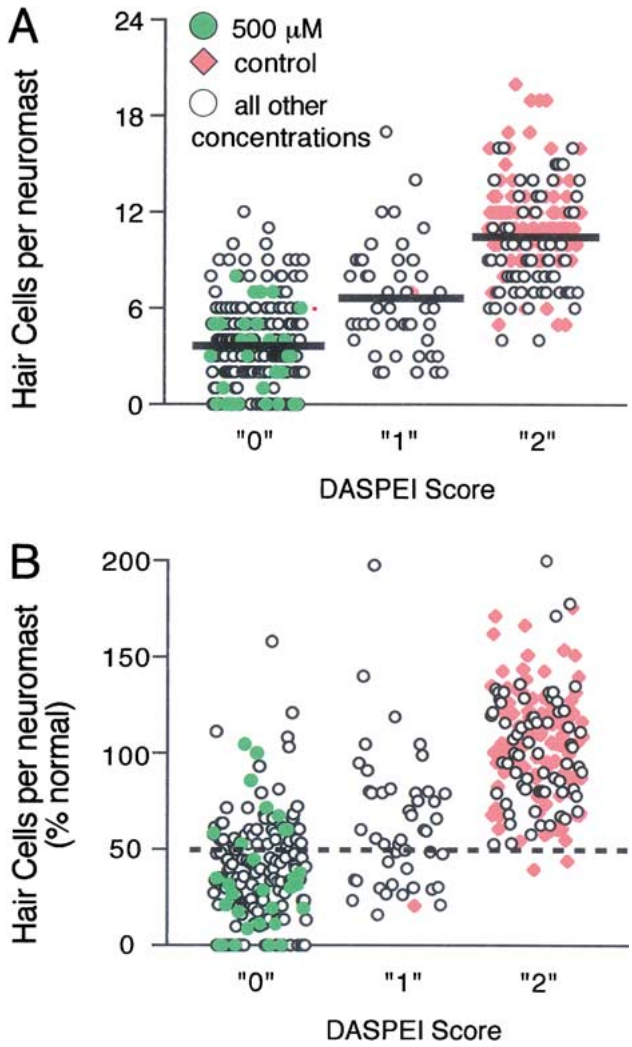

FIG. 5. Correspondence between DASPEI and phalloidin staining. Expanded scatterplots of live DASPEI scores from neuromasts (n = 388) that were also stained with phalloidin after fixation. **A.** Neuromasts are grouped by DASPEI score and the number of phalloidin-labeled hair cells counted in each neuromast is shown on the Y axis. The mean number of hair cells per neuromast for each scoring category is shown by the black lines. These means (± SD) are 3.9 ± 2.70, 6.5 ± 3.37, and 10.8 ± 3.07 hair cells, respectively. The means are all significantly different from each other by ANOVA ($p <$ 0.001). **B.** Neuromasts are grouped by DASPEI score. The percent of the normal number of hair cells per neuromast is shown on the Y axis. This percentage was determined by dividing the number of phalloidin-labeled hair cells observed in each neuromast (data in A) by the normal mean number of hair cells in each specific neuromast based on its lateral line location (from Table 1).

200, and 250 μM neomycin). This difference was small, usually 3–4 neuromasts, but statistically significant (data not shown). There were no reliable differences in neuromast hair cell survival as a function of age following the highest neomycin doses (300 and 500 μM). It is possible that p5 neuromasts retain more DASPEI-labeled hair cells at intermediate concentrations because they have a greater number of mature hair cells to begin with. We checked this by comparing phalloidin-labeled hair cell counts for identical neuromasts at p4 and p5 in untreated larvae. The average number of hair cells contained within a specific neuromast of the lateral line at p5 was slightly greater than at p4 in almost all neuromasts. The mean difference between ages was 0.71 phalloidin-labeled hair cells per neuromast. However, this difference was not statistically significant (p = 0.06).

We also assessed the possibility that individual neuromasts of the head and trunk lateral lines are differentially sensitive to neomycin treatment. Dose–response curves were constructed based on DASPEI staining of neuromasts of the head or trunk separately and for each of the 27 neuromasts. Neuromasts

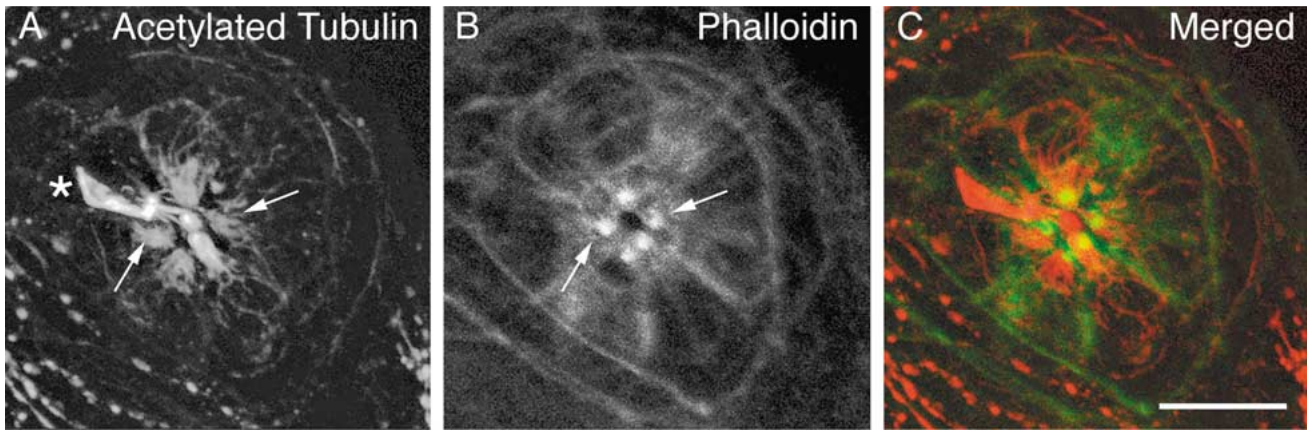

FIG. 6. A representative neuromast from a control zebrafish double-labeled with antiacetylated tubulin and phalloidin. **A.** Acetylated tubulin labels the kinocilia (indicated with an asterisk) and extends toward the nuclei of the cells. Two labeled hair cells are indicated with arrows. **B.** Phalloidin labels the stereocilia bundles. The same two hair cells indicated in A are shown here labeled with phalloidin indicated by the arrows. **C.** A composite image of acetylated tubulin label (red) and phalloidin (green). Scale bar = 10 μM.

of the head and trunk did not differ in their susceptibility to neomycin-induced damage, nor did any single neuromast show reliably greater sensitivity than any other neuromast (data not shown).

Correspondence between DASPEI, actin, and acetylated tubulin staining

We assessed the validity of DASPEI labeling as an indicator of the number of hair cells present in three ways: (1) f-actin staining of stereocilia with phalloidin, (2) immunocytochemistry with a hair cell selective antibody, acetylated tubulin (Raible and Kruse 2000), and (3) scanning electron microscopy (SEM) observations. Representative examples of normal neuromasts and neuromasts treated with 500 μM neomycin are shown in Figure 4. Figures 4A and B show a representative neuromast from an untreated (control) larva that had received a live DASPEI score of 2 and a representative neuromast that had received a DASPEI score of 0 before being fixed and labeled with phalloidin. Many more phalloidin-labeled hair cells are observed in neuromasts scored as 2 than 0 using DASPEI. Figures 4C and D show representative neuromasts from a control animal and an animal 4 h after exposure to 500 μM neomycin (DASPEI score = 0) labeled with acetylated tubulin. Hair cell bodies as well as stereocilia bundles are lost in proportion to the loss of DASPEI labeling.

As a third method of DASPEI validation, untreated and neomycin-treated neuromasts were examined using scanning electron microscopy. A normal neuromast that had received a DASPEI score of 2 is shown in Figure 4E. The neuromast has a characteristic dome- or volcano-shaped structure protruding from the body surface. Extending from the ''crater'' are the long kinocilia from the resident hair cells. Less apparent at this level of scanning electron microscopy examination are the short stereocilia bundles of the hair cells. All of the normal, untreated neuromasts observed had this same morphology regardless of position in the lateral line system at this age. Figure 4F shows a representative neuromast that was fixed 4 h following the initiation of a 1 h 500 μM neomycin treatment and had received a DASPEI score of 0. The long kinocilia have been eliminated. Additional studies by transmission electron microscopy confirm the loss of kinocilia and hair cells following 500 μM neomycin treatment (Pujol et al. 2002). These experiments support the validity of using DASPEI in a screening protocol to quickly assess lateral line hair cell integrity in living animals.

Using each method, more hair cells were present in neuromasts that had previously received a DASPEI score of 2 rather than 1 or 0. On the other hand, it is important to note that screening for hair cells using DASPEI is not perfect; some neuromast hair cells were present in the majority of neuromasts scored as 0. To quantify these relationships, DASPEI staining was scored in live animals' neuromasts at relatively low magnification under a fluorescence dissection microscope and then the number of phalloidin-stained hair cells were counted in the same neuromasts after the fish were fixed. Fish from all neomycin dosage groups were included in this analysis. Phalloidin cell counts were grouped according to their previous DASPEI scores, as shown in Figure 5A. The mean number (± SD) of phalloidin-labeled hair cells per neuromast for a DASPEI score of 0, 1, or 2 was 3.9 ± 2.70, 6.5 ± 3.37, and 10.8 ± 3.07 hair cells, respectively. While there is some overlap in the distri-

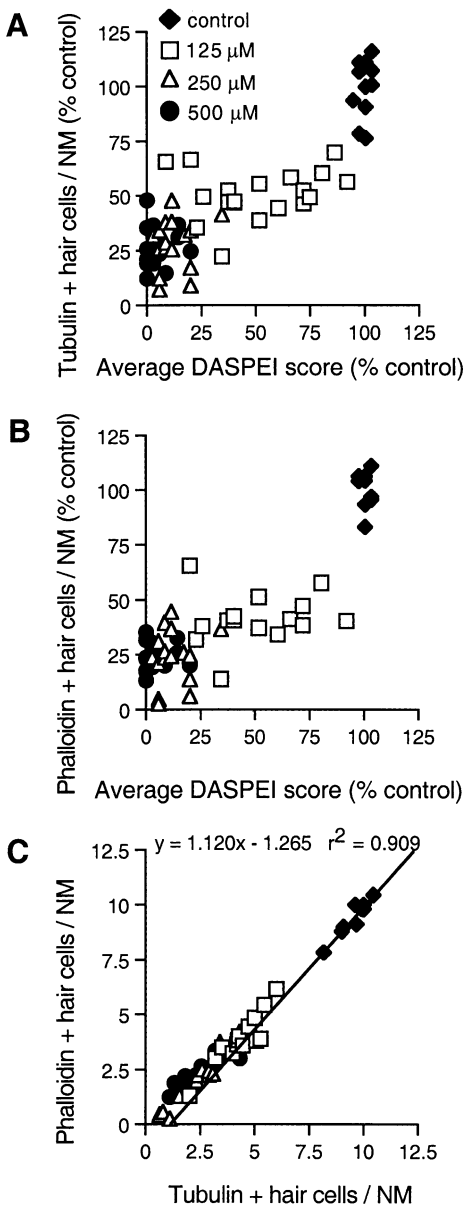

FIG. 7. Scatterplot comparisons of three methods used to assess neuromast hair cell loss. **A.** The average DASPEI score is shown plotted against the average number of acetylated tubulin-positive (+) hair cells per neuromast for each larva at all concentrations (0, 125, 250, and 500 μM). Both measures are expressed as a percentage of the mean control value for that analysis. **B.** The average DASPEI score is shown plotted against the average number of phalloidin-positive (+) hair cells per neuromast for each larva at all concentrations. Both measures are expressed as a percentage of the mean control value for that analysis. **C.** The average number of acetylated tubulin-positive (+) hair cells per neuromast is plotted against the number of phalloidin-positive (+) hair cells per neuromast for larvae at all concentrations. This plot was fitted with a linear regression. There is a high correlation between the number of hair cells counted using phalloidin and acetylated tubulin labeling ($r^2 = 0.909$).

butions of these scoring categories, the means are all significantly different from each other ($p < 0.001$). The overlap among categories is considerably reduced when data points from control and 500 μM-treated neuromasts are the only groups included for analysis as shown in Figure 5A represented by the green- and red-filled symbols.

The number of hair cells per neuromast in untreated zebrafish at p4 and p5 varies according to neuromast position in the lateral line system (shown in Table 1). To more accurately represent changes in hair cell number, we used the mean number of hair cells for each neuromast position (Table 1) to recalculate the data in Figure 5A. An expanded scatterplot of DASPEI scoring category against the percentage of normal hair cells per neuromast is shown in Figure 5B. Importantly, there were only 2 out of 167 neuromasts scored as 2 that contained less than 50% of the normal mean hair cell number. The majority of neuromasts (128 out of 175; 73%) scored as 0 contained less than 50% of normal hair cell numbers for that neuromast.

In an additional experiment we directly compared DASPEI scores of the head neuromasts to the number of hair cells counted in the same neuromasts after fixation and double labeling by phalloidin and acetylated tubulin. A representative control neuromast containing hair cells double-labeled using acetylated tubulin and phalloidin is shown in Figure 6. The scatterplots shown in Figures 7A and B indicate that DASPEI scores change in proportion to hair cell counts made using both acetylated tubulin and phalloidin labeling across the dose–response range. Interestingly, DASPEI does seem to slightly overestimate the number of hair cells lost after treatment with the highest doses of neomycin, consistent with the observations made above and seen in Figure 5 for a DASPEI score of 0.

One potentially confounding factor in our analysis of hair cell numbers using phalloidin to label stereocilia bundles is that damaged hair cells could lose their bundles but may not die. We addressed this issue by comparing the number of acetylated tubulin-labeled hair cells to the number of phalloidin-labeled hair cells in the same neuromasts as shown in Figure 7C. There is a high correlation ($r^2 = 0.909$) between these two independent means of counting hair cells indicating that ''bundleless'' hair cells are not likely to be a factor in our analyses.

Finally, the overall validity of using our DASPEI scoring system as a rapid and efficient method for assessing hair cell loss in a large number of fish was assessed using the same data as above by comparing the dose–response relationships constructed from DASPEI scores to average hair cell counts per neuromast using acetylated tubulin and phalloidin labeling. These three independently derived dose–response relationships are shown in Figure 8. The three methods yield very similar dose–response rela-

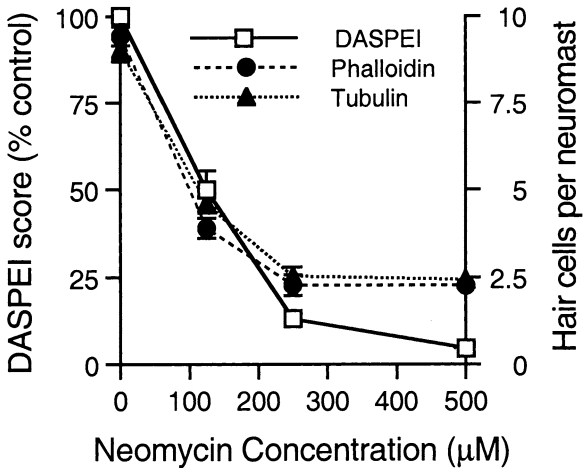

FIG. 8. Dose–response relationships of neuromast hair cell survival and neomycin concentration determined by three different methods (from the same data as shown in Fig. 7). The average DASPEI score expressed as a percentage of the control value is plotted on the left *Y* axis. The average number of hair cells per neuromast counted using phalloidin and acetylated tubulin labeling is plotted using the right *Y* axis. The overall decrease in DASPEI scores and hair cell counts was highly significant ($p < 0.001$) and all doses were significantly different from each other (p's < 0.001), except scores for 250 vs. 500 μM using phalloidin and acetylated tubulin staining. There was no significant difference between phalloidin and acetylated tubulin cell counts at any concentration. DASPEI scores differed significantly from both other methods at the highest doses, 250 and 500 μM (p's $<$ 0.001), but they did not differ at the intermediate dose, 125 μM. Hence the change in DASPEI scores is proportional to hair cell counts across the dose–response range but slightly overestimates hair cell loss at the high doses.

tionships at low to midrange concentrations. However, at the highest concentrations some cells appear to be resistant to neomycin-induced death when studied by phalloidin or acetylated tubulin labeling. These conclusions were supported by statistical analysis using two-way ANOVA (dosage by analysis method) followed by appropriate individual comparisons between groups. The overall analysis showed significant effects of dosage ($p < 0.001$), analysis method ($p <$ 0.001), and the interaction term ($p < 0.01$). There were no significant differences between DASPEI score and hair cell counts by either method after 0 and 125 μM neomycin exposure. Individual comparisons revealed that the methods do yield significant differences at the highest doses, 250 and 500 μM, with a mean of approximately 2.5 hair cells per neuromast remaining by both acetylated tubulin and phalloidin labeling, while average DASPEI scores are 13% and 4.6%, respectively, of the average DASPEI control score. Scores as a function of neomycin concentration yielded significant differences at each successive dosage ($p < 0.001$) except for the comparisons between 250 and 500 μM using phalloidin or acetylated tubulin labeling, which revealed equivalent effects.

Lateral line hair cells at 12 and 24 h after neomycin treatment

Time course of DASPEI- and phalloidin-stained lateral line hair cell loss and recovery. It has been demonstrated previously that lateral line hair cells can regenerate following damage (Jorgensen 1991; Song et al. 1995; Jones and Corwin 1996). We examined neuromast hair cells at 12 and 24 h after neomycin exposure using DASPEI to further understand the changes in hair cell populations in p4 and p5 zebrafish larvae after aminoglycoside treatments. Figures 9A and B show DASPEI staining in representative larvae 4 h after initial incubation in either control media (Fig. 9A) or media containing 500 μM neomycin (Fig. 9B). The neomycin treatment eliminated all DASPEI staining. When animals were allowed an additional 20 h of recovery time and stained with DASPEI, the staining pattern looked remarkably normal, as illustrated in Figure 9C.

We quantified neuromast hair cell loss and recovery using the DASPEI scoring system at 12 and 24 h for four different doses of neomycin (0, 125, 250, and 500 μM). We also analyzed phalloidin labeling at these times for fish treated with 500 μM neomycin. Figure 10A shows that DASPEI-stained lateral line neuromasts increased in each treatment group as a function of the period of recovery. At 12 h following neomycin exposure, DASPEI scores were already greater than those at 4 h for intermediate concentrations. Animals that had received the highest neomycin dose (500 μM) did not show any recovery of DASPEI staining when analyzed 12 h after the initial aminoglycoside exposure. Hair cell staining recovered to control levels by 24 h after 125 μM neomycin treatments. Allowing the zebrafish to recover for 24 h following initial treatment with either 250 or 500 μM neomycin resulted in DASPEI labeling of approximately 60% of control levels.

Figure 10B shows the mean number of phalloidin-labeled hair cells per neuromast from the 500 μM-treated groups at 4, 12, and 24 h after aminoglycoside treatment. In the 500 μM-treated group, there was no further loss in the average number of hair cells per neuromast between 4 and 12 h. The number of phalloidin-labeled hair cells returned to the average control level after 24 h of recovery time. There appears to be more recovery of hair cell numbers when assessed using phalloidin labeling compared with DASPEI staining at 24 h after 500 μM neomycin exposure.

BrdU incorporation in neuromast cells.. To further explore the events during the 24 h following aminoglycoside toxicity, we assessed the relative amount of cell proliferation occurring in neuromasts in control animals and at 4, 12, and 24 h following 500 μM

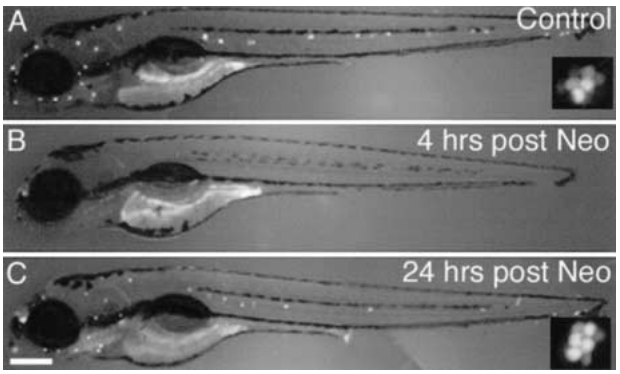

FIG. 9. Hair cell regeneration in neomycin-treated neuromasts. **A.** An untreated larva showing typical DASPEI staining of neuromasts. **B.** A DASPEI-stained larva 4 h after exposure to 500 μM neomycin. DASPEI staining is eliminated in neuromasts. **C.** A DASPEI-stained larva 24 h following exposure to 500 μM neomycin. Note the return of a normal DASPEI staining pattern. **A, C insets:** DASPEI-labeled control (A) and regenerated (C) neuromasts appear similar at higher magnification. Scale bar = 0.5 mm for all panels excluding insets.

neomycin treatment. This was accomplished by a single exposure of larvae to BrdU in the embryo medium for 1 h at the time points of interest. The fish were fixed immediately after the BrdU exposure (pulse-fix protocol). This BrdU-labeling protocol will assess only cells that are in S-phase during the 1 h exposure. The number of neuromast cells in S-phase was counted. Figures 11A and B show representative neuromasts from a control animal and an animal 12 h after 500 μM neomycin treatment. Treated animals had many more BrdU-labeled cells within individual neuromasts than untreated animals. The labeled cells occurred predominantly in the periphery of the neuromast structure, suggesting that they are likely mantle support cells because of their location in the neuromast as well as the large size of the nuclei in comparison to the hair cell nuclei. A minority of the labeled cells in the periphery appear to be much larger than expected for BrdU-labeled nuclei. This has been observed previously (Williams and Holder 2000) and interpreted as BrdU labeling in the cytoplasm as well as the nuclei. As expected, there is ongoing proliferation in neuromasts of control animals.

Figure 12 shows the average number of neuromast cells in S-phase from control larvae and at 4, 12, and 24 h following 500 μM neomycin treatment. In control animals the mean number of BrdU-labeled cells after 1 h of BrdU exposure was approximately 1 cell per neuromast. At 4 h after neomycin exposure, there was a slight increase in the number of BrdU-labeled cells per neuromast relative to control levels, but this difference was not significant. However, proliferation was dramatically upregulated at 12 h following neomycin exposure with an approximate four-fold increase over control levels of neuromast cells in S-phase. By 24 h after aminoglycoside toxicity, the number of proliferative cells remained higher than control levels but lower than the number of cells incorporating BrdU at 12 h following 500 μM neomycin exposure. Both the neomycin treatment and the interaction effect of treatment and time of recovery on the number of BrdU-labeled cells were highly significant ($p < 0.005$). Differences between treatment and controls were significant at 12 and 24 h ($p < 0.005$), and treatment groups differed significantly at each recovery time ($p < 0.001$).

We also assessed whether the increase in neuromast cells in S-phase at 12 h after neomycin exposure resulted in the production of new hair cells. Larvae were exposed to BrdU for 1 h 12 h following 500 μM neomycin treatment. They were then allowed to recover for an additional 0, 12, or 36 h. Hair cells were identified using acetylated tubulin immunocytochemistry. Neuromast cells double-labeled for acetylated tubulin and BrdU represented hair cells assumed to be ''born'' around 12 h after neomycin exposure. We did not observe double-labeled cells in neuromasts from either control or neomycin-treated larvae in the 12 h pulse-fix experiment as exemplified in Figures 13 A and B. As shown previously, BrdU incorporation at 12 h after 500 μM neomycin exposure is significantly greater than the amount of ongoing proliferation seen in a control neuromast (compare BrdU label in 11B to 11A). A very low number of neuromasts containing cells double-labeled for acetylated tubulin and BrdU were observed in both control and neomycin-treated animals at 24 h after neomycin exposure or experimental initiation (12 h after BrdU exposure), and there appeared to be more neuromasts containing new hair cells in the drug-treated group. By 36 h after BrdU exposure in untreated animals, approximately one-third of all neuromasts contained 1–2 double-labeled cells. In neomycin-treated animals that recovered for 36 h after BrdU exposure (48 h after neomycin treatment), 1–4 double-labeled hair cells were observed within most neuromasts. Figures 13C and D show two examples from animals exposed to neomycin that were allowed to recover for 48 h (36 h after BrdU). In summary, the upregulation of proliferation observed during this particular 1 h period, 12 h after aminoglycoside exposure, results in the production of some new hair cells 12 h later. By 36 h after S-phase, a large number of these cells are expressing a hair cell phenotype.

DISCUSSION

The goal of our experiments was to develop and communicate an efficient preparation for studying

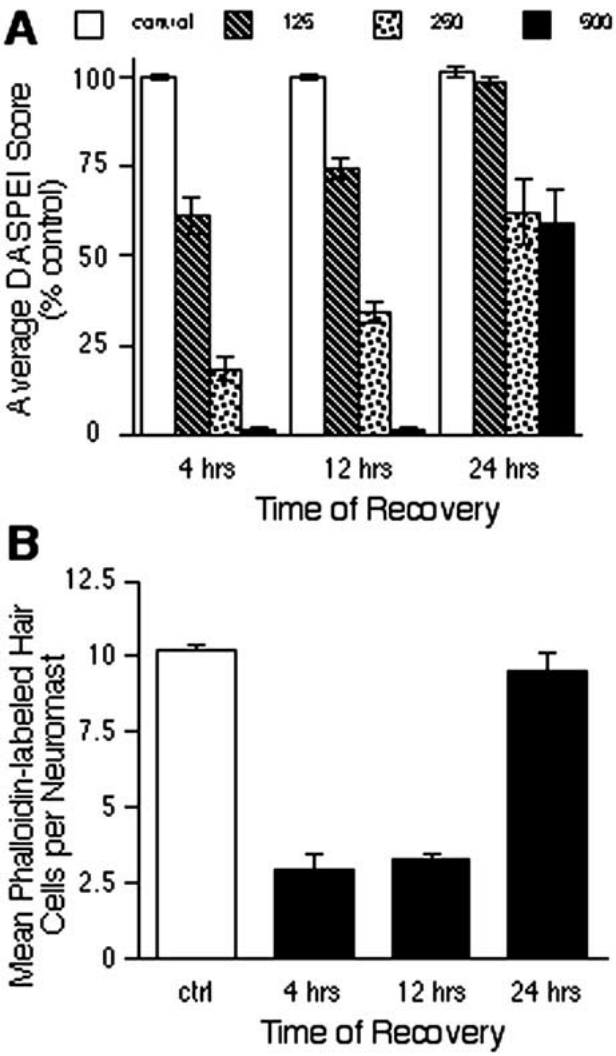

FIG. 10. Time course of neuromast hair cell regeneration following neomycin exposure. **A.** Neuromast hair cell survival quantified with DASPEI scores relative to control are plotted at 4, 12, and 24 h postexposure for larvae treated with various doses of neomycin (0, 125, 250, and 500 μM, n =11–29 fish/group/time point). DASPEI scores significantly increased as a function of recovery time following neomycin exposure ($p < 0.001$). **B.** Mean phalloidin-labeled hair cell counts per neuromast are plotted at 4, 12, and 24 h postexposure to 500 μM neomycin ($n > 26$ neuromasts/group). Similar to the DASPEI scores, there is significant recovery of hair cells between 4 and 24 h and between 12 and 24 h ($p < 0.001$). The mean number of hair cells per neuromast is also plotted from a control, untreated group for comparison (white bar). Bars are mean ± SEM.

genes involved in the process of hair cell death and survival following aminoglycoside toxicity. We have demonstrated that the larval zebrafish lateral line hair cells degenerate in response to neomycin exposure in a dose-dependent manner. We have also begun to describe the time course of some of the important events such as neuromast hair cell loss and regeneration following neomycin exposure in larval zebrafish.

Aminoglycoside-induced hair cell death

To our knowledge the present study is the first to standardize and quantify larval teleost fish lateral line hair cell death in response to ototoxic drugs by determining the dose–response relationship. The concentrations of aminoglycoside used in the present study to produce hair cell death are generally lower than those used in other *in vivo* vertebrate preparations that utilize systemic injections. These doses are more similar to direct application of gentamicin-soaked pledgets to the round window in chicks (Weisleder and Rubel 1993; Carey et al. 1996; Husmann et al. 1998; Muller and Smolders 1998). This is presumably due to the superficial location of the larval neuromasts; hair cells are exposed directly to the drug in the embryo medium. One recent report studied aminoglycoside-induced lateral line hair cell death in zebrafish at 10 days postfertilization (Williams and Holder 2000). Those authors reported that a much lower dose of neomycin (10 μM for 1.5 h) was sufficient to kill lateral line hair cells as assessed by acridine orange staining. We have no explanation for the large difference between the results of our studies.

Dose-dependent aminoglycoside-induced inner ear hair cell death has been described previously in a variety of vertebrate species both *in vivo* and *in vitro* (Yan et al. 1991; Lombarte et al. 1993; Cotanche et al. 1994; Nakagawa et al. 1998; Matsui et al. 2000). There have been fewer quantitative studies of hair cell loss in the fish lateral line system. In one study the ability to localize a wave source, a function of the lateral line, was described in an adult teleost fish, Cyprinodontidae, following exposure to a range of streptomycin and neomycin concentrations (Kaus 1987). This behavior declines in a dose-dependent manner, suggesting that aminoglycosides impair lateral line hair cell function as they do inner ear hair cell function.

We were rarely successful in killing every hair cell within a neuromast 4 h after treatment, even with a high dose of neomycin. Neuromasts that did not stain with DASPEI were not always completely devoid of hair cells when assessed by other methods. There are several potential explanations for this observation. First, DASPEI is a voltage-sensitive dye. It is possible that remaining hair cells identified with phalloidin staining had lost their membrane potential. We would expect that these hair cells would go on to die, although it is possible that some persist and recover. A second possibility is that the failure to detect the few remaining hair cells using DASPEI could have been a technical issue. The signal from only a few cells may be too weak to detect using an epifluorescent dissecting microscope, which had a lower maximum resolution than that used to count hair cells in fixed tissue. This could not have always been the case,

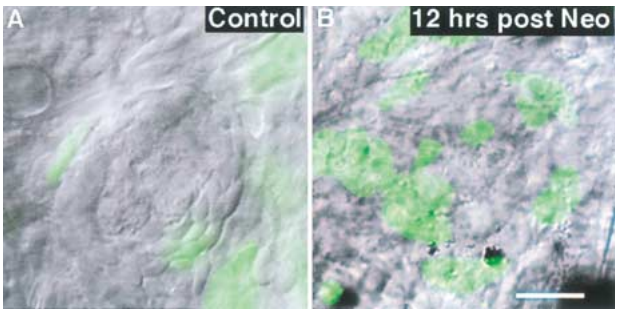

FIG. 11. Neomycin-induced upregulation of proliferation in neuromasts. **A.** A representative neuromast from an untreated control larva that was exposed to BrdU 12 h after the initiation of the experiment contains few BrdU-labeled cells. **B.** A neuromast from an animal treated with 500 μM neomycin contains many more S-phase cells than the control neuromast when exposed to BrdU for 1 h 12 h after treatment. The BrdU nuclei (green) are overlaid onto a gray DIC image of the neuromast. Each fluorescent image is from a flattened *z*-series through the neuromast. Scale bar = 10 μm.

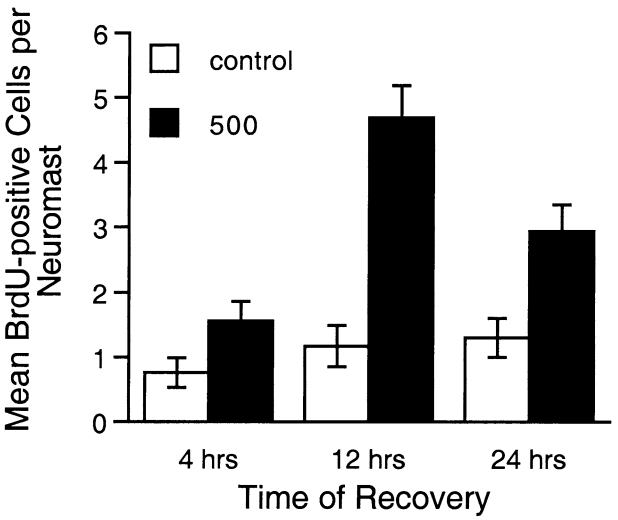

FIG. 12. Time course of neuromast cells entering S-phase following 500 μM neomycin exposure. Mean BrdU-positive cells per neuromast are plotted for neuromasts exposed to BrdU in the embryo medium for 1 h 4, 12, and 24 h after neomycin exposure (n = 173 neuromasts, 19–41 neuromasts/group and 3–7 neuromasts/fish). The number of S-phase cells in a neuromast is upregulated at 12 and 24 h after neomycin exposure. The time of BrdU exposure and neomycin treatment had significant effects on the number of proliferating cells (two-way ANOVA, $p < 0.005$). Bars are mean ± SEM.

however, because as few as 2 cells were able to give a DASPEI signal scored as 1 (see Fig. 5A).

A third possible explanation is that ongoing proliferation is occurring in neuromasts during our experimental paradigm. As a result it could be presumed that there are hair cells at several stages of development within any given neuromast at the time they are treated with neomycin. Aminoglycosides may not be effective in inducing death in hair cells that are immature (Rubel 1978; Duckert and Rubel 1990; Hashino and Salvi 1996). Therefore, we might expect to see a low number of hair cells, possibly the ''youngest'' within a given neuromast, that survive drug treatment. A final possibility is that there may be hair cell heterogeneity within a neuromast at this point in lateral line development. Based on differential ototoxic sensitivity, type I and type II hair cells have been described in the fish inner ear as well as in canal and superficial neuromasts, respectively, of the adult lateral line (Yan et al. 1991; Song et al. 1995). Differentiating among these possibilities will require further research. In summary, DASPEI scores recorded from low-power images through a dissecting microscope did not perfectly predict the number of hair cells present within a neuromast as assessed by these other methods. However, DASPEI scores are proportional to the number of hair cells present using a variety of methods, including phalloidin and acetylated tubulin labeling. Of particular importance

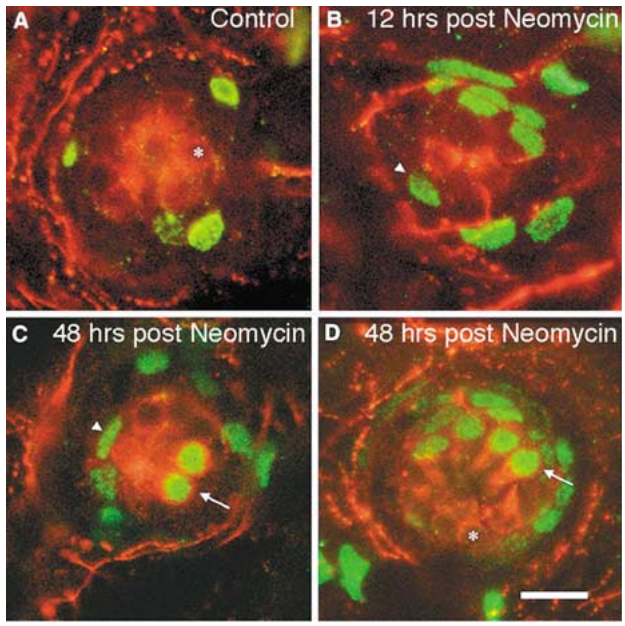

FIG. 13. New hair cells produced by neuromast cells in S-phase at 12 h after 500 μM neomycin exposure. Hair cells were identified using immunocytochemistry (ICC) for acetylated tubulin (shown in red). BrdU-positive cells were also detected with ICC (shown in green). **A.** A representative neuromast from a control animal pulsed with BrdU for 1 h 12 h after the experiment was initiated and fixed immediately. There are few S-phase cells within the neuromast. **B.** A representative neuromast exposed to 500 μM neomycin, pulsed with BrdU 12 h later, and then immediately fixed. This neuromast contains many more S-phase cells but few hair cells. **C, D.** Two examples of neuromasts treated with 500 μM neomycin, exposed to BrdU for 1 h 12 h after neomycin exposure, and then fixed 36 h later (48 h after neomycin treatment). These neuromasts contained new hair cells double-labeled for acetylated tubulin and BrdU as well as hair cells without BrdU labeling and BrdU-labeled cells without acetylated tubulin labeling. Asterisks indicate hair cells, arrowheads indicate BrdU labeling, and the arrows indicate ''new'' hair cells. Scale bar = 10 μm.

is the fact that none of the control fish were given a DASPEI score of 0. Therefore, DASPEI scoring provides a rapid, reliable screening method for assessment of lateral line hair cell survival.

Lateral line hair cell regeneration

Lateral line hair cells of larval zebrafish regenerate following aminoglycoside-induced degeneration. There are at least two possible mechanisms for replacing hair cells in sensory epithelia. New hair cells can be produced by proliferation of precursor cells that then differentiate into hair cells. Alternatively, postmitotic cells can be induced to differentiate into hair cells with no intervening mitosis in a process termed direct transdifferentiation (Adler and Raphael 1996; Baird et al. 1996; Jones and Corwin 1996; Roberson et al. 1996; Steyger et al. 1997).

We investigated the potential role of proliferation in generating new hair cells following maximal hair cell loss. Neuromast cell entry into S-phase is dramatically upregulated 12 h after the onset of aminoglycoside toxicity. We observed that some cells labeled with the S-phase marker BrdU 12 h after neomycin exposure assumed a hair cell phenotype within the next 12–36 h. Therefore, it is likely that death-induced upregulation of proliferative events contributes to at least some of the new hair cells seen at 24 h using DASPEI and phalloidin labeling. A more intensive study involving BrdU exposure at times between 4 and 12 h will be necessary to determine what proportion of hair cell replacement observed by 24 h is due to induced proliferation versus ongoing proliferation or induced transdifferentiation.

The observed time course of larval lateral line hair cell regeneration is extraordinarily fast in comparison to other systems. In the chick basilar papilla, new hair cells are detected by 90–100 h after noise-induced damage and several days following drug administration (Girod et al. 1989; Duckert and Rubel 1990; Cotanche et al. 1994; Matsui et al. 2000; Stone and Rubel 2000b). Jones and Corwin (1996) observed mature hair cells arising over one week in the axolotl after laser-induced damage. However, in the adult oscar, a teleost fish, new hair cell stereocilia bundles located in canal neuromasts were observed as early as two days following prolonged aminoglycoside exposure (Song et al. 1995). In addition, behavioral recovery in adult Cyprinodontidae fish is reported to occur one to three days after aminoglycoside treatment (Kaus 1987). Regeneration may occur more quickly in these systems as opposed to the chick basilar papilla because cells are not mitotically quiescent under normal developmental circumstances. In addition, cell cycle length increases during zebrafish development, suggesting that proliferation and differentiation could occur more quickly in the larva than in adult systems (Kane and Kimmel 1993; Kimmel et al. 1994).

Supporting cells appear to be the most likely candidate for hair cell progenitors in the avian system after damaging stimuli (Corwin and Cotanche 1988; Girod et al. 1989; Weisleder and Rubel 1993; Stone et al. 1996; Warchol and Corwin 1996; Stone and Rubel 2000b). In the axolotl lateral line, Jones and Corwin (1996) have provided direct evidence that new hair cells arise from supporting cell divisions in neuromasts after laser ablation of all preexisting hair cells. In the zebrafish lateral line, we observed that BrdU-labeled cells were generally located in the periphery of the neuromast following aminoglycoside exposure, similar to the observations of Williams and Holder (2000). This location suggests that they may be support cells, although other cell types are possible. Further investigation, including double-labeling transmission electron microscopy studies, are in progress to accurately and carefully describe hair cell regeneration and the progenitors in this model system (Pujol et al. 2002).

One application of this study is to begin a genetic screen using randomly mutagenized zebrafish to search for genes that modulate hair cell susceptibility to ototoxicity. Genetic screens in zebrafish have already been successful in pulling out classes of genes that are important for hair cell function and development, including myosin VIIA (Whitfield et al. 1996; Nicolson et al. 1998; Ernest et al. 2000). The final mechanism of hair cell death following ototoxic insults appears to be similar in a variety of vertebrates including fish (Forge and Li 2000; Pirvola et al. 2000; Williams and Holder 2000). Therefore, we expect both exogenous and endogenous molecules that influence lateral line hair cell death, survival, and regeneration in the zebrafish are likely to be effective in the inner ear of other vertebrate species, including humans.

ACKNOWLEDGMENTS

The authors thank Dale Cunningham for his help with scanning electron microscopy, Laurie Johnson for manuscript preparation assistance, and Melinda Modrell and Tiffany Kao for their excellent technical support. We also thank Dr. Eric Bauer and Setsuko Murakami for additional histology and valuable help. This work was supported by NIDCD grants DC04661, DC00018, and DC02854, and the University of Washington RRF.

REFERENCES

ADLER HJ, RAPHAEL Y. New hair cells arise from supporting cell conversion in the acoustically damaged chick inner ear. Neurosci. Lett. 205:17–20, 1996.

ALEXANDRE D, GHYSEN A. Somatotopy of the lateral line projection in larval zebrafish. Proc. Natl. Acad. Sci. U.S.A. 96:7558–7562, 1999.

BAIRD RA, STEYGER PS, SCHUFF NR. Mitotic and nonmitotic hair cell regeneration in the bullfrog vestibular otolith organs. Ann. N.Y. Acad. Sci. 781:59–70, 1996.

BANG PI, SEWELL WF, MALICKI JJ. Morphology and cell type heterogeneities of the inner ear epithelia in adult and juvenile zebrafish (Danio rerio). J. Comp. Neurol. 438:173–190, 2001.

CAREY JP, FUCHS AF, RUBEL EW. Hair cell regeneration and recovery of the vestibuloocular reflex in the avian vestibular system. J. Neurophysiol. 76:3301–3312, 1996.

CHARDIN S, ROMAND R. Regeneration and mammalian auditory hair cells. Science 267:707–711, 1995.

CHENG AG-L, SHANG J, RUBEL EW. Hair cell death in vitro in the avian BP: characterization of the model and caspase inhibition. Assoc. Res. Otolaryngol. 24:Abstr. 466, 2001.

CORWIN JT, COTANCHE DA. Regeneration of sensory hair cells after acoustic trauma. Science 240:1772–1774, 1988.

COTANCHE DA. Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. Hear. Res. 30:181–195, 1987.

COTANCHE DA, LEE KH, STONE JS, PICARD DA. Hair cell regeneration in the bird cochlea following noise damage or ototoxic drug damage. Anal. Embryol. (Berl.) 189:1–18, 1994.

CRUZ RM, LAMBERT PR, RUBEL EW. Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. Arch. Otolaryngol. Head Neck Surg. 113:1058–1062, 1987.

CUNNINGHAM L, CHENG AG, RUBEL EW. Caspase activation in hair cells of the mouse utricle exposed to neomycin. J. Neurosci. 22:8532–8540, 2002.

DUCKERT LG, RUBEL EW. Ultrastructural observations on regenerating hair cells in the chick basilar papilla. Hear. Res. 48:161–182, 1990.

ERNEST S, RAUCH GJ, HAFFTER P, GEISLER R, PETIT C, NICOLSON T. Mariner is defective in myosin VIIA: a zebrafish model for human hereditary deafness. Hum. Mol. Genet. 9:2189–2196, 2000.

FISCHEL–GHODSIAN N. Genetic factors in aminoglycoside toxicity. Ann. N.Y. Acad. Sci. 884:99–109, 1999.

FORGE A, LI L. Apoptotic death of hair cells in mammalian vestibular sensory epithelia. Hear. Res. 139:97–115, 2000.

FORGE A, SCHACHT J. Aminoglycoside antibiotics. Audiol. Neurootol. 5:3–22, 2000.

GIROD DA, DUCKERT LG, RUBEL EW. Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hear. Res. 42:175–194, 1989.

HADDON C, MOWBRAY C, WHITFIELD T, JONES D, GSCHMEISSNER S, LEWIS J. Hair cells without supporting cells: further studies in the ear of the zebrafish mind bomb mutant. J. Neurocytol. 28:837–850, 1999.

HASHINO E, SALVI RJ. Regenerated hair cells exhibit a transient resistance to aminoglycoside toxicity. Brain Res 720:172–182, 1996.

HUSMANN KR, MORGAN AS, GIROD DA, DURHAM D. Round window administration of gentamicin: a new method for the study of ototoxicity of cochlear hair cells. Hear. Res. 125:109–119, 1998.

JONES JE, CORWIN JT. Regeneration of sensory cells after laser ablation in the lateral line system: hair cell lineage and macrophage behavior revealed by time-lapse video microscopy. J. Neurosci. 16:649–662, 1996.

JORGENSEN JM. Regeneration of lateral line and inner ear vestibular cells. Ciba Found. Symp. 160:151–163, 1991.

KALMIJN AD. Functional evolution of lateral line and inner ear sensory systems. In: COOMBS S, GORNER P, MUNZ H (eds) The mechanosensory lateral line. Springer-Verlag, New York, 1989, pp 187–215.

KANE DA, KIMMEL CB. The zebrafish midblastula transition. Development 119:447–456, 1993.

KAUS S. The effect of aminoglycoside antibiotics on the lateral line organ of *Aplocheilus lineatus* (Cyprinodontidae). Acta Otolaryngol. 103:291–298, 1987.

KIMMEL CB, WARGA RM, KANE DA. Cell cycles and clonal strings during formation of the zebrafish central nervous system. Development 120:265–276, 1994.

LI L, NEVILL G, FORGE A. Two modes of hair cell loss from the vestibular sensory epithelia of the guinea pig inner ear. J. Comp. Neurol. 355:405–417, 1995.

LOMBARTE A, YAN HY, POPPER AN, CHANG JS, PLATT C. Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. Hear. Res. 64:166–174, 1993.

MATSUI J, OESTERLE E, STONE J, RUBEL E. Characterization of damage and regeneration in cultured avian utricles. J. Assoc. Res. Otolaryngol. 1:46–63, 2000.

MATSUI JI, OGILVIE JM, WARCHOL ME. Inhibition of caspases prevents ototoxic and ongoing hair cell death. J. Neurosci. 22:1218–1227, 2002.

METCALFE WK, KIMMEL CB, SCHABTACH E. Anatomy of the posterior lateral line system in young larvae of the zebrafish. J. Comp. Neurol. 233:377–389, 1985.

MONTGOMERY J, CARTON G, VOIGT R, BAKER C, DIEBEL C. Sensory processing of water currents by fishes. Philos. Trans. R. Soc. Lond. B Biol. Sci. 355:1325–1327, 2000.

MULLER M, SMOLDERS JW. Hair cell regeneration after local application of gentamicin at the round window of the cochlea in the pigeon. Hear. Res. 120:25–36, 1998.

NAKAGAWA T, YAMANE H, TAKAYAMA M, SUNAMI K, NAKAI Y. Dose-dependent response of vestibular hair cells of guinea pigs following streptomycin ototoxiation. Acta Otolaryngol. 118:530–533, 1998.

NICOLSON T, RUSCH A, FRIEDRICH RW, GRANATO M, RUPPERSBERG JP, NUSSLEIN–VOLHARD C. Genetic analysis of vertebrate sensory hair cell mechanosensation: the zebrafish circler mutants. Neuron. 20:271–283, 1998.

PIRVOLA U, XING-QUN L, VIRKKALA J, SAARMA M, MURAKATA C, CAMORATTO AM, WALTON KM, YLIKOSKI J. Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. J. Neurosci. 20:43–50, 2000.

PUJOL R, RAIBLE DW, CUNNINGHAM DE, RUBEL EW. Ultrastructure of normal and neomycin-exposed zebrafish lateral line hair cells. Assoc. Res. Otolaryngol. 25:Abstr. 577, 2002.

RAIBLE DW, KRUSE GJ. Organization of the lateral line system in embryonic zebrafish. J. Comp. Neurol. 421:189–198, 2000.

ROBERSON DW, RUBEL EW. Cell division in the gerbil cochlea after acoustic trauma. Am. J. Otol. 15:28–34, 1994.

ROBERSON DW, KREIG CS, RUBEL EW. Light microscopic evidence that direct transdifferentiation gives rise to new hair cells in regenerating avian auditory epithelium. Aud. Neurosci. 2:195–205, 1996.

RUBEL EW. Ontogeny of structure and function in the vertebrate auditory system. In: Jacobson M (ed) Handbook of Sensory Physiology, Vol. IX, Development of Sensory Systems. Springer-Verlag, New York, 1978, pp 135–237.

SCHUKNECHT H. Pathology of the Ear. Harvard University Press, Boston, 1974.

SMOLDERS JW. Functional recovery in the avian ear after hair cell regeneration. Audiol. Neurootol. 4:286–302, 1999.

SONG J, YAN HY, POPPER AN. Damage and recovery of hair cells in fish canal (but not superficial) neuromasts after gentamicin exposure. Hear. Res. 91:63–71, 1995.

STEYGER PS, BURTON M, HAWKINS JR, SCHUFF NR, BAIRD RA. Calbindin and parvalbumin are early markers of non-mitotically re-

generating hair cells in the bullfrog vestibular otolith organs. Int. J. Dev. Neurosci. 15:417–432, 1997.

STONE JS, RUBEL EW. Cellular studies of auditory hair cell regeneration in birds. Proc. Natl. Acad. Sci. U.S.A. 97:11714–11721, 2000a.

STONE JS, RUBEL EW. Temporal, spatial, and morphologic features of hair cell regeneration in the avian basilar papilla. J. Comp. Neurol. 417:1–16, 2000b.

STONE JS, LEANO SG, BAKER LP, RUBEL EW. Hair cell differentiation in chick cochlear epithelium after aminoglycoside toxicity: in vivo and in vitro observations. J. Neurosci. 16:6157–6174, 1996.

WARCHOL ME, CORWIN JT. Regenerative proliferation in organ cultures of the avian cochlea: identification of the initial progenitors and determination of the latency of the proliferative response. J. Neurosci. 16:5466–5477, 1996.

WEISLEDER P, RUBEL EW. Hair cell regeneration after streptomycin toxicity in the avian vestibular epithelium. J. Comp. Neurol. 331:97–110, 1993.

WESTERFIELD M. The zebrafish book: a guide for the laboratory use of zebrafish (*Danio rerio*), 4th ed. University of Oregon Press, Eugene, OR, 2000.

WHITFIELD TT, GRANATO M, VAN EEDEN FJ, SCHACH U, BRAND M, FURUTANI–SEIKI M, HAFFTER P, HAMMERSCHMIDT M, HEISENBERG YJ, JIANG YJ, KANE DA, KELSH RN, MULLINS MC, ODENTHAL J, NUSSLEIN–VOLHARD C. Mutations affecting development of the zebrafish inner ear and lateral line. Development. 123:241–254, 1996.

WILLIAMS JA, HOLDER N. Cell turnover in neuromasts of zebrafish larvae. Hear. Res. 143:171–181, 2000.

YAN HY, SAIDEL WM, CHANG JS, PRESSON JC, POPPER AN. Sensory hair cells of a fish ear: evidence of multiple types based on ototoxicity sensitivity. Proc. R. Soc. Lond. B Biol. Sci. 245:133–138, 1991.

YLIKOSKI J, XING-QUN L, VIRKKALA J, PIRVOLA U. Blockade of c-Jun N-terminal kinase pathway attenuates gentamicin-induced cochlear and vestibular hair cell death. Hear. Res. 163:71–81, 2002.